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Symbiotic Efficiency and Genotypic Characterization of Variants of *Bradyrhizobium* spp. in Commercial Inoculants for Soybeans

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ABSTRACT: High yields obtained from soybean [*Glycine max* (L.) Merrill], and consequent profits, are related to efficient symbiosis of soybean with nitrogen fixing bacteria of the *Bradyrhizobium* genus. Inoculation with strains belonging to the *Bradyrhizobium japonicum*, *B. elkanii*, and *B. diazofficiens* species, approved by the Brazilian Ministry of Agriculture (MAPA), is a common practice for this crop. However, variations in the composition of inoculants can change the quality of the product. The aim of this study was to genetically identify and to evaluate the symbiotic efficiency of isolates with cultural characteristics different from those of strains approved by MAPA and which are present in two commercial inoculants for soybean. Isolates obtained from inoculants and the strains SEMIA 5079 (CPAC 15), SEMIA 5080 (CPAC 7), SEMIA 587 (BR 96), and SEMIA 5019 (BR 29), indicated in the formulation of these inoculants, were tested for symbiotic efficiency in soybeans (cultivar: COODETEC 5G 830 RR) in a greenhouse. Identification by partial sequencing of the 16S rRNA gene, together with BOX-PCR analysis, showed that variant isolates were not similar to the inoculant strains indicated in the commercial products, despite belonging to the same genus and/or species. No loss was observed in the nodulation ability of the isolates; however, significant differences were reported in the symbiotic efficiency of the isolates compared to the inoculant strains approved by MAPA. Nevertheless, the presence of morphological variants did not influence the efficiency of inoculant products.

Keywords: *Glycine max*, 16S rRNA gene, cultural characterization, rhizobia, genetic diversity.

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INTRODUCTION

Brazil is one of the world's largest soybean [*Glycine max* (L.) Merril] producers, with production of 102.4 million tons and yield of 3 tons per ha in the 2016/17 harvest (Conab, 2016). Symbiosis between N_2 fixing bacteria (NFB) of the *Bradyrhizobium* genus and soybean is one of the factors that affect these results.

Seed inoculation in soybean crops is always recommended, and seeds should be inoculated in each crop season. Thus, there is a market for industrial production of commercial inoculants. However, the agricultural potential of inoculants may be affected by factors such as high application rates of nitrogen fertilization in the soil, inadequate inoculation of the seeds, and use of low quality commercial inoculants.

Inoculants commercialized in Brazil for soybean crops are formulated with strains of the species *Bradyrhizobium japonicum* (SEMIA 5079/CPAC 15), *B. elkanii* (SEMIA 587 and SEMIA 5019/ BR 29), and *B. diazofficiens* (SEMIA 5080/ CPAC 7). These inoculant strains completely meet the nitrogen demand of the soybean crop (Franco, 2009). However, the whole inoculation process may be compromised if inoculant products do not meet quality control standards. Therefore, establishing parameters that define the quality of inoculants is paramount for product standardization (Castroux et al., 2001; Herridge et al., 2002; Brasil, 2011).

Genetic stability and integrity, one of the characteristics of *Bradyrhizobium*, are among the characteristics of an ideal inoculant. However, several studies have reported variations in the morphological, physiological, genetic, and symbiotic characteristics of *Bradyrhizobium* isolated from soils inoculated with commercial inoculants, and they attribute these variations to genetic adaptation to native microorganisms and recombination processes with them (Ferreira and Hungria, 2002; Galli-Terasawa et al., 2003; Barcellos et al., 2007; Batista et al., 2007; Giongo et al., 2008). However, inoculant strains of *B. japonicum* or *B. elkanii*, cultured under laboratory conditions, also produced colonies with distinct morphology and genotypes (Sylvester-Bradley et al., 1988; Basit et al., 1991; Kober et al., 2004), and they can change the symbiotic relationships with the host plant (Fuhrmann, 1990; Melchiorre et al., 2011; Torres et al., 2012). According to the intensity of this variation, rhizobia may lose nodulation ability or become less efficient in N_2 fixation. Greenhouse experiments revealed that variability in the phenotypic characteristics of *B. japonicum* or *B. elkanii* strains of commercial inoculants affected the competitiveness of nodule formation, nodulation, and the efficiency of nitrogen fixation in soybean (Kober et al., 2004). Therefore, this is an important factor to consider in selection and approval of strains to be used as inoculants, as well as in quality control of the inoculants.

Inoculant quality control programs consider the viable cell count and the presence of contaminants as the main evaluation criteria (Brasil, 2011; Herrmann and Lesueur, 2013), and cultural and genetic characterization of the strains of the inoculants assist in description and analysis of the products. The hypothesis of this work is that the commercial inoculants present strains with distinct morphological characteristics in relation to the strains indicated in the labels of the products and these strains can alter the symbiotic relationships. Thus, the objectives of this study were to morphologically and genetically characterize the colonies with characteristics distinct from those of *B. japonicum*, *B. elkanii*, and *B. diazofficiens* strains present in Brazilian commercial inoculants for soybean, and to evaluate the symbiotic efficiency of isolates and soybean products in a greenhouse.

MATERIALS AND METHODS

Origin and growing conditions of isolates

Eleven commercial soybean inoculants were analyzed for the presence of bacterial colonies with different cultural characteristics. The criteria of absorption of Congo red dye and other cultural characteristics were used to differentiate the isolates. Two commercial products (products E

and D) had colonies with distinct cultural characteristics. One of the products had *B. japonicum* (SEMIA 5079/CPAC 15) and *B. diazofficiens* (SEMIA 5080/CPAC 7) in its composition, and the other had *B. elkanii* (SEMIA 587/BR 96) and *B. japonicum* (SEMIA 5079/CPAC15).

Table 1 shows the origin and characteristics of the inoculant products containing distinct strains. A total of 12 isolates were selected from inoculant samples at dilutions equal to or greater than 10^{-6}, since the presence of unspecified microorganisms up to 10^{-5} dilution in commercial inoculants is authorized by Normative Instruction No. 13 (Brasil, 2011).

