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# Screening of tropically derived, multi-trait plant growth- promoting rhizobacteria and evaluation of corn and soybean colonization ability

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ABSTRACT

The present study assessed the plant growth-promoting (PGP) traits and diversity of culturable rhizobacteria associated with guarana (*Paullinia cupana*), a typical tropical plant. Ninety-six bacteria were isolated, subjected to biochemical tests, and identified by partial or total 16S rDNA sequencing. *Proteobacteria* and *Firmicutes* were the dominant rhizospheric phyla found, and *Burkholderia* and *Bacillus* were the most abundant genera. Thirteen strains exhibited the four PGP traits evaluated, and most of them belonged to the genus *Burkholderia*. Two multi-trait PGP strains, RZ2MS9 (*Bacillus* sp.) and RZ2MS16 (*Burkholderia ambifaria*), expressively promoted corn and soybean growth under greenhouse conditions. Compared to the non-inoculated control, increases in corn root dry weight of 247.8 and 136.9% were obtained with RZ2MS9 and RZ2MS16 inoculation, respectively, at 60 days after seeding. The dry weights of corn and soybean shoots were significantly higher than those of non-inoculated plants, showing increases of more than 47% for both strains and crops. However, soybean root dry weight did not increased after bacterial inoculation with either strain. The colonization behavior of RZ2MS16 was assessed using GFP-labeling combined with fluorescence microscopy and a cultivation-based approach for quantification. RZ2MS16:*gfp* was able to colonize the roots and shoots of corn and soybean, revealing an endophytic behavior.

## 1. Introduction

Global demand for agricultural crops is increasing and may continue to do so for decades (Tilman et al., 2011). However, the impacts and limits of fertilizer and insecticide applications have raised public concerns about the sustainability and security of the food supply (Liu et al., 2015; Pingali, 2012), providing motivation for identifying alternatives to chemical fertilizers, such as bio-fertilizers. Bio-fertilizers, or microbial inoculants, are living microorganisms that when applied to the seeds or plant surfaces adjacent to soil, can colonize the rhizosphere and/or the inner regions of plant tissues to promote plant growth (Bhattacharyya and Jha, 2012).

The rhizosphere, characterized as the soil surrounding a root in which physical, chemical and biological properties are influenced by root growth and activity (McCully, 2005), has been the focus of agricultural research for many years due to its importance in crop productivity, soil health and sustainable agriculture (Jha et al., 2013; Ordookhani et al., 2011; Ryan et al., 2009). Bacteria associated with the rhizosphere at different stages of plant development are termed rhizobacteria. Approximately 2–5% of the total rhizospheric bacteria are able to positively influence plant growth and are generally referred to as plant growth-promoting rhizobacteria (PGPRs) (Antoun and Prévost, 2006). The potential role of PGPRs as bio-fertilizers in agriculture offers an economically and environmentally beneficial way to reduce the use of chemical fertilizers, antibiotics, herbicides, pesticides and other artificial supplements (Adesemoye et al., 2009; Ahemad and Khan, 2009; Bhattacharyya and Jha, 2012; Lucy et al., 2004).

PGPRs may promote plant growth through several biological mechanisms (Glick, 2012), such as the production of plant growth hormones such as auxins (Patten and Glick, 1996), production of siderophores (Kloepper et al., 1980; Tian et al., 2009), phosphate solubilization (Rodriguez et al., 2004; Vikram and Hamzehzarghani, 2008), and nitrogen fixation (Ashraf et al., 2011). Their beneficial effects have been demonstrated in important crops, such as wheat

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(*Triticum aestivum*) (Govindasamy et al., 2014; Turan et al., 2012), rice (*Oryza sativa*) (Cassán et al., 2014), sunflower (*Helianthus annuus*) (Ambrosini et al., 2012), rapeseed (*Brassica napus*) (Farina et al., 2012), bean (*Phaseolus coccineus*) (Stefan et al., 2013), corn (*Zea mays*) (Arruda et al., 2013; Krey et al., 2013), and others (Hayat et al., 2010).

Interestingly, PGPRs that induce growth in one plant species do not necessarily have similar effects on other species. Some bacteria exert general growth-promotion effects on several plant species, whereas other bacteria show strong host-plant selectivity and colonize a single plant species or a limited variety of species (Long et al., 2008; Zeller et al., 2007). These PGPR-plant relationships may involve specific interactions and recognition processes (Benizri et al., 2001). Furthermore, it has been reported that several bacteria originating from the rhizosphere not only colonize the rhizosphere and/or the rhizoplane but also may enter plants and colonize internal tissues (Raaijmakers et al., 1995), where many bacterial species have demonstrated plant growth-promoting effects. Endophytic colonization may have advantages over root-surface associations since microbes can establish themselves in a sheltered environment (Castanheira et al., 2017). A thorough understanding of plant colonization by PGPRs is important to better predict how bacteria interact with plants and whether they are likely to assert themselves in the plant environment after field application as bio-fertilizers or biocontrol agents (Compant et al., 2010).

