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Full Length Research Paper

Low diversity diazotrophic of culturable *Burkholderia* species associated with sorghum

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Sorghum is an important crop around the World. *Burkholderia* genus has emerged as an important plant associated bacteria in the last years. However, it is important to understand these processes and how they took place in the environment. In this study, we evaluated the occurrence of *Burkholderia* species associated with five varieties of sorghum cultivated in different places of Mexico. *Burkholderia* species were isolated using a selective medium for diazotrophic species and identified using a semi-selective set of primers and clustered by restriction analysis of 16S rRNA sequence. It was found a low geographical distribution of species *Burkholderia tropica* and *Burkholderia vietnamiensis*. Due to the low diversity of diazotrophic *Burkholderia* found, colonization and competition assays were performed in the rhizosphere of sorghum plants. By colonization assays we inoculated populations of 1 × 0^8 cfu/ml of *B. tropica*, *Burkholderia unamae*, *Burkholderia silvatantlica* and *Burkholderia xenovorans*. We showed that all strains could colonize the rhizosphere. In competition assays, *B. unamae* and *B. tropica* did not show any antagonistic effect among them, but we showed that both species colonized the rhizosphere with the same number of bacteria in sorghum plants. The present study confirms the low geographic distribution of diazotrophic *B. tropica* and *B. vietnamiensis* associated with sorghum field-grown in México.

Key words: β-rhizobia, *Burkholderia*, sorghum, nitrogen fixation.

INTRODUCTION

The genus *Burkholderia* includes over 60 described species which are widely distributed in the natural environment over different places and conditions (Coenye and Vandamme, 2003). For example, some species of *Burkholderia* are found in nosocomials environments, especially species belonging to the *B. cepacia* complex (Bcc), isolated from patients with cystic fibrosis disease (Mahenthiralingam et al., 2005). Nevertheless, most of the *Burkholderia* species are known as soil-borne bacteria, many exhibit non-pathogenic interactions with plants (Compant et al., 2008) and some are plant endophytes association (Caballero-Mellado et al., 2004; Reis et al., 2004).

Historically, the ability of fixing N_2 in bacteria of the genus *Burkholderia* was identified only in *Burkholderia vietnamiensis* (Gillis et al., 1995), a member of the Bcc (Mahenthiralingam et al., 2005). Nevertheless, analysis of important crop plants grown under field conditions revealed the richness of the genus *Burkholderia* in unknown diazotrophs (Estrada-de los Santos et al., 2001). At present 9 diazotrophic plant-associated *Burkholderia* species have been properly described, such as *Burkholderia unamae* (Caballero-Mellado et al., 2004), *Burkholderia xenovorans* (Goris et al., 2004), *Burkholderia tropica* (Reis et al., 2004), and *Burkholderia*

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silvatlantica (Perin et al., 2006a). These species are capable to colonize the rhizosphere and/or the endophytic environment of a wide range of taxonomically unrelated host plants such as maize, sorghum, pineapple, and coffee (Caballero-Mellado et al., 2004, 2007; Gillis et al., 1995; Perin et al., 2006a, b; Reis et al., 2004). Recently, analysis of tomato plants field-grown revealed a high diversity of well-known and unknown diazotrophic *Burkholderia* species (Caballero-Mellado et al., 2007). In addition, two legume nodulating N_2-fixing strains were recently classified as *Burkholderia phymatum* and *B. tuberum* (Vandamme et al., 2002). Other species like *Burkholderia mimosarum* and *Burkholderia nodosa* are N_2-fixing and legume nodulating bacteria, have been also recently described as novel species (Chen et al., 2007, 2006). Currently, un-named strains with the ability to form nodules on *Macroptilium atropurpureum*, *Mimosa* species and other mimosoid legumes probably represent novel *Burkholderia* species (Barrett and Parker, 2005, 2006).

Burkholderia strains are considered as promising candidates for biotechnological applications (Chiarini et al., 2006; O'Sullivan and Mahenthiralingam, 2005), unfortunately, most of them belong to Bcc species involved in human infections, hindering potential applications. On the other hand, all of the diazotrophic *Burkholderia*, excluding *B. vietnamiensis*, comprise a group of closely related species that are phylogenetically distant from the Bcc species (Caballero-Mellado et al., 2007). In addition, transmissibility factors such as *cblA* and *esmR* genes, identified among clinical and environmental isolates of opportunistic pathogens of *B. cenocepacia* and other Bcc species, have not been detected thus far in plant-associated diazotrophic *Burkholderia* species (Perin et al., 2006a).