Cultural characterization

Isolates were characterized in culture medium 79 (Fred and Waksman, 1928), known as YMA (Vicent, 1970), with pH 6.8. Solutions of bromothymol blue (0.5 %) or Congo red (0.25 %) dyes were added to the culture medium for evaluation of change in pH and for detection of contaminating bacteria, respectively.

Colonies were evaluated in relation to the diameter, color, production, and consistency of exopolyssacharide, time of appearance of the first isolated colonies, alkaline/acid/neutral reaction, and absorption of Congo red dye over 15 days of cultivation in a greenhouse at 28 °C. Daily analyses were performed from the time of appearance of the first isolated colonies. To confirm the morphological characteristics of the original colony, isolates were cultured five times in succession on plates with medium 79.

The strains used in Brazil as commercial inoculants for soybean, *B. japonicum* (SEMIA 5079/CPAC 15), *B. diazofficiens* (SEMIA 5080/CPAC 7), and *B. elkanii* (SEMIA 587/BR 96 and SEMIA 5019/BR 29), approved by the Brazilian Ministry of Agriculture (MAPA), were also included for cultural characterization to verify their similarity with the strains analyzed in the present study. These strains belong to the laboratory of Biology, Microbiology, and Biological Soil Processes collection of the *Universidade Federal de Lavras*.

Identification of isolates by 16S rRNA partial gene sequencing

The 12 isolates with distinct cultural characteristics were subjected to 16S rRNA partial gene sequencing. The DNA was extracted using the alkaline lysis method, according to Niemann et al. (1997), from cells grown for five days in solid medium at 28 °C.

Table 1. Origin of the isolates with cultural characteristics different from those of the strains listed in commercial soybean inoculant products

Isolate	Inoculant product	Product composition[1]	Type of inoculant	Viable cells in the inoculant[2]	Dilution[3]
				cells per g or mL	
UFLA 06-51	Product D	SEMIA 587 and 5079	Liquid	8.8×10^8	10^{-6}
UFLA 06-52	Product D	SEMIA 587 and 5079	Liquid	8.8×10^8	10^{-6}
UFLA 06-55	Product D	SEMIA 587 and 5079	Liquid	8.8×10^8	10^{-7}
UFLA 06-57	Product D	SEMIA 587 and 5079	Liquid	8.8×10^8	10^{-7}
UFLA 06-58	Product D	SEMIA 587 and 5079	Liquid	8.8×10^8	10^{-6}
UFLA 06-48	Product E	SEMIA 5079 and 5080	Peat	1.2×10^9	10^{-8}
UFLA 06-49	Product E	SEMIA 5079 and 5080	Peat	1.2×10^9	10^{-8}
UFLA 06-50	Product E	SEMIA 5079 and 5080	Peat	1.2×10^9	10^{-7}
UFLA 06-53	Product E	SEMIA 5079 and 5080	Peat	1.2×10^9	10^{-6}
UFLA 06-54	Product E	SEMIA 5079 and 5080	Peat	1.2×10^9	10^{-7}
UFLA 06-56	Product E	SEMIA 5079 and 5080	Peat	1.3×10^9	10^{-8}
UFLA 06-59	Product E	SEMIA 5079 and 5080	Peat	1.2×10^9	10^{-8}

[1] Inoculant strains indicated in the product formulation. [2] Number of viable bacterial cells in the product. [3] Dilution of the inoculant sample in which the isolate was obtained.

Partial amplification of the 16S rRNA gene was performed with a final volume of the PCR reaction of 50 µL containing 5 µL DNA, 5 µL deoxynucleoside triphosphates (dNTP) (2 mmol L^{-1}), 5 µL PCR buffer (10 ×), 4 µL $MgCl_2$ (2.5 mmol L^{-1}), 1 µL of each of the primers (10 mmol L^{-1}) - 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane, 1991) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991), 0.4 µL Taq DNA polymerase (5U $µL^{-1}$), and sterile ultrapure water. The reaction was carried out using the Eppendorf Mastercycler thermal cycler (Eppendorf AG, Hamburg, Germany) with the following cycles: initial denaturation (94 °C for 5 min), 35 denaturation cycles (94 °C for 40 s), annealing (55 °C for 40 s), extension (72 °C for 1.5 min), and final extension (72 °C for 7 min). Amplification of the PCR product and its concentrations was verified by electrophoresis in 1 % agarose gel, and then it was stained with SYBR Green (Invitrogen). A molecular marker (SmartLadder-Eurogentec) was included to estimate the lengths of the amplified products. Subsequently, PCR products were sent to the laboratory of Macrogen Inc. (Macrogen Inc., Seoul, Korea) for purification and sequencing.

The quality of the sequences was verified using the Bionumerics 7.1 (Applied Maths, Sint-Martens-Latem) and the sequences were subjected to BLAST for comparison with the sequences of GenBank (National Center for Biotechnology Information). All the sequences analyzed in the experiment had a number of base pairs greater than or equal to 227.

The sequences determined in this study were deposited in GenBank under the accession numbers KT943949 to KT943960.

Genetic diversity by BOX-PCR

The genetic diversity of 11 of the 12 isolates in the present study was evaluated by BOX-PCR analysis, together with the reference strains of *B. elkanii* (SEMIA 587 and SEMIA 5019), *B. japonicum* (SEMIA 5079), and *B. diazofficiens* (SEMIA 5080), in addition to type strains USDA 6^T (*B. japonicum*) and USDA 76^T (*B. elkanii*). Only the isolate UFLA 06-52 was not evaluated at this stage since the band profile was not amplified when performing BOX-PCR analysis.

For DNA extraction, isolates were cultured in medium 79 (pH 6.8) for five days. Subsequently, colonies isolated from strains were placed in microtubes containing 1 mL of sterile ultrapure water. Samples were homogenized and heated at 95 °C for 10 min. The tubes were transferred to an ice box, so that cells would be lysed with the heat shock, finalizing the DNA extraction process. Genetic diversity was evaluated by the BOX-PCR method, as described in Guimarães et al. (2012), where 0.4 µL of bovine serum albumin (BSA) and 5 µL of DNA were used, which is an adaptation of the original procedure that used 0.2 µL of BSA and 1 µL of DNA (Versalovic et al., 1994; Rademaker et al., 1998). Amplified fragments were separated by electrophoresis at 90 V on 1.5 % agarose gel in 0.5X TAE buffer for 15 hours at room temperature. The 1 kb Plus DNA Ladder (Invitrogen™) marker was used. Finally, the gel was stained with ethidium bromide and photographed.