According to the World Trade Organization (WTO), Brazil is the 3rd largest agricultural producer in the world (Kaschuk et al., 2010). Considered a valuable resource of biodiversity in the world, Amazonia is one of the major rainforests and plays an important role in the discovery of new plant, animal and microorganism species (Gaston, 2000). The microbial diversity of Amazonian is poorly understood, and the data available from tropical regions are scarce (Huang et al., 2014; Kim et al., 2007; Peay et al., 2013). Thus, the goals of this work were to isolate, identify and physiologically characterize culturable rhizobacteria from guarana (*Paullinia cupana* var. *sorbilis*), a typical native crop in the Amazon, and to evaluate the *in planta* growth-promoting effects on corn (*Zea mays* L.) and soybean [*Glycina max* (L.) Merr.] of two selected multi-trait strains. One strain was also *gfp*-marked to enable tracking during plant colonization.

## 2. Material and methods

### 2.1. Bacterial isolation

Samples of rhizospheric soil from ten adult guarana trees, clone 800, were collected by uprooting the plant, collecting the root and the soil in clean, sterile bags and transporting them back to the laboratory. The sample collection occurred at 'Santa Helena' farm, Maués, Amazonas, Brazil (3° 32′ 44″ S and 57° 41′ 30″ W) in November 2010.

The bacterial isolation was performed according to Araújo et al. (2014): ten grams of rhizospheric soil was placed in individual Erlenmeyer flasks containing 90 ml of sterile phosphate-buffered solution (PBS) (NaCl, 8.0 g; KCl, 0.2 g; $Na_2HPO_4$, 1.44 g; $KH_2PO_4$, 0.24 g; distilled water, 1000 ml; pH 7.4). Samples were incubated under agitation (150 rpm) for 30 min at 28 °C. Aliquots of 0.1 ml of three-fold serial dilutions were inoculated in duplicate onto petri dishes plates containing 20 ml of 10% triptone soya agar (TSA) (Oxoid®) supplemented with benomyl (50 μg $ml^{-1}$) to avoid fungal growth. The plates were incubated at 28 °C for 48 h. Different bacterial colonies were selected according to their color and shape, numbered, and purified. Bacterial cultures were preserved in 15% glycerol at −80 °C until further study.

### 2.2. Bacterial molecular identification

Bacterial cells were inoculated in 5 ml of liquid 100% triptone soya broth (TSB) medium (Oxoid®) under constant agitation (150 rpm) for 48 h at 28 °C. Subsequently, the cells were subjected to centrifugation for 10 min at 10,000 g. DNA was extracted from the precipitate using a DNeasy® Blood & Tissue Kit (Qiagen®) following the manufacturer's recommendations. Partial 16S rDNA was amplified using the primers R1387 (5′-CGGTGTGTACAAGGCCCGGGAACG-3′) and PO27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) (Heuer et al., 1997). PCR was performed using 3.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 M each primer, 2.5 U Taq DNA polymerase, and 10× buffer in a final volume of 50 μl. Amplifications were carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems) with an initial denaturation step at 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 62.5 °C for 1 min, and 72 °C for 30 s; and one final elongation cycle at 72 °C for 7 min. As a positive control, we used the DNA from *Escherichia coli* strain TOP 10 (Invitrogen). As a negative control, we replaced the DNA template with sterile DNase-free water. The 16S rDNA gene PCR products were purified using the polyethylene glycol method described by Lis (1980) and sequenced at the *Instituto do Genoma Humano* (University of São Paulo, São Paulo, Brazil). Sequencing was made using the primer 1387R.

Bacterial identification was performed by comparing the obtained sequences against those deposited in the following public databases: Ribosomal Database Project II (RDPII) (https://rdp.cme.msu.edu/) using the Classifier program with 95% confidence (Wang et al., 2007) and GenBank from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) using the BLASTn tool (Altschul et al., 1997). All of the bacterial sequences presented in this study were submitted to GenBank (accession numbers KT699773–KT699868).

The two strains assayed for growth promotion under greenhouse conditions were completely 16S rDNA-sequenced using the 16S rDNA internal primers proposed by Thompson et al. (2001).