MATERIALS AND METHODS

Sorgum plants obtaninig

Bacteria were isolated from fields of: Guanajuato, México (101° W, 20° N), which is an important sorghum-producing state and Morelos, México (99° W, 18.5° N) which has a small production of sorghum. The samples of rhizosphere/ rhizoplane of 21 sorghum (*Sorghum vulgare*) plants (var. D65, D72 and Ambar) field-grown in Yecapixtla and Cuautla, Morelos, México, were analyzed 4 h after collection, and 15 plants (var. D65, D66 and Pioner 8641) cultivated in Irapuato, Guanajuato, Mexico, were analyzed 48 h after collection.

Isolation of strains and culture conditions

From each location, five sorghum plants of each variety were randomly collected with a distance of 10 m between plants. Samples of rhizosphere-rhizoplane, as well as surface-sterilized roots and stems of sorghum were analyzed for recovery of diazotrophic *Burkholderia* isolates as described previously (Perin et al., 2006a). Briefly, for diazotrophic *Burkholderia* isolates, sorghum roots were excised from plants and loosely adhering soil was removed; Afterward, a portion of root was cut into small pieces (1 cm) and five grams of each root sample was vortexed at 3000 rpm for 3 min in 10 mM $MgSO_4 \cdot 7H_2O$ (Mgsol) containing 0.01% (v/v) Tween 20. This suspension was considered to contain bacteria from the rhizosphere-rhizoplane (Rh-Rz), and the solution was serially diluted and used for the bacterial isolation; 100 µl aliquots were placed in vials containing 5 ml of N-free semisolid BAz medium, used as an enrichment culture of N_2-fixing *Burkholderia* (Estrada-de los Santos et al., 2001), and incubated at 29°C for four to five days. Vials showing a fine subsurface pellicle were transferred to fresh semi-solid BAz medium, and after incubation for 4 to 5 days growth was transferred once more to fresh BAz medium, and new growth streaked out on semi-selective BAc agar plates (Estrada-de los Santos et al., 2001). Colonies with different morphology were purified and assayed for nitrogenase activity by the acetylene reduction activity (ARA) method (Burris, 1972). ARA positive colonies were maintained in 20% glycerol at 80°C prior to characterization.

PCR-amplification of *nifH* genes

Primers IGK (Poly et al., 2001) and NDR-1 (Valdés et al., 2005) were used for the amplification of the nitrogenase reductase *nifH* gene using the PCR conditions described previously (Perin et al., 2006a). The reaction amplified a 1.2-kb fragment comprising the complete *nifH* gene, the intergenic spacer region, and the 5' end of the *nifD* gene (Valdés et al., 2005).

Clustering of Burkholderia isolates using amplified 16S rDNA restriction analysis (ARDRA)

fD1 and rD1 primers were used for the amplification of the rRNA gene (Weisburg et al., 1991) with the PCR conditions described previously (Estrada-de los Santos et al., 2001). The PCR-amplified 16S rRNA genes (ca 1.5 kb) were restricted with *Alu* I, *Dde* I, *Hae* III, *Hha* I, *Hinf* I, *Msp* I, and *Rsa* I. The restriction fragments were separated by electrophoresis in 3% agarose gels, and the restriction patterns were compared. Each isolate was assigned to an amplified 16S rRNA gene restriction analysis (ARDRA) genotype, defined by the combination of the restriction patterns obtained with the seven restriction endonucleases (Estrada-de los Santos et al., 2001).

PCR-identification of isolates with 16S rRNA species-specific primers

ARA positive isolates were subjected directly to identification using species-specific primers and conditions described (Wong et al., 2009); PCR-amplified products were sequenced at the Biotechnology Institute, UNAM (México).

16S rRNA gene sequencing and phylogenetic analysis

Representative ARA positive isolate, were identify with primers fD1 and rD1 were used for amplifying the 16S rRNA gene (ca. 1.5 kb) (Weisburg et al., 1991) using the PCR conditions previously described. To obtain 16S rRNA sequences, PCR products were cloned as described previously (Perin et al., 2006a), and the gene sequences were determined at the Biotechnology Institute, UNAM (Mexico). The 16S rRNA gene sequences were deposited in the EMBL/GenBank database. These sequences were compared with previously published 16S rRNA from *Burkholderia* species and the closely related genus *Pandoraea*. The multiple alignments of the

Table 1. Isolation of N_2 fixing *Burkholderia* species associated with Sorghum varieties.