The genetic diversity of the strains was analyzed by the presence or absence of polymorphic bands in the gel. Data were clustered using the UPGMA (unweighted pair group mean arithmetic) algorithm and Jaccard's coefficient in the BioNumerics 7.1 software (Aplied Maths, Sint-Martens-Latem).

Isolates authentication and N_2 fixation

The 12 isolates were evaluated for their ability to nodulate and fix N_2 in soybean. The experiments were carried out in a greenhouse for 30 days, from October to November 2012, under controlled conditions of temperature, and humidity. The experiment was conducted in a completely randomized design with three replications in sterile and recyclable long neck glass bottles (500 mL), using filter paper as a support for the

development of plant roots (Florentino et al., 2009). Bottles were coated with foil and filled with Hoagland and Arnon solution (1950) diluted four times.

Isolates were compared with four positive controls separately inoculated with the reference strains *Bradyrhizobium elkanii* (SEMIA 5019 or SEMIA 587), *B. japonicum* (SEMIA 5079), and *B. diazofficiens* (SEMIA 5080) and two negative controls without inoculation, with high or low mineral N concentration. A solution with 5.25 mg L^{-1} of N was used in the inoculated treatments and in the control treatment without inoculation with low mineral N concentration. In contrast, a solution with 52.5 mg L^{-1} of N was used in the control without inoculation with high mineral N concentration. In addition to the controls, samples of commercial inoculants (products D and E) were used to determine if the presence of the isolate in the product changes its efficiency.

The soybean cultivar used was COODETEC 5G 830 RR. Seeds were previously disinfected with 70 % alcohol for 30 seconds, transferred to 2 % sodium hypochlorite solution for 2 min, and washed six times in sterile distilled water. Afterwards, seeds were pre-germinated in a Petri dish containing sterilized filter paper and moistened cotton, and incubated at 28 °C for two days. Each treatment received a seedling, and inoculated treatments received 1.0 mL of inoculum. The inoculum was composed of the bacterial isolate grown in liquid medium and contained approximately 10^9 cells per mL, or aliquots of products D and E. In the case of the peat inoculant (product E), the peat was diluted 10 times in sterile distilled water and shaken for 30 seconds in a vortex. Afterwards, 1.0 mL of the solution was inoculated on the seedling. At 30 days after inoculation, plants were harvested, and the following parameters were analyzed: number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), shoot dry matter (SDM), total dry matter (TDM), and efficiency in relation to the control with mineral N (ECN). The relative efficiency of each treatment was calculated by the formula: ECN = (inoculated SDM/SDM with N) × 100.

Symbiotic efficiency in Leonard jars

From the results obtained on nodulation and N_2 fixation in glass bottles, the 12 isolates were evaluated for symbiotic efficiency in soybean in Leonard jars (Vicent, 1970). The experiment was carried out in a greenhouse for 55 days (January to March 2014) in a completely randomized design with three replications. The plant species used and the germination conditions of the experiment were the same as previously described. After germination, seedlings were transplanted into sterilized Leonard jars, with a mixture of sand and vermiculite (1:1) in the upper part, supplemented with sterilized nutrient solution (Hoagland and Arnon, 1950) diluted four times in the lower part. Each jar received four seedlings, which were inoculated with 1.0 mL of inoculum per plant. Plants were thinned at five days after planting, leaving two plants per jar.

Positive and negative controls were used as described in the authentication experiment. A solution with 5.25 mg L^{-1} of N was used for the inoculated treatments and for the control treatment without inoculation with low N concentration. In contrast, a complete solution of 52.5 mg L^{-1} of N was used for the control without inoculation with high N concentration. During the experiment, the contents of the jars were periodically completed with autoclaved nutrient solution, according to the uptake rate of the plants. Plants were harvested at the beginning of flowering, and the number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), shoot dry matter (SDM), and relative efficiency (ECN) were determined.

Statistical analysis

Data from the experiments for authentication of N_2 symbiotic efficiency were subjected to analysis of variance (Anova), respecting the premises of normal distribution of residues and homoscedasticity, using the Sisvar 4.3 software (Ferreira, 2011). Treatment means were evaluated by the Scott-Knott test at 5 % significance.

RESULTS

Cultural characterization

After five successive cultures in medium 79, the isolates maintained the main characteristics of the *Bradyrhizobium* strains indicated on the inoculant labels. All isolates had slow growth (5-7 days), white colonies in culture medium 79 with bromothymol blue, gummy consistency of exopolyssacharide, and alkaline pH of the medium, which are typical characteristics of bacteria of the genus *Bradyrhizobium* (Table 2).

However, among the isolates, all colonies were smaller (diameter <2.5 mm) than the inoculant strains described on the product labels (diameter >2.5 mm), and three isolates of the D package had colonies different from the inoculant strains described on the product label (circular). The exopolyssacharide yield of four isolates from package D and of three isolates from package E was little or scarce, unlike the inoculant strains described on the product label that produce moderate and abundant exopolyssacharide. All isolates and inoculant strains absorbed Congo red dye (colonies with different shades of pink color), except for the isolate UFLA 06-57, which exhibited white colonies under these culture conditions (Figure 1).