### 2.3. Bacterial PGP traits

Analyses of auxin production, biological nitrogen fixation (BNF) potential, phosphate solubilization, and siderophore production were carried out using the bacterial strains initially grown in TSA medium at 28 °C for 24 h as inocula.

#### 2.3.1. Auxin production

The auxin production by rhizobacteria strains was measured using the quantitative method developed by Bric et al. (1991) and adapted for the quantitative method described by Husen (2013). Each bacterial culture was grown in liquid TSB medium at 28 °C and agitated at 150 rpm for 24 h. A bacterial suspension (100 μl) of each fully grown bacterial culture was inoculated in 5 ml liquid TSB medium amended with 5 mM L-tryptophan and placed in the dark for 72 h in an incubating shaker (150 rpm) at 28 °C. Centrifugation (10,000 × g) of bacterial cultures was done at 150 rpm for 5 min, and the supernatant (1 ml) was mixed with 2 ml of the Salkowski reagent (Gordon and Weber, 1951). The development of a pink color indicated indole acetic acid (IAA) production, and the optical density (OD) was read at 520 nm using a spectrophotometer. The OD values obtained were interpolated from a standard curve to determine the IAA concentration per ml basis (Sarwar and Kremer, 1995). Two independent experiments were performed in triplicate. Additionally, as a positive control, we measured the auxin produced by *Pantoea agglomerans* strain 33.1, which was previously reported as an IAA producer (Quecine et al., 2012).

#### 2.3.2. Growth capacity of the isolates in nitrogen-free culture medium

The evidence of the strains to fix atmospheric nitrogen was assessed as described by Döbereiner et al. (1995) by growing the strains on semisolid nitrogen-free medium seven consecutive times. After 96 h of incubation at 28 °C in the dark, the formation of a white growth film near the surface of the tubes indicated a positive result. *P. agglomerans* strain 33.1 was used as a positive control (Quecine et al., 2012). This procedure was performed in triplicate.

#### 2.3.3. Phosphate solubilization

The ability of bacterial strains to solubilize inorganic calcium

phosphate was indicated by a halo obtained after cultivation of the bacteria on culture medium supplemented with $Ca_3(PO_4)_2$ at 28 °C for 72 h according to methods described by Verma et al. (2001). *P. agglomerans* strain 33.1 was used as a positive control for phosphate solubilization (Quecine et al., 2012). The ratio between the halo diameter (cm) and the colony diameter (cm) was calculated, generating an index of phosphate solubilization (IPS) that was useful for ranking the bacteria into three categories (low, medium and high IPS) as proposed by Silva Filho and Vidor (2000).

#### 2.3.4. Siderophore production

The production of siderophores by rhizobacteria was analyzed by observing the ability of the strains to produce an orange halo zone on chrome azurol S agar (CAS agar) medium (Schwyn and Neilands, 1987). At the same $OD_{600}$, the strains were spot-inoculated onto CAS agar plates and incubated at 28 °C for 24 h *Pseudomonas fluorescens* strain Pf-5 was used as a positive control for siderophore production (Hartney et al., 2011). An estimation of the halo diameter (cm) divided by the colony diameter (cm) generated the metal chelation index (MCI) that was used to classify the strains by siderophore production (Batista, 2012).

### 2.4. Plant growth-promotion assay

Evaluation of plant growth-promotion by the selected rhizobacterial strains was carried out under greenhouse conditions at the College of Agriculture "Luiz of Queiroz", University of São Paulo, Piracicaba – SP, Brazil (22° 42′ 30″ S and 47° 38′ 30″ W), in January 2012.

Bacterial inocula containing $10^8$ CFU $ml^{-1}$ were prepared by growing each of the selected bacteria in TSB medium at 28 °C with 150 rpm agitation for 24 h. The inocula were kept at room temperature prior to seed inoculation. Treatments consisted of control (containing only the TSB culture medium), RZ2MS9 – *Bacillus* sp. and RZ2MS16 – *Burkholderia ambifaria*.

Seeds of soybean variety BRS 133 and of corn variety 'Alto Agrícola' were washed with distilled water and passed through a sterile sucrose solution (10% w/v) before agitation and draining. The seeds were immersed, maintained in bacterial suspensions for 30 min and then sown. A randomized block experimental design with a total of 30 replicates per treatment was used for each crop species to assess the treatment effects on plant growth.

The corn and soybean seeds were planted in pots with 1.6 kg of the thick, branny substrate Bioplant® (http://www.bioplant.com.br/), which is composed of peat, correctives, vermiculite, charcoal and pine bark (from Bioplant Agrícola Ltda.). The plants were automatically irrigated 3 times per day. No nutrient solution was added. At thirty and sixty days after seeding (DAS), the plants were harvested. Roots and shoots were separated and washed under running water. Shoot height was measured. The root system and the shoots of each plant were placed in paper bags in a drying oven with forced ventilation at 60 °C until they achieved a constant weight. Then, the dry shoot and root weights were determined according to Huang et al. (2017).