Crop and locality	Variety	Species	No. of isolates	Source
Cuatla, Morelos	D-65	*B. tropica*	5	Rhizosphere
			3	Rhizoplane
Yecapixtla, Morelos	D-65	*B. tropica*	6	Rhizosphere
			3	Rhizoplane
		B. vietnamiensis	1	Rhizosphere
Yecapixtla, Morelos	Ambar	*B. tropica*	5	Rhizosphere
			4	Rhizoplane
Cuatla, Morelos	D-72	*B. tropica*	4	Rhizosphere
			2	Rhizoplane
Irapuato, Guanajuato	D-65	*B. tropica*	5	Rhizosphere
			1	Rhizoplane
			3	Roots
Irapuato, Guanajuato	D-66	*B. tropica*	5	Rhizosphere
			4	Rhizoplane
			2	Roots
		B. vietnamiensis	1	Rhizosphere
Irapuato, Guanajuato	Pioner 8641	*B. tropica*	5	Rhizosphere
			1	Rhizoplane

Burkholderia species were isolated from the rhizosphere and rhizoplane of sorghum varieties collected in the regions of Cuatla, Morelos and Irapuato, Guanajuato, Mexico.

sequences were performed with CLUSTAL W software (Thompson et al., 1994). The tree topology was inferred by the neighbor-joining method (Saitou and Nei, 1987), based on 1348 DNA sites, and distance matrices were performed according to Jukes and Cantor (1969) using the program MEGA version 2.1 (Kumar et al., 2001).

Colonization of sorghum roots by diazotrophic *Burkholderia*

Sorghum var. D65 seeds were surface sterilized in 25% commercial bleach solution (Clorox; 6% sodium hypochlorite) for 20 min, and then washed three times with distilled sterile water. Sorghum seeds were germinated on 0.75% water agar plates for 24 h at 29°C; thereafter seeds were sown in pots filled with sterile river sand, which was previously watered with 200 ml of farheus nutritive solution. Germinated seeds were inoculated with 1 ml bacterial suspension (1×10^8 cfu/ml) in 10 mM $MgSO_4.7H_2O$. Sorghum plants were grown under greenhouse conditions for 40 days; and roots were rinsed in sterile water. Serial dilutions were made in 10 mM $MgSO_4.7H_2O$. This suspension was considered to contain bacteria from the rhizosphere-rhizoplane (Rh-Rz), and the solution was serially diluted and used for the bacterial isolation; 100 µl aliquots were placed in BAc agar and incubated at 29°C for three days; Colonies recovered on BAc agar plates from the highest dilutions were purified and identified using species-specific primers.

Essays in competition for colonization of sorghum plants

Six sorghum plants were inoculated with 1 ml of 10^8 cfu/ml *B. tropica* and *B. unamae*. The plants were kept in greenhouse for 21 days, after then viable accounts of the rhizosphere-rhizoplane of plants were made using the methods described (Estrada-de los Santos et al., 2001). Selection of species was made based on the ability of *B. unamae* of growing on phenol as carbon source (Caballero-Mellado et al., 2007).

RESULTS

Diazotrophic *Burkholderia* isolation

Enrichment cultures for N_2-fixing *Burkholderia* were made in N-free semi-solid BAz medium followed by further isolation and colony purification on BAc agar plates (Estrada-de los Santos et al., 2001). Screening of 100 colonies from sorghum plants allowed the recovery of 60 isolates (Table 1), which showed consistent nitrogenase activity as measured by the ARA method and yielded a PCR product of the expected size (1.2-kb) corresponding to the *nifH* gene (data not shown). These results confirmed the nitrogen-fixing ability of the *Burkholderia* isolates. No attempt was made to determine the taxonomic position of ARA negative colonies, and they were excluded from further analysis. 58 of the 60 diazotrophic isolates associated with sorghum plants (96.7%), were identified by ARDRA profiles and yielded