Identification of isolates by 16S rRNA partial gene sequencing

Analysis of the 16S rRNA partial gene sequences showed that among the 12 isolates, nine showed high similarity with *Bradyrhizobium japonicum*, two with *Bradyrhizobium* sp., and one with *B. elkanii* (Table 3). The similarity between the isolates and the sequences deposited in GenBank was 99 or 100 %. The isolates that had sequences 100 % similar to those of inoculant strains, but with distinct phenotypic characteristics,

Table 2. Cultural characterization of inoculant strains and of variant colonies isolated from commercial soybean inoculants in solid medium, with bromothymol blue or Congo red dye

Isolate	Days[1]	Diameter[2]	pH[3]	Form[4]	Consist. EPS[5]	EPS production[6]	Color[7]	Dye Absorption [8]	
								7 days	8-10 days
		mm							
UFLA 06-48	7	1.5 - 2.0	Alk	C	G	M	W	W	P
UFLA 06-49	7	2.0 - 2.5	Alk	C	G	M	W	P	P
UFLA 06-50	5	2.0 - 2.5	Alk	C	G	L	W	W	P
UFLA 06-51	6	<1.5	Alk	P	G	L	W	W	P
UFLA 06-52	5	<1.5	Alk	P	G	L	W	P	P
UFLA 06-53	5	2.0-2.5	Alk	C	G	L	W	W	P
UFLA 06-54	5	2.0-2.5	Alk	C	G	M	W	P	P
UFLA 06-55	5	1.5-2.0	Alk	P	G	S	W	P	P
UFLA 06-56	5	1.5-2.0	Alk	C	G	L	W	W	P
UFLA 06-57	6	2.0-2.5	Alk	C	G	L	W	W	P
UFLA 06-58	5	2.0-2.5	Alk	C	G	A	W	W	P
UFLA 06-59	5	2.0-2.5	Alk	C	G	M	W	W	W
SEMIA 587	5	>2.5	Alk	C	A	A	W	W	P
SEMIA 5019	5	>2.5	Alk	I	A	A	W	W	P
SEMIA 5079	5	>2.5	Alk	C	G	A	W	W	P
SEMIA 5080	5	>2.5	Alk	C	G	M	W	W	P

[1] Time for emergence of the colonies isolated. [2] Colony diameter evaluated at the time of growth of colonies isolated. [3] Reaction of culture medium 79 with bromothymol blue after colony growth: alkaline (Alk). [4] Colony form: punctiform (P), circular (C), and irregular (I). [5] Consistency of exopolyssacharide: gummy (G) and aqueous (A). [6] Exopolyssacharide production: scarce (S), little (L), moderate (M), and abundant (A). [7] Color of colony in medium 79 with bromothymol blue: white (W). [8] Color of colony in medium 79 with Congo red dye at 7 days of cultivation and from 8 to 10 days of cultivation (>7 days): white (W) and pink (P).

were denominated “variants”. Isolates that did not have sequences 100 % similar to the inoculant strains and with distinct phenotypic characteristics were denominated “exotic” rhizobia. Of the isolates from product D, only UFLA 06-57 had sequences similar to those of the strain approved as an inoculant in Brazil (SEMIA 5079) and indicated on the product label. Isolates UFLA 06-51 and UFLA 06-55 were identified as *Bradyrhizobium* sp. and showed high similarity to UFLA 06-27 (*Bradyrhizobium* sp.), isolated from an area with a history of soybean cultivation and of use of a commercial inoculant (Ribeiro et al., 2015). The UFLA 06-52 showed high similarity with the strain *B. elkanii* (USDA 76^{T}). The UFLA 06-58 was similar to the strain identified as *B. japonicum* (KG2-80), isolated from a soil sample collected in Japan using soybean as a trap plant (Shiina et al., 2014). The isolates from product E had sequences very similar to those of the inoculant strain SEMIA 5079, indicated on the product label, and are considered cultural variants of *B. japonicum*. The absence of isolates similar to the strain SEMIA 5080 (*B. diazoefficiens*) may have occurred due to high similarity between these two species (*B. japonicum* e *B. diazoefficiens*) based on 16S gene sequencing, being recently separated (Delamuta et al., 2013).

Genetic diversity by the BOX-PCR analysis

The 16S rRNA gene sequencing alone did not allow genetic discrimination of the 11 *Bradyrhizobium*. Therefore, the BOX-PCR technique was used.