### 2.5. Transformation of RZ2MS16 with pCM88 for gfp labeling

RZ2MS16 behavior during corn and soybean colonization was evaluated by genetically labeling this strain with *gfp*. The plasmid pCM88 carrying the *gfp* and tetracycline resistance genes was kindly provided by Dr. Christopher James Marx and Dr. Mary Lidstrom (University of Washington). DH5-α cells were used as pCM88 host cells. The plasmid extraction was performed using a miniprep kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations.

For electroporation, RZ2MS16 competent cells were obtained according to the modified protocol described by Ferreira et al. (2008). One loop of bacterial culture was inoculated in 5 ml of SOB liquid medium (Sambrook and Russell, 2001) and incubated overnight at 28 °C with shaking (200 rpm). The resulting cells were transferred to 50 ml of SOB liquid medium and grown at 28 °C with shaking (200 rpm) to the mid-exponential growth phase ($OD_{600}$nm = 0.6 to 0.8). Cells were collected by centrifugation (10,000g) at 4 °C for 10 min. The cell pellets were washed once with chilled sterilized water, washed with 10% glycerol at 4 °C and resuspended in 10% (v/v) glycerol at a concentration of approximately $10^9$ cells $ml^{-1}$. Aliquots with 100 µl of cells were frozen in liquid nitrogen and stored at −80 °C. RZ2MS16 was electroporated (Gene Pulser, BioRad − 2.5 kV, 25 µF, 400, 0.2 cm cuvettes) with 100 ng of the pCM88 plasmid. After incubation at 28 °C (150 rpm) for 4 h, the transformed cells were plated on Luria-Bertani (LB) medium (Sambrook and Russell, 2001) supplemented with tetracycline (50 µg $ml^{-1}$). One colony was randomly selected, stored at −80 °C and named RZ2MS16:*gfp* for further study.

### 2.6. In vitro target-bacterium growth

The growth of RZ2MS16:*gfp* in liquid LB medium was compared to the wild-type strain to determine the metabolic burden of pCM88. After 24 h, liquid cultures of the original RZ2MS16 strain and RZ2MS16:*gfp* were diluted ($OD600_{600nm}$ = 0.2) in 50 ml media and incubated at 28 °C in a rotary shaker (200 rpm). The *gfp*-tagged strain was grown with tetracycline (50 µg $ml^{-1}$). The $OD_{600nm}$ was used to monitor bacterial growth (Spectronic 20+, Thermo Spectronic) at 1 h intervals for 24 h. Bacterial growth was determined in four replicates for each strain.

### 2.7. Plant colonization assay

RZ2MS16:*gfp* cells were transferred to 50 ml of LB medium containing tetracycline (50 µg $ml^{-1}$) in a 125 ml Erlenmeyer flask and incubated at 28 °C on a shaker (150 rpm) until the late log phase. The inoculum concentration was then adjusted to approximately $10^5$ CFU $ml^{-1}$ with PBS based on the $OD_{600nm}$ and was confirmed by colony counting.

The corn and soybean seeds were surface-disinfected with 70% ethanol for 1 min, 2.5% sodium hypochlorite for 2 min, 70% ethanol for 1 min and at least two subsequent rinses in sterile distilled water. The seeds were cultured *in vitro* and placed in 50 ml tubes containing 7 ml of MS medium (Murashige and Skoog, 1962). After 15 days, the seedlings were transferred to liquid containing the bacterial inoculum and incubated at 28 °C with a 16 h photoperiod. In the control treatment, only PBS was added. The seedlings were kept in the bacterial inocula/PBS (control) for 1 day and then transferred to tubes with 290 $cm^3$ of Bioplant® commercial substrate. Three and nine days after inoculation (DAI), three plants per treatment were sampled for fluorescence microscopy (FM), and four plant seedlings were sampled for quantification using a cultivation-based approach.

### 2.8. Fluorescence microscopy (FM)

For microscopical observations, the roots, stem, and leaves of the plants inoculated with RZ2MS16:*gfp*, and the control mock-inoculated plants were hand-cut and visualized immediately. The magnifications used for the microscopy ranged from 100 to 400 ×. Plant tissues were analyzed using the epifluorescence microscope Axiophot II (Zeiss, Germany) with the following filter sets (excitation/emission): 365/397 nm for blue, 450/515 nm for green and 546/590 nm for red. Images were digitalized through a PCO CCD camera using ISIS Metasystems software (Metasystems, Germany).