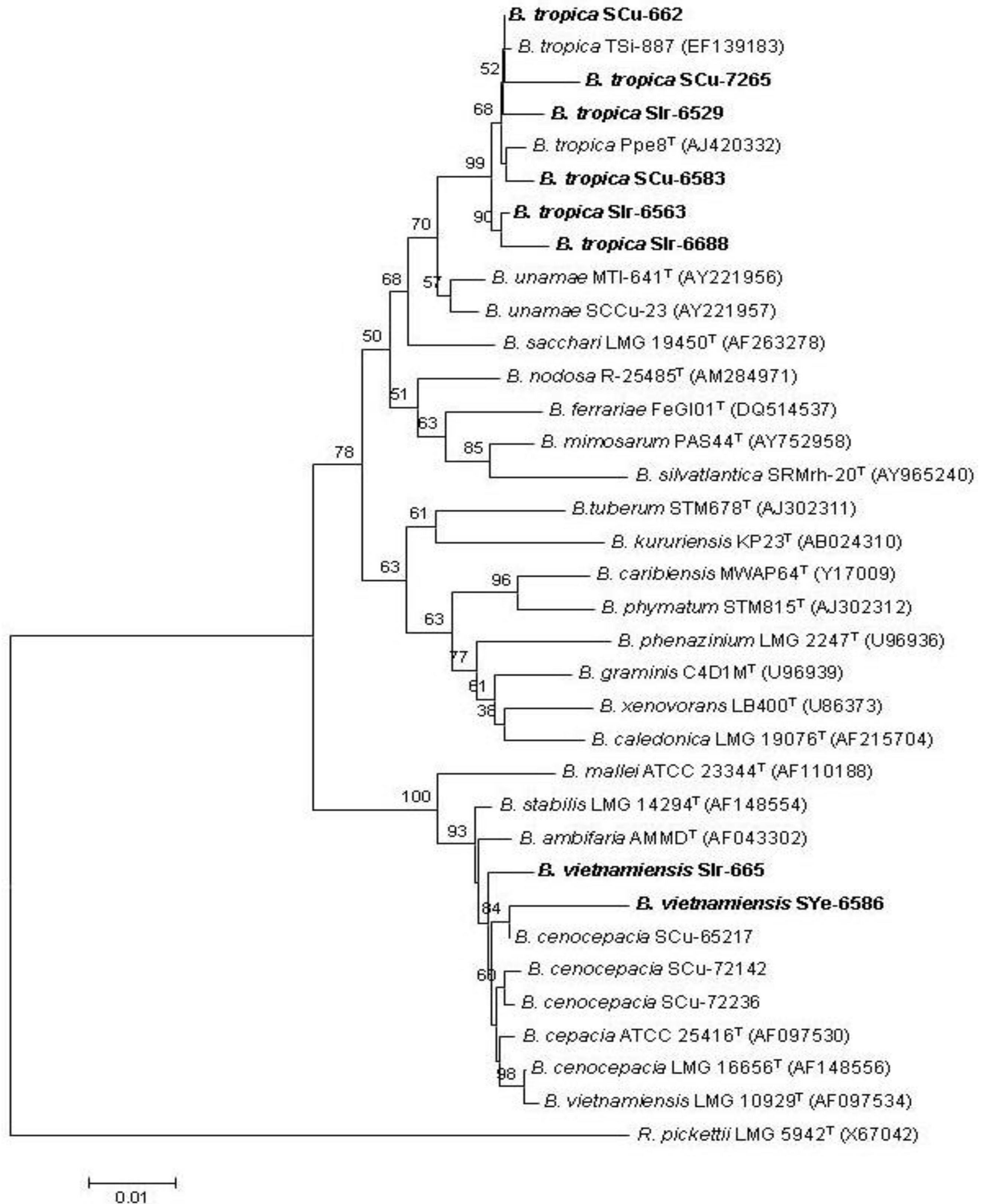

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the relatedness among the N_2-fixing plant-associated *Burkholderia* species. The bar represents 1 nucleotide substitution per 100 nucleotides. Nodal robustness of the tree was assessed using 1000 bootstrap replicates. The NCBI GenBank accession number for each type strain tested is shown in parentheses. Phylogenetic relationship of the 16SrRNA gene sequence of isolates of *Burkholderia vietnamiensis* and *Burkholderia tropica* in sorghum plants.