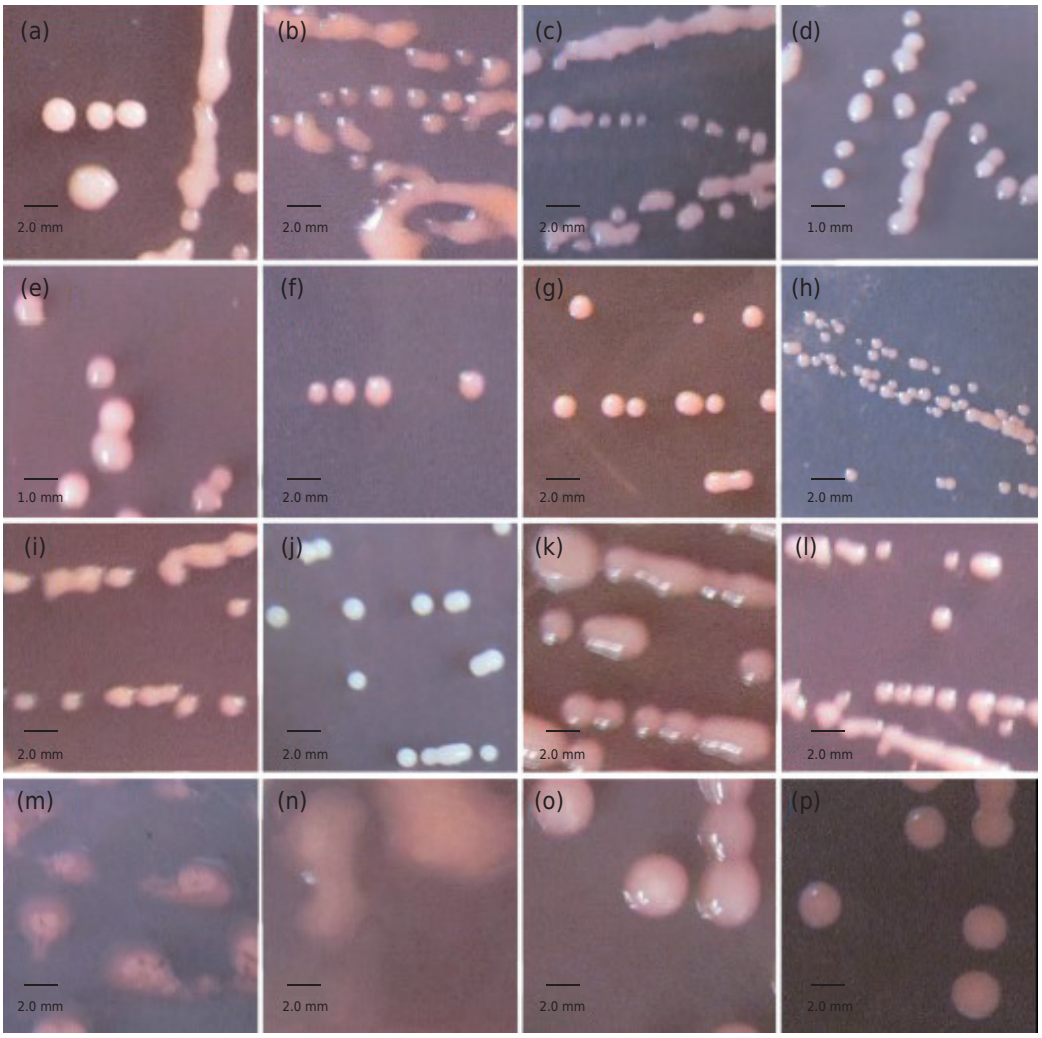

Figure 1. Morphological characteristics of the colonies in medium 79 with Congo red dye after 8 days of cultivation. UFLA 06-48 (a), UFLA 06-49 (b), UFLA 06-50 (c), UFLA 06-51 (d), UFLA 06-52 (e), UFLA 06-53 (f), UFLA 06-54 (g), UFLA 06-55 (h), UFLA 06-56 (i), UFLA 06-57 (j), UFLA 06-58 (k), UFLA 06-59 (l), SEMIA 5019 (m), SEMIA 587 (n), SEMIA 5079 (o), and SEMIA 5080 (p).

Table 3. Identification by 16S rRNA partial gene sequencing of variant strains isolated from commercial soybean inoculants

Isolate	Inoculant product	Inoculant composition	Length (bp) of 16S rRNA sequences	Most similar sequences found in GenBank (NCBI)		
				Species	Similarity	Accession number
					%	
UFLA 06-51	Product D	SEMIA 587 and 5079	706	*Bradyrhizobium sp.* - UFLA06-27	99	KJ739912
UFLA 06-52	Product D	SEMIA 587 and 5079	881	*B. elkanii* - USDA 76^T	100	NR_036953
UFLA 06-55	Product D	SEMIA 587 and 5079	808	*Bradyrhizobium sp.* - UFLA06-27	100	KJ739912
UFLA 06-57	Product D	SEMIA 587 and 5079	591	*B. japonicum* - SEMIA 5079	100	CP007569
UFLA 06-58	Product D	SEMIA 587 and 5079	419	*B. japonicum* - KG2-80	100	AB984344
UFLA 06-48	Product E	SEMIA 5079 and 5080	794	*B. japonicum* - SEMIA 5079	100	CP007569
UFLA 06-49	Product E	SEMIA 5079 and 5080	867	*B. japonicum* - SEMIA 5079	100	CP007569
UFLA 06-50	Product E	SEMIA 5079 and 5080	867	*B. japonicum* - SEMIA 5079	100	CP007569
UFLA 06-53	Product E	SEMIA 5079 and 5080	784	*B. japonicum* - SEMIA 5079	100	CP007569
UFLA 06-54	Product E	SEMIA 5079 and 5080	817	*B. japonicum* - SEMIA 5079	100	AF234888
UFLA 06-56	Product E	SEMIA 5079 and 5080	850	*B. japonicum* - SEMIA 5079	100	CP007569
UFLA 06-59	Product E	SEMIA 5079 and 5080	227	*B. japonicum* - SEMIA 5079	100	CP007569

After clustering the profiles formed by the BOX-PCR analysis, nine genotypes were observed, considering 70 % of similarity (Figure 2). One genotypic group containing six isolates (UFLA 06-59, UFLA 06-54, UFLA 06-48, UFLA 06-56, UFLA 06-50, and UFLA 06-53, belonging to product E); one with three isolates (UFLA 06-58 and UFLA 06-57, belonging to product D, and UFLA 06-49, belonging to product E); and two with only one isolate each (UFLA 06-55 and UFLA 06-51, belonging to product D) were observed. Reference strains SEMIA 587 and SEMIA 5019 were clustered together, while strains SEMIA 5079, SEMIA 5080, USDA 6^T, and USDA 76T were isolated from each other.

Authentication of isolates and N_2 fixation

In the authentication and N_2 fixation experiment, no nodulation was observed in the controls without inoculation and with low or high mineral N concentration, indicating ideal conditions of asepsis in conducting the experiment. Positive controls inoculated with *B. elkanii* (SEMIA 587 and 5019), *B. japonicum* (SEMIA 5079), and *B. diazofficiens* (SEMIA 5080), as well as the inoculant products D and E, efficiently nodulated soybean, manifesting that the culture conditions were favorable to the occurrence of nodulation and N_2 fixation.

All the isolates nodulated the soybean and these data are presented in table 4. Products D and E, as well as seven isolates tested, exhibited number of nodules (NN) similar or superior to that of the inoculant strains (SEMIA 587, SEMIA 5079, and SEMIA 5080). For NDM, the values observed for products D and E were similar to those observed for strains SEMIA 5019 and SEMIA 5079, followed by strains SEMIA 587 and SEMIA 5080, which had values similar to those of eight isolates tested in the study. Seven isolates (five variant isolates and two exotic isolates) had mean values of SDM, TDM, and ECN similar to at least three inoculant strains, and these means were lower than those of products D and E, and those of the control with N mineral. Thus, all isolates and inoculant strains were less efficient than the control with mineral N and the inoculant products D and E. This indicates that the presence of isolates with low symbiotic efficiency in the inoculant products did not interfere with the efficiency of the product under the conditions tested in this study.