### 2.9. Cultivation-based approach for quantification

One gram of rhizosphere soil was placed in a new sterile tube containing 3 ml of PBS and agitated at 120 rpm for 1 h. The cell suspension was diluted and plated on 10% TSB media for bacterial

quantification. The seedlings were washed with running tap water and surface-disinfected according to Araújo et al. (2014). To confirm the efficiency of the disinfection process, aliquots of the sterile distilled water used in the final washing were spread onto plates of 10% TSB media and examined for surface contaminants after 3 days of incubation at 28 °C. The surface-disinfected samples were macerated in PBS, and the appropriate dilutions were plated onto 10% TSB plates containing tetracycline (50 μg $ml^{-1}$) to select only RZ2MS16:*gfp* and benomyl (50 μg $ml^{-1}$) to avoid fungal growth.

### 2.10. Statistical analysis

We conducted one independent plant growth-promotion experiment to each crop, corn and soybean, under greenhouse conditions. The corn experiment was conducted using a randomized block design with 3 blocks containing 10 replicates of each treatment per block, while the soybean experiment was conducted using a randomized block design with 2 blocks containing 15 replicates of each treatment per block.

At 30 and 60 DAS, we measured the response variables related to plant development: dry weights of shoot and root and shoot height. The data was submitted to analysis of variation, followed by Tukey's test to compare the means obtained in each treatment.

For the plant colonization experiment, the obtained data were standardized to gram of fresh tissue and log-transformed to log CFU + 2 to normalize the data according to Quecine et al. (2012). The means of four replicates per treatment were compared using Tukey's test.

All analysis were performed in Statistical Analysis Software (SAS) Program for Windows (SAS Institute Inc., Cary, NC) and the significance level adopted in all tests was 5%.

## 3. Results

### 3.1. Bacterial isolation and molecular identification

After rhizosphere isolation, the culturable bacterial load in the guarana rhizosphere was approximately $4.6 \times 10^7$ CFU g of $soil^{-1}$. A total of 96 morphologically diverse colonies were selected for this study. The partial 16S rDNA gene sequencing enabled the classification of bacterial strains into 13 genera from four phyla: *Proteobacteria* [*Betaproteobacteria* (45.8%) and *Gammaproteobacteria* classes (9.4%)], *Firmicutes* (34.4%), *Actinobacteria* (3.1%) and *Bacteroidetes* (7.3%). *Betaproteobacteria*, the most abundant class, was composed mostly of the genus *Burkholderia*. *Bacillus* was the most abundant genus belonging to the phylum *Firmicutes* (Table 1).

### 3.2. Plant growth-promoting traits

All 96 rhizobacterial strains were biochemically tested for four PGP traits. Most strains (93%) produced IAA in the presence of the precursor tryptophan. The IAA production ranged from 0.62 to 185.03 μg $ml^{-1}$. *Burkholderia* strains produced an average of 47.5 μg $ml^{-1}$, while *Bacillus* strains produced an average of 20.9 μg $ml^{-1}$ of IAA (Table S1). Evidence of nitrogen-fixing ability was observed in 23 strains, and the majority of them also belonged to the *Burkholderia* genus (65%) (Fig. 1). Among the 96 strains, 41 were able to solubilize phosphate *in vitro*, and 63% of these belonged to the *Burkholderia* genus (Fig. 1). Six phosphate solubilizers, all *Burkholderia*, showed the highest IPS ( > 3.0) (Table S1). Twenty-four strains were able to produce siderophores, with 83% from the *Burkholderia* genus (Fig. 1). The higher siderophore producing strains with the highest MCI belonged to the *Burkholderia* genus, with a single exception (strain RZ2MS9) that belonged to the *Bacillus* genus (Table S1).

Thirteen strains showed the four PGP traits evaluated. Other than strains RZ4MS6 (*Delftia* sp.) and RZ2MS9 (*Bacillus* sp.), all of them belong to the genus *Burkholderia*. Fifteen strains demonstrated three of the PGP traits evaluated (Fig. 2). Only 5 strains did not show any of PGP traits evaluated (Table S1).

### 3.3. Plant growth-promotion assay

We selected two strains for evaluation under greenhouse conditions. These strains, *Bacillus* sp. (RZ2MS9) and *Burkholderia ambifaria* (RZ2MS16), represented the most abundant genera found in the guarana rhizosphere and had their 16S rDNA completely sequenced. RZ2MS9 could not be identified at species level with 16S rDNA gene sequencing, but it belongs to the *Bacillus cereus* sensu lato group (Fig. S1), while RZ2MS16 was identified as *Burkholderia ambifaria* (Fig. S2). The first strain displayed all the PGP traits evaluated, whereas the second was not capable of phosphate solubilization but showed a high production of IAA (175 μg $ml^{-1}$, highly above the average IAA produced by *Burkholderia* spp. in this study) (Table 2).

