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# Genomic identification and characterization of the elite strains *Bradyrhizobium yuanmingense* BR 3267 and *Bradyrhizobium pachyrhizi* BR 3262 recommended for cowpea inoculation in Brazil

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ABSTRACT

The leguminous inoculation with nodule-inducing bacteria that perform biological nitrogen fixation is a good example of an "eco-friendly agricultural practice". *Bradyrhizobium* strains BR 3267 and BR 3262 are recommended for cowpea (*Vigna unguiculata*) inoculation in Brazil and showed remarkable responses; nevertheless neither strain was characterized at species level, which is our goal in the present work using a polyphasic approach. The strains presented the typical phenotype of *Bradyrhizobium* with a slow growth and a white colony on yeast extract-mannitol medium. Strain BR 3267 was more versatile in its use of carbon sources compared to BR 3262. The fatty acid composition of BR 3267 was similar to the type strain of *Bradyrhizobium yuanmingense*; while BR 3262 was similar to *Bradyrhizobium elkanii* and *Bradyrhizobium pachyrhizi*. Phylogenetic analyses based on 16S rRNA and three housekeeping genes placed both strains within the genus *Bradyrhizobium*: strain BR 3267 was closest to *B. yuanmingense* and BR 3262 to *B. pachyrhizi*. Genome average nucleotide identity and DNA–DNA reassociation confirmed the genomic identification of *B. yuanmingense* BR 3267 and *B. pachyrhizi* BR 3262. The *nod*C and *nif*H gene analyses showed that strains BR 3267 and BR 3262 hold divergent symbiotic genes. In summary, the results indicate that cowpea can establish effective symbiosis with divergent bradyrhizobia isolated from Brazilian soils.



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## Introduction

Bacteria collectively known as rhizobia form an important part of the soil microbiota and perform biological nitrogen fixation (BNF) through nitrogenase activity when in symbiosis with leguminous plants. This ecological phenomenon has great biotechnological impact on biomass and grain production. For example, in the soybean crop production in Brazil it is estimated to save over US$ 10 billion annually by the use of *Bradyrhizobium* inoculation instead of chemical fertilization.[1]

The Brazilian Ministry of Agriculture has a list of rhizobial strains recommended for more than 50 leguminous grain-producing crops, forage and green manure.[2] In recent years efforts have been made to better characterize these strains recommended for inoculation in Brazil and at least five new species have been described.[3–6]

Brazil is the world's third leading cowpea producer, with an estimated production of 500,000 tons per year.[7] The crop yield varies from 400 to 2000 kg $ha^{-1}$, depending on the system and the region of cultivation.[7] This yield has been rising in recent years with improvements in cultivation management, such as the inoculation of seeds with nitrogen-fixing bacteria, a practice applied to approximately 100,000 hectares.

The strains BR 3267 and BR 3262 are considered as "elite" for inoculation of cowpea plants in Brazil[2] and several studies under controlled and field conditions have shown that both strains make significant contributions to crop yields, including more than 50% N accumulation via BNF.[8] BR 3267 strain was isolated from the semiarid northeastern region of the country, using cowpea as trap plants, while BR 3262 strain was isolated from an Atlantic Forest area in southeastern Brazil using the same strategy.[1,9] Although these strains were isolated more than a decade ago, they were only partially characterized through assessment of the growth rate, colony morphology and 16S rRNA phylogeny,[11] but not classified at the species level.

Zilli and colleagues[11] have characterized the 16S rRNA genes of the BR 3267 and BR 3262 strains and concluded that both are members of the genus *Bradyrhizobium*. This genus was created in the early 1980s to accommodate root nodule-inducing bacteria with slow growth on media containing mannitol and yeast extract.[12] Since the turn of the century, two major subgroup divisions (I and II) have been recognized within this genus based on DNA–DNA hybridization.[12–14] The *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* were the first species assigned to subgroup I and II, respectively.[15,16] According to Zilli and colleagues,[10] BR 3267 clustered within the subgroup *B. japonicum* and BR 3262 within the subgroup *B. elkanii*. However, in the light of current knowledge, those results can be considered inconclusive because new *Bradyrhizobium* species was recently described. Thus, further investigation employing the latest molecular techniques is needed for the correct positioning of these strains.

In the past five years, the use of housekeeping genes as powerful phylogenetic markers for bacteria has led to the description of over 15 new species within the genus *Bradyrhizobium*[17] and enabled the separation of genetically close strains into different species. Examples are the definition of the species *Bradyrhizobium diazoefficiens* based on *B. japonicum* and *Bradyrhizobium pachyrhizi* from *B. elkanii*.[3,18] Furthermore, new methods for genome-to genome comparison, like Average Nucleotide Identity (ANI) and Genome Blast Distance Phylogeny (GBDP) have been introduced and are contributing to improve bacterial taxonomy.[19,20]

Therefore, the goal of our study was to characterize at a finer the taxonomic level both BR 3267 and BR 3262 strains using a polyphasic approach.

## Materials and methods

### Strains used

The cowpea strains BR 3267 and BR 3262, and the type strain *B. elkanii* USDA $76^T$ were obtained from the Johanna Döbereiner Biological Resource Center (CRB-JD, Embrapa Agrobiologia, Seropédica-Rio de Janeiro, Brazil). The type strains *B. pachyrhizi* PAC $48^T$ (=LMG $24246^T$) and *Bradyrhizobium yuanmingense* CCBAU $10071^T$ (=LMG 21827) were obtained from the LMG Culture Collection (Belgium). The strains were grown on yeast extract-mannitol agar medium (YMA) and were incubated at 28 °C[21] for seven days until they reached sufficient colony growth levels to observe morphological features and purity.

### Phenotypic and physiologic characterizations

Inoculum preparation for both BR 3267 and BR 3262 strains was carried out using YMA medium at 28 °C. Carbon source utilization was assessed with Biolog GN2 microplates (Biolog Inc., Hayward, CA) following the manufacturer's instructions, except that cell concentration was adjusted to 5 on the McFarland scale. Plates were incubated in the dark at 28 °C for ten days. The biochemical features were assessed using API 20 NE strips (bioMérieux, Marcy-L'Etoile, France) following a standard protocol that uses a saline solution (0.85% NaCl) for bacterial suspension. Additionally, the tolerance to abiotic stress, such as temperature, pH and salinity (NaCl), was determined by examining the growth in YMA medium. The temperature tolerance was evaluated at 15, 20, 25, 28, 30, 32 and 37 °C, and the pH tolerance was tested in a range from 4 to 10. The salinity tolerance was examined at 28 °C in YMA medium supplemented with 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 or 2.5% (w/v) of NaCl. The resistance to antibiotics was determined using YMA medium and the disk diffusion method for ampicillin (25 μg), chloramphenicol (50 μg), erythromycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (10 μg), penicillin (10 μg), streptomycin (10 μg) and tetracycline (30 μg). All tests were run in triplicate.

### Fatty acid composition

The BR 3267 and BR 3262 and type strains *B. elkanii* USDA $76^T$, *B. pachyrhizi* PAC $48^T$ and *B. yuanmingense* CCBAU $10071^T$ type strains were characterized based on whole-cell fatty acids, derived using the methyl esters' method (FAME) and

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