For root dry matter (RDM), the isolate UFLA 06-59 was similar to the control with mineral N, exhibiting the highest means. Nine isolates were similar to the inoculant strains and to products D and E. The exotic isolate UFLA 06-52 was the closest to the inoculant strains and to products D and E in the variables analyzed in this study.

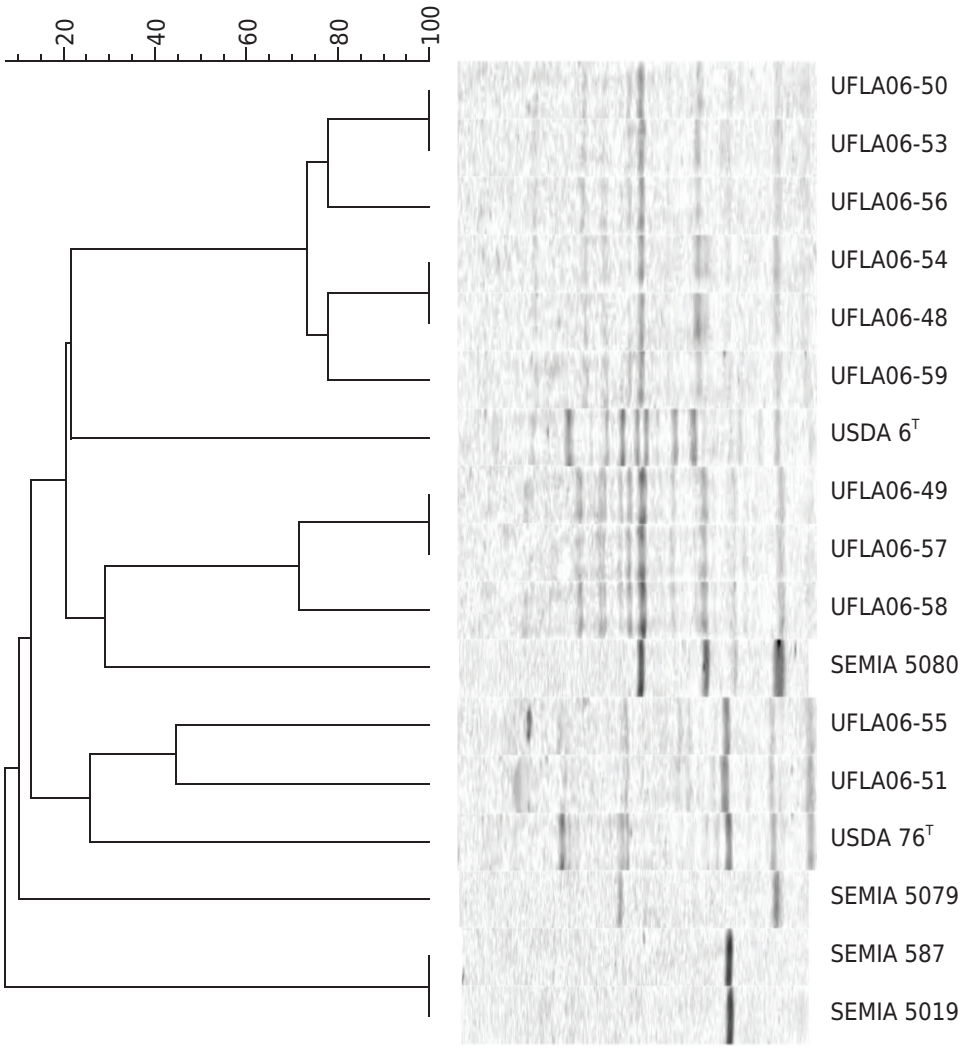

Figure 2. Dendrogram obtained by comparison of profiles obtained by the BOX-PCR technique of the isolates of commercial inoculants, of the type strains of these species, and of the strains approved as inoculants to soybean.

Symbiotic efficiency in Leonard Jars

Symbiotic efficiency data in soybean in the previous experiment in long neck bottles with nutrient solution showed that, despite having nodulation capacity, 41 % of the isolates were poorly efficient in biological N_2 fixation (SDM, TDM, and ECN) compared to the inoculant strains, to products D and E, and to the control with mineral N. The isolates were also evaluated in Leonard jars (Table 5) to verify if the cultivation time and substrate volume influenced symbiotic efficiency. As in the previous experiment, nodulation was not observed in the controls without inoculation and with low or high mineral N concentration, indicating ideal conditions of asepsis in the experiments. Positive controls inoculated with *B. elkanii* (SEMIA 587 and 5019), *B. japonicum* (SEMIA 5079), and *B. diazofficiens* (SEMIA 5080) were efficient in nodulation and in N_2 fixation, demonstrating that the culture conditions were favorable for symbiosis.

Among the isolates studied, three isolates had NN similar to the strain SEMIA 587. These isolates as well as SEMIA 587 had NN lower than the other inoculant strains. For NDM, only the exotic isolate UFLA 06-52 had results similar to those of three inoculant strains, and results inferior to those of SEMIA 5019. Although they stimulated the production of nodules in soybean root, NDM values for the variant isolates UFLA 06-59 and UFLA 06-48 were very low, similar to those of the controls without inoculation. For the variables SDM

Table 4. Number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), and relative efficiency (ECN) of soybean plants cultivated in glass bottles with different treatments: strains isolated from inoculants, inoculant products D and E, two uninoculated controls, one with high mineral N concentration (52.5 mg L^{-1}) and one with low mineral N concentration (5.25 mg L^{-1}), and four controls inoculated with strains approved as soybean inoculants: *B. japonicum* (SEMIA 5079), *B. diazofficiens* (SEMIA 5080), and *B. elkanii* (SEMIA 587 and SEMIA 5019)