The selected bacterial strains had different effects on biomass production in the evaluated crops (Fig. 3A). Corn plants showed increases of 35.5 and 35.2% in the shoot height when inoculated with RZ2MS9 and RZ2MS16, respectively, at 30 DAS (Figs. 3B and C). Similar results

**Table 1**
The taxonomic distribution of the isolated bacterial strains from the guarana rhizosphere.

| Phylum | Class | Genus | Number of strains | Strains |
|---|---|---|---|---|
| *Proteobacteria* | *Betaproteobacteria* | *Burkholderia* | 29 | RZ1MS3, RZ1MS4, RZ1MS6, RZ1MS11, RZ1MS13, RZ1MS15, RZ1MS19, RZ1MS21, RZ1MS22, RZ2MS2, RZ2MS4, RZ2MS7, RZ2MS8, RZ2MS16, RZ2MS20, RZ3MS8, RZ3MS9, RZ3MS19, RZ3MS22, RZ3MS24, RZ3MS26, RZ4MS8, RZ4MS14, RZ4MS15, RZ4MS17, RZ5MS1, RZ5MS4, RZ5MS5, RZ5MS8 |
| | | *Ralstonia* | 8 | RZ1MS1, RZ1MS2, RZ1MS8, RZ2MS6, RZ2MS12, RZ2MS14, RZ2MS19, RZ2MS21 |
| | | *Cupriavidus* | 4 | RZ1MS5, RZ1MS17, RZ3MS7, RZ3MS12 |
| | | *Delftia* | 3 | RZ4MS18, RZ4MS6, RZ4MS12 |
| | *Gammaproteobacteria* | *Dyella* | 7 | RZ1MS10, RZ2MS3, RZ2MS11, RZ3MS28, RZ4MS10, RZ4MS13, RZ4MS16 |
| | | *Pantoea* | 1 | RZ1MS7 |
| | | *Enterobacter* | 1 | RZ3MS23 |
| *Firmicutes* | *Bacilli* | *Bacillus* | 30 | RZ1MS9, RZ1MS12, RZ1MS16, RZ1MS18, RZ1MS20, RZ2MS5, RZ2MS9, RZ2MS10, RZ2MS13, RZ2MS17, RZ2MS18, RZ2MS23, RZ3MS5, RZ3MS6, RZ3MS10, RZ3MS14, RZ3MS15, RZ3MS20, RZ3MS21, RZ3MS25, RZ3MS27, RZ4MS1, RZ4MS4, RZ4MS19, RZ5MS2, RZ5MS3, RZ5MS9, RZ5MS10, RZ5MS11, RZ5MS13 |
| | | *Paenibacillus* | 3 | RZ1MS23, RZ2MS1, RZ3MS2 |
| *Actinobacteria* | *Actinobacteria* | *Flexivirga* | 1 | RZ3MS16 |
| | | *Curtobacterium* | 1 | RZ3MS3 |
| | | *Kitasatospora* | 1 | RZ5MS7 |
| *Bacteroidetes* | *Flavobacteria* | *Cryseobacterium* | 7 | RZ3MS13, RZ3MS17, RZ3MS18, RZ4MS2, RZ4MS5, RZ4MS9, RZ5MS6 |

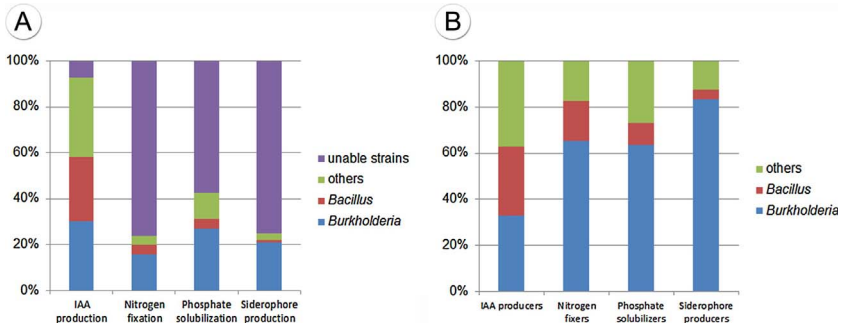


**Fig. 1.** Multiple PGP traits of guarana-derived rhizobacteria according to genera distribution of the 96 bacterial strains based on their PGP traits (A) and genera distribution of the bacterial strains positive for each PGP trait (B).