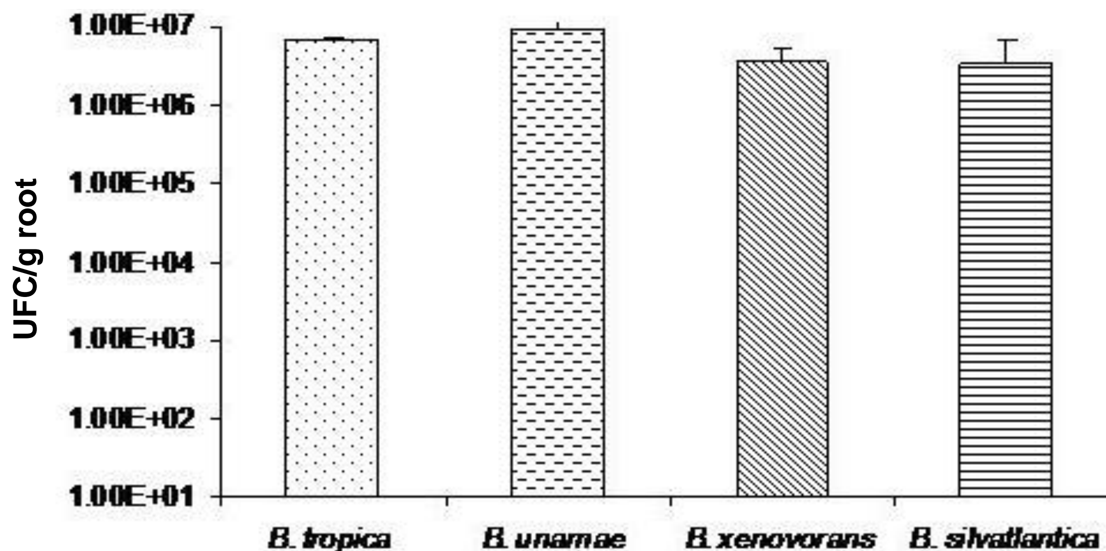

Figure 2. Number of bacteria recovered per gram of root of the rhizosphere, 21 days after inoculation of sorghum plants with the different species: *B. unamae*, *B. tropica*, *B. xenovorans* and *B. silvatlantica*.

the fragment of the expected size for *B. tropica*. Also two more isolates (SCu-665 and SYe-6586) were identified by ARDRA and 16S sequencing as *B. vietnamiensis* (Table 1).

ARDRA analysis

A total of two ARDRA profiles, clearly different were identified from ARA-positive isolates recovered from the rhizosphere-rhizoplane and from surface-sterilized roots of sorghum.

Phylogenetic analysis of 16S rDNA gene sequences

The 16S rRNA gene of six diazotrophic isolates from sorghum plants, identified with ARDRA profiles and species-specific primers as *B. tropica,* were sequenced and then compared with available 16S rRNA sequences from all of the *Burkholderia* species. Analysis of strains SCu-662 (GenBank Acc. No. FJ436048), SIr-6529 (Acc. No. FJ436050), SIr- 6563 (Acc. No. FJ436051), SCu-7265 (Acc. No. FJ436052), SCu-6583 (Acc. No. FJ436053) and SIr-6688, (Acc. No. FJ436054) recovered from sorghum plants showed 99% identity with previously described *B. tropica* strains (for example, acc. nos. EF139182, EF139183 and AY128105) (Reis et al., 2004; Caballero-Mellado et al., 2007). In addition, two diazotrophic isolates SCu-665 (acc. no. FJ436049) and SYe-6586 (Acc. No. FJ436055), recovered from sorghum plants cultivated in two distant geographic regions, matched at 99% identity with *B. vietnamiensis* G4 (CP000614, CP000615 chromosomes 1 and CP000616, chromosomes 2) (Figure 1).

Sorghum plants inoculation with diazotrophic *Burkholderia*

As described aforementioned, we found a predominance of *B. tropica* association with sorghum plants, thus the ability of *B. unamae B. silvatlantica* and *B. xenovorans* to colonize the rhizospheres of the sorghum plants was evaluated. All species were able to colonize with populations greater than 10^7 cfu/g of root (Figure 2).

Assays in competition for colonization of sorghum plants

Competition tests were conducted to determine whether *B. tropica* could cause antagonism on the colonization of *B. unamae* plant sorghum. The results confirmed that both species can coexist on the same plants, as seen in other studies (Perin et al., 2006). The results also show that no antagonistic effects of *B. unamae* against *B. tropica* mediated radical exudates released by roots of sorghum were observed (Figure 3).