Treatment	NN	NDM	SDM	RDM	TDM	ECN
		g per plant				%
UFLA 06-48	6.60 f	0.033 d	0.463 g	0.120 d	0.586 g	35.99 g
UFLA 06-49	18.30 e	0.060 b	0.440 g	0.173 c	0.673 f	34.40 g
UFLA 06-50	16.30 e	0.040 c	0.610 e	0.196 c	0.850 d	47.42 e
UFLA 06-51	5.30 f	0.020 d	0.176 i	0.093 d	0.293 i	13.60 i
UFLA 06-52	43.30 c	0.070 b	0.713 c	0.236 b	1.020 c	55.40 c
UFLA 06-53	39.30 c	0.043 c	0.616 e	0.200 c	0.860 d	47.94 e
UFLA 06-54	55.60 b	0.040 c	0.653 d	0.193 c	0.883 d	50.73 d
UFLA 06-55	49.60 b	0.043 c	0.560 f	0.176 c	0.783 e	43.64 f
UFLA 06-56	35.00 d	0.096 e	0.650 d	0.190 c	0.910 d	50.66 d
UFLA 06-57	28.60 d	0.073 b	0.656 d	0.206 c	0.926 d	51.08 d
UFLA 06-58	56.33 b	0.060 b	0.650 d	0.186 c	0.896 d	50.63 d
UFLA 06-59	2.66 f	0.006 d	0.440 g	0.126 a	0.573 g	34. 00 g
Product D	41.60 c	0.076 a	0.770 b	0.276 b	1.120 b	59.86 b
Product E	41.00 c	0.083 a	0.803 b	0.270 b	1.156 b	62.45 b
SEMIA 587	41.30 c	0.066 b	0.713 c	0.243 b	1.040 b	56.91 c
SEMIA 5019	65.30 a	0.076 a	0.723 c	0.216 c	1.016 c	56.23 c
SEMIA 5079	29.30 d	0.086 a	0.666 d	0.253 b	1.006 c	51.83 d
SEMIA 5080	35.30 d	0.050 c	0.613 e	0.233 b	0.896 d	47.68 e
52.5 mg L^{-1} de N	0.00 f	0 d	1.283 a	0.413 a	1.700 a	100.00 a
5.25 mg L^{-1} de N	0.00 f	0 d	0.353 h	0.143 d	0.496 h	27.40 h
CV (%)[(1)]	13.77	19.91	4.78	10.24	4.90	4.36

Means of 3 replications. Means followed by the same letters in the columns do not differ from each other by the Scott-Knott test at 5 % probability. [(1)] Coefficient of variation.

and ECN, the exotic isolate UFLA 06-52 was similar to the inoculant strain SEMIA 5079 and inferior to the others. This same isolate was similar to at least three inoculant strains for RDM and TDM. The other isolates tested, except for UFLA 06-51, showed means similar or inferior to the mean of the control without inoculation and without mineral N.

DISCUSSION

Selection of rhizobia strains with high agricultural potential requires a series of experimental measures for their approval as commercial inoculant products. Although several countries have specific legislation for recommendation, production, and commercialization of inoculants, the low quality of these products may compromise inoculation technology (Herrmann and Lesueur, 2013). Variations in the morphological and genetic characteristics of the reference strains used in the inoculants not only hinder evaluation of the product, but can also interfere with the symbiotic processes of nodulation and N_2 fixation efficiency in the host plant (Bloem et al., 2002; McInnes et al., 2005).

In the present study, isolates with cultural characteristics different from those of the inoculant strains from the same product were observed. The cultivation of these isolates in medium 79 with Congo red dye, used to differentiate colonies of *Bradyrhizobium* from contaminants (Brasil, 2011), allowed visualization of *Bradyrhizobium* colonies with different

Table 5. Number of nodules (NN), nodule dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), and relative efficiency (ECN) of soybean plants cultivated in Leonard pots with different treatments: strains isolated from inoculant products isolated from commercial inoculants, two uninoculated controls, one with high mineral N concentration (52.5 mg L^{-1}) and one with low mineral N concentration (5.25 mg L^{-1}), and four control inoculants with strains approved as soybean inoculants: *B. japonicum* (SEMIA 5079), *B. diazofficiens* (SEMIA 5080), and *B. elkanii* (SEMIA 587 and SEMIA 5019)

Treatment	NN	NDM	SDM	RDM	TDM	ECN
		g per plant				%
UFLA 06-48	10.1 f	0.013 g	1.746 g	0.666 c	2.423 e	24.01 h
UFLA 06-49	120.0 d	0.113 e	1.773 g	0.743 c	2.633 e	24.40 h
UFLA 06-50	65.3 e	0.066 f	1.980 g	0.836 c	2.876 e	27.20 h
UFLA 06-51	98.0 d	0.250 c	2.596 e	0.800 c	3.646 d	35.66 f
UFLA 06-52	144.0 c	0.400 b	3.773 c	1.226 b	5.433 b	52.32 d
UFLA 06-53	118.3 d	0.183 d	2.303 f	0.856 c	3.343 e	31.69 g
UFLA 06-54	116.6 d	0.101 e	2.166 f	0.883 c	3.150 e	29.80 g
UFLA 06-55	138.6 c	0.083 e	1.860 g	0.810 c	2.753 e	25.58 h
UFLA 06-56	102.0 d	0.096 e	1.883 g	0.750 c	2.726 e	25.87 h
UFLA 06-57	142.3 c	0.170 d	2.163 f	0.816 c	3.146 e	29.73 g
UFLA 06-58	70.3 e	0.056 f	1.800 g	0.716 c	2.573 e	24.73 h
UFLA 06-59	9.6 f	0.01 g	2.040 f	0.803 c	2.846 e	28.06 g
SEMIA 587	118.0 c	0.396 b	4.160 b	1.113 b	5.666 b	57.200 c
SEMIA 5019	178.3 b	0.563 a	4.480 b	1.016 b	5.833 b	62.96 b
SEMIA 5079	202.0 a	0.373 b	3.946 c	1.0566 b	5.376 b	54.25 d
SEMIA 5080	169.3 b	0.350 b	3.133 d	0.946 b	4.470 c	43.83 e
52.5 mg L^{-1} de N	0.0 f	0 g	7.273 a	2.323 a	9.600 a	100.00 a
5.25 mg L^{-1} de N	0.0 f	0 g	2.260 f	0.803 c	3.060 e	31.09 g
CV (%)[(1)]	14.38	20.19	7.23	13.23	9.46	4.54