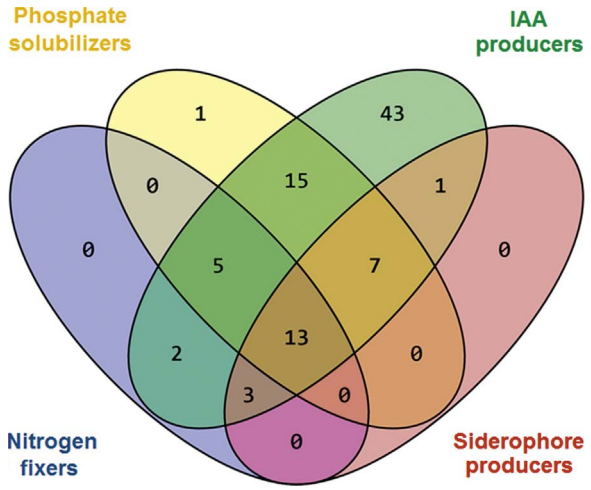


**Fig. 2.** Venn diagram summarizing the number of guarana rhizobacteria showing multiple PGP traits. Yellow – phosphate solubilizers, green – IAA producers, blue – nitrogen-fixers and red – siderophore producers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
PGP traits displayed by selected bacterial strains for *in vivo* assays.

| Strains | Identification[a] | PGP traits[b] | | | |
|---|---|---|---|---|---|
| | | IAA | BNF | IPS | MCI |
| RZ2MS9 | *Bacillus* sp. | 067.4 | + | 2.6 | 3.4 |
| RZ2MS16 | *Burkholderia ambifaria* | 175.0 | + | 0.0 | 2.7 |

IPS = index of phosphate solubilization and MCI = metal chelation index.

[a] The identification was done according to BLASTn analysis of complete 16S rDNA sequences and phylogenetic analysis.

[b] IAA = indole acetic acid production (μg ml). BNF = biological nitrogen fixation (with '+' = positive).

were also observed in soybean plants, which showed significant shoot height increases of 25.9 and 22.5% compared to those of the control when inoculated with RZ2MS9 and RZ2MS16, respectively, at 30 DAS (Figs. 3D and E). The dry weight of the corn and soybean shoots were significantly higher than those of non-inoculated plants, showing increases of more than 47% in both strains, crops and times of evaluation. The dry weight of corn shoots inoculated with RZ2MS9 increased by 235.5% compared to the control at 60 DAS. RZ2MS9 and RZ2MS16 inoculation significantly increased corn root weight by 247.8 and 136.9%, respectively, which was significantly greater than that of non-inoculated control plants, at 60 DAS. However, the soybean root dry weight did not increase following the bacterial inoculation of either strain (Fig. 3A).

### 3.4. Bacterial labeling

Plasmid pCM88 was introduced into the RZ2MS16 strain with a transformation efficiency of $4 \times 10^4$ transformants .μg of $DNA^{-1}$. One clone was randomly selected for further assays and named RZ2MS16:*gfp*. Wild-type RZ2MS16 did not exhibit any auto-fluorescence, and the heterologous *in vitro* expression of the *gfp* gene by the RZ2MS16:*gfp* strain was observed by epifluorescence microscopy (Fig. 4B). The growth behaviors of the *gfp*-target strain and the wild-type strain were assayed *in vitro* for 24 h in TSB medium. The transformation of RZ2MS16 did not significantly affect the bacterial growth of the transformant compared to wild-type growth according to regression analysis ($p \geq 0.05$) (Fig. 4A).

### 3.5. Colonization of corn and soybean by RZ2MS16:gfp

Fluorescence microscopy and quantitative assays revealed that the RZ2MS16:*gfp* strain was able to colonize corn and soybean as an endophyte. At 9 DAI, no fluorescent bacterial cells were observed in non-inoculated plants (Figs. 5A, B, C and 6A, B). In the inoculated corn plants, fluorescent bacterial cells were mostly observed in chlorenchyma, in both palisade and spongy parenchyma, and were easily differentiated from plant auto-fluorescence. Some colonies were associated with bundle sheath cells (Fig. 5D, E). Few bacterial fluorescent cells were found in corn root cells (Fig. 5F). In soybean, RZ2MS16:*gfp* cells were concentrated in the palisade cells and were less abundant in the paraveinal mesophyll cells (Fig. 6C). Very rarely, bacterial cells were observed in the soybean roots (Fig. 6D).

