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Symbiotic efficiency and genetic diversity of soybean bradyrhizobia in Brazilian soils

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ABSTRACT

The symbiotic N_2-fixing genus *Bradyrhizobium* includes 29 species distributed throughout different geographic regions. Only five species have recently been described based on isolates from tropical soils, three species from Brazil (*B. manausense*, *B. ingae*, *B. neotropicale*) and two species from Peru (*B. paxllaere e B. icense*), although tropical region is considered to be the origin of legume rhizobia symbiosis. Besides, some authors suggested that *Bradyrhizobium* was introduced in Brazil with first soybeans inoculants from USA. In this work, 46 *Bradyrhizobium* strains were isolated from soils collected in different regions of Brazil (Midwest, Northeast, Southeast, and South), using soybean as a trap plant. These strains were characterized genetically by analyzing the 16S rRNA gene and five housekeeping genes (*atpD, gyrB, dnaK, recA*, and *rpoB*). They were also characterized in terms of their symbiotic efficiency with soybean plants grown under axenic conditions in Leonard jars. The phylogenetic analysis of housekeeping genes revealed the possible presence of novel species in the Northeast and Southeast soils, some of which exhibited high symbiotic efficiency with soybean plants. These results emphasize the great diversity among native strains belonging to *Bradyrhizobium* genus in Brazilian soils as well as potential ones to be used as inoculants. They also indicate that symbiotically efficient native bradyrhizobia occur in Brazilian soils and are independent of strains introduced as soybean inoculant.

1. Introduction

The genus *Bradyrhizobium* encompasses N_2-fixing bacteria that can live symbiotically with legumes or associated with non-legumes. In Brazil, efficient symbiotic N_2-fixing strains were already selected and are available to be used as inoculants for soybean and several other forest, forage, and green manure legume species (Moreira and Siqueira, 2006). Soybean is an important commodity in Brazil, USA and Argentina, which are the largest producers in the world. Inoculation with N_2-fixing *Bradyrhizobium* strains is a common practice that contributes largely to decreasing the production costs of this crop by replacing mineral N-fertilizers. This success arose from the successful breeding of this crop for symbiotic N_2-fixation. However, some authors attributed the origin of selected inoculant strains to the first inoculants introduced in Brazil from USA, which lead some authors to consider the absence of *Bradyrhizobium* strains in Brazilian soils and or horizontal transfer of symbiotic genes (Lopes et al., 1976; Martínez-Romero and Caballero-Mellado, 1996; Ferreira and Hungria 2002; Torres et al., 2012).

The genus *Bradyrhizobium* includes slow growing bacteria that alkalinize culture medium containing mannitol as a carbon source (Jordan, 1982). Currently, there are 29 described *Bradyrhizobium* species: *B. japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1992), *B. liaoningense* (Xu et al., 1995), *B. yuanmingense* (Yao et al., 2002), *B. betae* (Rivas et al., 2004), *B. canariense* (Vinuesa et al., 2005a), *B. denitrificans* (Van Berkum et al., 2006), *B. pachyrhizi* and *B. jicamae* (Ramírez-Bahena et al., 2009), *B. iriomotense* (Islam et al., 2008), *B. cytisi* (Chahboune et al., 2011), *B. lablabi* (Chang et al., 2011), *B. daqingense* (Wang et al., 2012), *B. huanghuaihaiense* (Zhang et al., 2012), *B. oligotrophicum* (Ramírez-Bahena et al., 2012), *B. rifense* (Chahboune et al., 2012), *B. arachidis* (Wang et al., 2013), *B. retamae* (Guerrouj et al., 2013), *B. diazoefficiens* (Delamuta et al., 2013), *B. ganzhouense*, (Lu et al., 2014), *B. paxllaeri* and *B.*

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icense (Durán et al., 2014a,b), *B. manausense* (Silva et al., 2014a), *B. ingae* (Silva et al., 2014b), *B. valentinum* (Durán et al., 2014a,b), *B. neotropicale* (Zilli et al., 2014), *B. ottawaense* (Yu et al., 2014), *B. erythrophlei* and *B. ferriligni* (Yao et al., 2015). The number of described novel species has increased in the past two years along with the use of appropriate molecular techniques, such as housekeeping gene analysis (Willems et al., 2001; Martens et al., 2008; Rivas et al., 2009), as well as the study of new host plants and unexplored geographic regions.

However, these species were described mainly based on strains from temperate regions, although diverse *Bradyrhizobium* strains have been isolated from various species in tropical ecosystems (Moreira et al., 1998, 1993; Lima et al., 2005, 2009; Doignon-Bourcier et al., 2000; Guimarães et al., 2012; Jaramillo et al., 2013). These authors applied phenotypic (SDS-PAGE of total proteins) and genotypic methods (AFLP, IGS PCR-RFLP and Rep-PCR) that showed also high interspecific variability. In tropical regions, only recently the analysis of housekeeping genes sequences has been applied, allowing for the reliable proposal of new species (Silva et al., 2014a, b; Zilli et al., 2014).

The high degree of conservation of the 16S rRNA gene sequence indicates low diversity within the genus *Bradyrhizobium*. A better resolution at the species level has been achieved by the analysis of housekeeping genes (Stepkowski et al., 2005; Menna et al., 2009; Rivas et al., 2009; Delamuta et al., 2012), which have been widely employed in bacterial taxonomic classification studies.