DISCUSSION

60 strains of diazotrophic *Burkholderia* from five sorghum varieties cultivated in two different locations in Mexico were isolated. Strains were isolated from the rhizosphere, rhizoplane, and inside the roots of plants. Based on analysis of ARDRA profiles, species-specific primers and

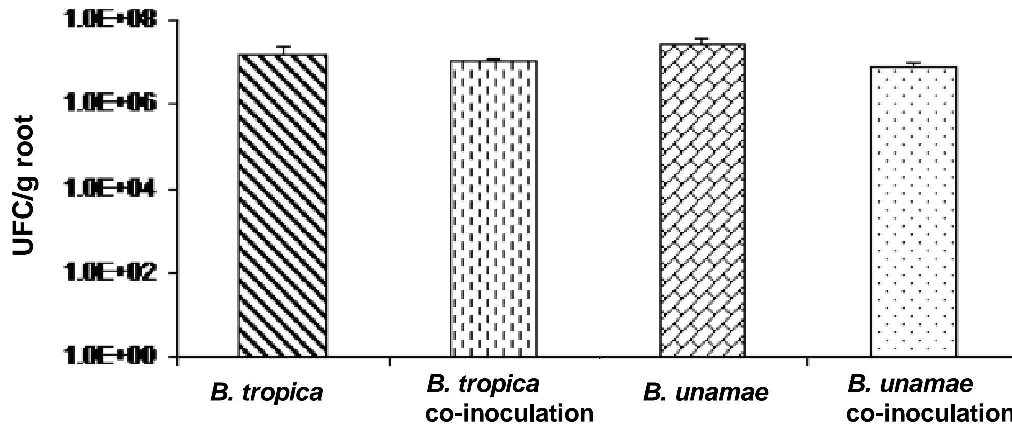

Figure 3. Number of bacteria recovered per gram of root in the rhizosphere of the sorghum plant co-inoculated with *B. tropica* and *B. unamae* 21 days after inoculation

16S rRNA gene sequencing, 58 of 60 strains (96.7%) were identified as *B. tropica* and two strains (3.3%) were identified by ARDRA profiles and 16S gene sequence as *B. vietnamiensis*. 58 strains of *B. tropica* were identified as 16 and 17 genotypes (Reis et al., 2004) (Table 1).

The prevalence of *B. tropica* associated with plants of sorghum in the 2 regions sampling, contrasts with other studies in which it has been observed a great diversity of nitrogen-fixing *Burkholderia* species associated with the cultivation of corn sugarcane (Perin et al., 2006) and tomato (Caballero-Mellado et al., 2007). *B. unamae, B. tropica* and *B. silvatlantica* species are found in rhizospheric and endophytic association with maize and sugarcane (Perin et al., 2006). Tomato plants have been founded in association rhizospheric *B. unamae, B. tropica, B. xenovorans* and 2 other unidentified species, including one closely related to the nitrogen-fixing *B. kururiensis* (Caballero-Mellado et al., 2007). Considering the wide prevalence of isolates of *B. tropica* associated with plants of sorghum from regions up to 400 km apart (from Guanajuato to Morelos, México), it is important to determine factors involved in such predominance. In addition, it could be of interest to evaluate the ability to colonize the sorghum roots by other species of diazotrophic *Burkholderia,* and their possible coexistence with *B. tropica* in the rhizospheric and/or endophytic environment (Perin et al., 2006a; Caballero-Mellado et al., 2007). Nevertheless, independent assays of sorghum inoculation with *B. unamae*, *B. xenovorans* and *B. silvatlantica*, as well as co-inoculation assays of these species with *B.tropica*, revealed that all of these type of species were capable of colonizing sorghum roots in similar numbers (around 1×10^7 CFU/g dry root), of *B. tropica* (Figure 2). Furthermore, co-inoculation of sorghum seeds with *B. tropica* and *B. unamae* colonized the sorghum roots in comparable numbers to *B. tropica* (1×10^7 CFU/g dry root) (Figure 3). These results suggest that sorghum does not exude any compound that promotes rhizosphere/rhizoplane colonization by *B. tropica* or inhibits colonization by other species. These effects have been reported for antagonist strains of *Gluconcacetobacter diazotrophicus* during colonization of sugarcane (Muñoz-Rojas et al., 2005), although it does not seem to be the case between *B. tropica* and *B. unamae*. Agricultural practices such as tillage and fertilization, may be key factors in changing the microbial community of soil (Salles et al., 2006), which could explain the wide prevalence of *B. tropica* in the rhizospheric environment of sorghum.

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