[(1)] CV: coefficient of variation.Means of 3 replications. Means followed by the same letters in the columns do not differ from each other by the Scott-Knott test at 5 % probability.

shades of pink. This result corroborates other studies (Bloem et al., 2002; Botha et al., 2004; Loureiro et al., 2007), and this variation is associated with interaction of the dye with structures on the surface of the rhizobia cell, which may be weak, with capsular polysaccharides (CPS) (pink - orange colony); strong, with cellulose fibrils (red colonies); and absent, with exopolysaccharides (EPS) (Zevenhuizen et al., 1986). The color of *Bradyrhizobium* colonies possibly varied because of the increase in the concentration of CPS during colony growth. For this bacterial group, CPS forms an adherent and cohesive layer on the cell surface, which facilitates the infection processes in the root of the host plant (Laus et al., 2005).

Isolates of product D were identified as *Bradyrhizobium* sp., *B. japonicum*, or *B. elkanii*, and only one isolate (UFLA 06-57) showed a sequence similar to that of the inoculant strain indicated on the product label (*B. japonicum* SEMIA 5079). Isolates of product E showed the same taxonomic identification as one of the inoculant strains included in the composition of the product (*B. japonicum* SEMIA 5079), indicating that the colonies suffered dimorphism. For these variant isolates, colonies were characterized as small or medium (diameter between 1.5 - 2.5 mm), with low to medium mucus production, very different from reference strains with big colonies (diameter >2.5 mm), with viscous aspect and high exopolysaccharide production. Colony dimorphism in *Bradyrhizobium* was also observed by other authors. Sylvester-Bradley et al. (1988) observed two distinct morphological groups, with aqueous or dry colonies, from the original *Bradyrhizobium* cultures. In the study of Torres et al. (2012), large modifications were observed in the

DNA profile between the parent strains *B. japonicum* and their soybean nodule variant isolates, under controlled experimental conditions.

Cultural and genetic variations in *Bradyrhizobium* populations under controlled culture conditions were evident. The cultural characterization combined with 16S rRNA gene sequencing and the BOX-PCR technique revealed variations in the strains of this genus.

Exotic rhizobia isolates of product D showed nodulation and N_2 fixation in symbiosis with soybean. In the N_2 authentication and fixation experiments, only the exotic isolate UFLA 06-51 exhibited lower nodulation than the strains and inoculants evaluated in this study. However, no isolates had efficiency results similar to those of the inoculant products. Nevertheless, the presence of exotic rhizobia did not compromise the efficiency of the inoculant product D (product of origin of isolates UFLA 06-51, UFLA 06-52, UFLA 06-55, UFLA 06-57, and UFLA 06-58), which had results of NN, NDM, RDM, SDM, TDM, and ECN similar to or higher than the results of the inoculant strains. Among the isolates derived from product D, only the isolate UFLA 06-52 had symbiotic efficiency results similar to those of the inoculant strains for both experiments. According to comparison of the 16S rRNA gene sequences, this isolate was not similar to the inoculant strains described on the product label.

For the variant isolates of *B. japonicum* from product E, cultural modifications did not alter nodulation capacity with soybean. In both experiments, all isolates induced nodule formation in the host plant, indicating that nodulation characteristics remained stable. Despite the cultural variations in the colonies, these isolates were able to infect and form nodules in soybean roots. McInnes et al. (2005) observed that, for variants of *Sinorhizobium meliloti* isolated from commercial inoculants in Australia, the presence of dry colonies or large mucus production did not interfere with nodulation of the host plant when grown under greenhouse conditions. Although they did not alter nodulation, differences in cultural characteristics may have influenced biological N_2 fixation with the host plant since the variant isolates, in general, showed less efficiency in relation to the inoculant strains. However, this did not alter the quality of product E, which had higher means than the variant isolates under the conditions tested.

Differences in cultural characteristics may have influenced biological N_2 fixation with the host plant, since the variant or exotic isolates, in general, showed less efficiency in relation to the inoculant strains and, in some cases, also inferior to that of the control with low mineral N concentration, which can be explained by some detrimental effect on plant growth. No conclusive evidence has been reported in which morphological variation in the *Bradyrhizobium* colony interfered with the symbiotic characteristics of the variant isolates. Other authors (Fuhrmann, 1990; Basit et al., 1991; Kober et al., 2004; Carvalho et al., 2005) observed that variations in the cultural characteristics of *Bradyrhizobium* influenced symbiosis with soybean; however, no pattern in the morphology of the colonies explained such influence. Nevertheless, none of these studies were performed with strains in inoculants. The presence of variant isolates is an important factor to consider in quality control programs and in maintenance of bacterial cultures in microbial collections, and this should be periodically monitored not only under these collections, but also in inoculants.

CONCLUSIONS

Commercial inoculants formulated with *Bradyrhizobium* were able to present rhizobia strains with morphological and symbiotic characteristics distinct from the strains indicated in the product. From the analyses of 16S rRNA partial gene sequencing and of the BOX-PCR analyses of the strains studied, it was possible to differentiate the variant isolates genetically from the strains present in the inoculants. The high genetic similarity of the variant isolates with strains of *B. japonicum* and *B. elkanii* from other regions (countries) may indicate that the commercial inoculants studied had strains not listed in their composition.

The efficiency in biological N_2 fixation of each independent isolate (variant or not) was, in general, lower than those of the commercial inoculants and of the strains *B. elkanii* (SEMIA 587 e 5019), *B. japonicum* (SEMIA 5079), and *B. diazofficiens* (SEMIA 5080). However, the presence of these variants in the commercial inoculant products analyzed in the present study did not, however, affect their symbiotic efficiency with the host plant. Results also show that cultural characterization can be used as a prior analysis in the differentiation of rhizobia strains different from those indicated in the inoculant product.

The information presented here may assist agencies involved with surveillance and standardization of commercial soybean inoculants in evaluating the quality of the product.

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