Considering the importance of the genus *Bradyrhizobium* as a source of genetically stable strains to be used as inoculants for soybeans and for many other species, bioprospection studies are carried out to map its occurrence and to evaluate its diversity in Brazilian soils. The present study aimed to analyze the symbiotic and genotypic diversity of 46 *Bradyrhizobium* bacterial strains, which were isolated from soils of different Brazilian regions, by performing nodulation and symbiotic efficiency tests in soybean plants (*Glycine max*), as well as a phylogenetic analysis of 16S rRNA and housekeeping genes (*atpD*, *dnaK*, *gyrB*, *recA*, and *rpoB*).

2. Materials and methods

2.1. Origin of soil samples for analysis

Soil samples were collected from different regions of Brazil: Midwest (Dourados, Mato Grosso do Sul state-MS), Northeast (Bom Jesus, Piauí state-PI), Southeast (Ijaci, Minas Gerais state-MG), and South (Campos Novos, Santa Catarina state-SC) (Fig. 1). Origin, geographic coordinates, altitude, soil classification, chemical and physical characteristics of samples are presented in the Table 1. All sampled areas have a history of inoculated soybean cropping, for at least five years.

The soil used in every experiment came from three composite samples, which consisted of five sub-samples each, collected from within the 0–20 cm depth layer. The samples were stored in sterilized plastic bags after collection and kept refrigerated (4 °C) until use. The experiments with soil samples collected from the different states were carried out in 2011, in the months of March (Ijaci-MG), May (Bom Jesus-PI and Dourados-MS), and October (Campos Novos-SC).

2.2. Isolation of bacterial strains using soybean as the trap plant

Bacterial strains were captured over four experiments conducted in a greenhouse at the Soil Biology, Microbiology and Biological Processes Laboratory of the Department of Soil Science, Federal University of Lavras.

The experiments were carried out in recyclable, sterilized long-neck bottles (500 mL) using filter paper as a support for plant root

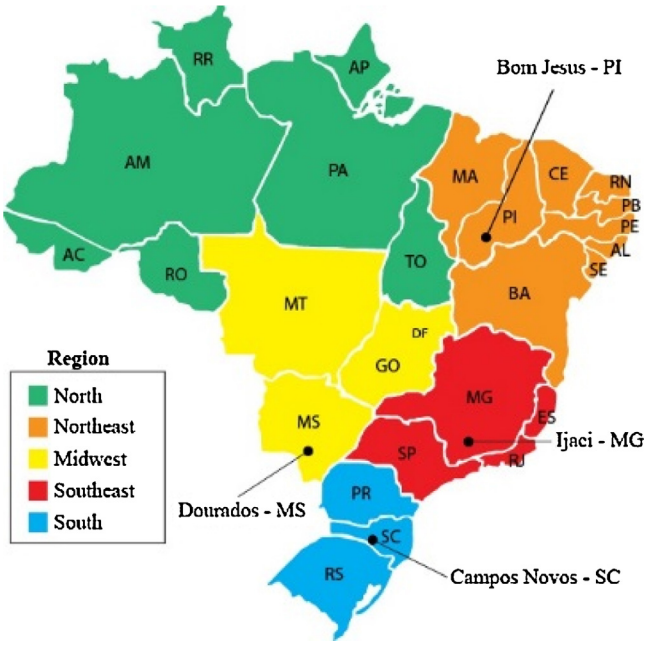

Fig. 1. Map showing the sites where the soil samples used in this study were collected: Midwest (Dourados-MS), Northeast (Bom Jesus-PI), Southeast (Ijaci-MG), and South (Campos Novos-SC).

development (Florentino et al., 2009). The bottles were covered in aluminum foil and filled with a 4-fold dilution of Hoagland and Arnon (1950) solution. Hoagland and Arnon (1950) solution with a low nitrogen concentration (5.25 mg L^{-1}, considered as a start-up dose for nitrogen fixation) was used in the inoculated treatments and the uninoculated control. In the uninoculated control with high nitrogen concentration, Hoagland and Arnon (1950) complete solution containing 52.5 mg L^{-1} of nitrogen was used.

Soybean was used as a trap plant. The cultivars used were Monsoy 7980 (Bom Jesus-PI), CD 235 RR (Dourados-MS), Favorita RR (Ijaci-MG, and Campos Novos-SC). The soybean seed surfaces were disinfected with 70% alcohol for 30 s, immersed in 2–3% sodium hypochlorite solution for 2 min, and rinsed six times with sterilized distilled water. The seeds were then germinated in a Petri dish containing filter paper and moistened sterile cotton and incubated at 28 °C for 2 days.

The soil samples were then subjected to a series of serial dilutions. The procedure was carried out by suspending 10 g of soil in 90 mL of sterile saline solution (0.85% NaCl), stirring for 30 min at 125 rpm (10^{-1} dilution), and conducting six successive steps of adding a 1.0 mL aliquot to a tube containing 9.0 mL of saline solution (dilutions from 10^{-2} to 10^{-7}).

The seedlings were transferred to the bottles containing the nutrient solution and inoculated with 1 mL of each soil sample serial dilution (three replicates per dilution). *B. elkanii* strain Br 29 (SEMIA 5019) approved as a soybean inoculant by the Ministry of Agriculture, Livestock and Supply (Brazil) was used as a positive control; two uninoculated negative controls were also used, one with low and one with high mineral N content (5.25 mg L^{-1} and 52.5 mg L^{-1}, respectively). The control with low mineral N content was used to test for possible contamination, while the control with high mineral N content was used to determine whether the conditions were adequate for plant growth.

After 35 days of growth, the presence or absence of nodules was evaluated to estimate the most probable number (MPN) of nodule forming nitrogen fixing bacteria in legumes (NNFBL), which were then isolated.