Bacterial cells were quantified from inner corn and soybean tissues. In both crops, we observed a higher number of RZ2MS16:*gfp* cells in the rhizosphere at 3 DAI than at 9 DAI. In corn, it was more evident that the strain had moved from the rhizosphere to the roots and to shoot parts by 9 DAI, which increased the bacterial cell density in these tissues (Fig. 7A). In soybean, we observed less colonization of the plant tissues by RZ2MS16:*gfp* (Fig. 7B).

## 4. Discussion

Although the richest biodiversity on earth is located in tropical rainforests, microorganisms associated with typical Brazilian Amazon plants have been studied only recently (Azevedo et al., 2000; Bogas et al., 2015). In our work, culturable bacterial isolates belonging to 13 different genera were isolated from the guarana rhizosphere. To the best of our knowledge, this is the first study reporting an approach to assess the diversity of culturable microorganisms associated with the rhizosphere of this typical Amazon crop.

Strains belonging to *Burkholderia* and *Bacillus* genera were the most abundant among all the bacterial genera identified and represented 61.5% of the total isolates. The predominance of *Bacillus* spp. in the rhizosphere of plants has been previously reported by Pedrinho (2009). Likewise, *Burkholderia* species are generally reported among the dominant bacteria in the rhizosphere of several plants (*e.g.*, rice, corn,

A

| | Dry weight (g) | | | | Shoot height (cm) | |
|---|---|---|---|---|---|---|
| | Shoot | | Root | | | |
| | 30 DAS | 60 DAS | 30 DAS | 60 DAS | 30 DAS | 60 DAS |
| **Corn** | | | | | | |
| Control | 1.53 b | 03.94 c | 0.28 b | 0.92 c | 61.90 b | 70.97 b |
| RZ2MS9 | 3.70 a | 13.22 a | 0.50 a | 3.20 a | 83.87 a | 98.93 a |
| RZ2MS16 | 3.47 a | 08.44 b | 0.44 a | 2.18 b | 83.67 a | 94.60 a |
| **Soybean** | | | | | | |
| Control | 0.87 b | 05.04 b | 0.14 a | 2.59 a | 37.07 b | 75.47 b |
| RZ2MS9 | 1.28 a | 09.87 a | 0.19 a | 3.69 a | 46.70 a | 88.20 a |
| RZ2MS16 | 1.28 a | 10.19 a | 0.19 a | 4.04 a | 45.43 a | 87.63 a |

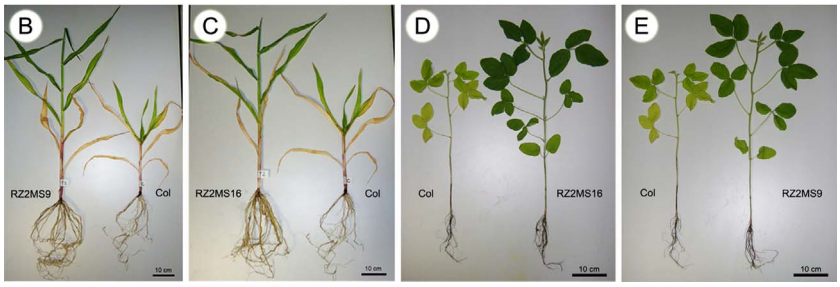


**Fig. 3.** Corn and soybean growth promotion by RZ2MS9 (*Bacillus* sp.) and RZ2MS16 (*B. ambifaria*). The data are the average dry weights of shoot and root and shoot heights at 30 and 60 DAS (days after seeding). Means values (30 replicates at each time for both crops) followed by different letters within the column differ significantly according to Tukey's test (A). Corn and soybean without (control treatment) and with RZ2MS9 (*Bacillus* sp.) and RZ2MS16 at 30 DAS (B, C, D and E, respectively).

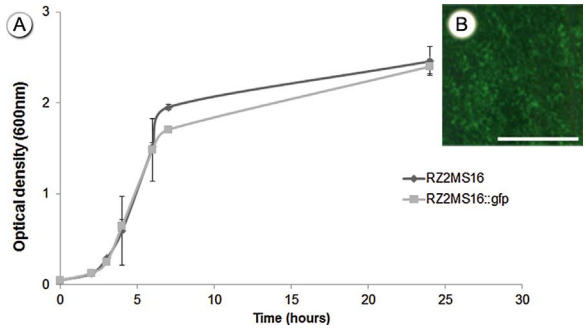


**Fig. 4.** Growth curves of wild-type bacterium *Burkholderia ambifaria* RZ2MS16 (dark gray line) and strain RZ2MS16 tagged with *gfp* (RZ2MS16:*gfp,* light gray line) in Luria-Bertani (LB) medium (A). These curves were obtained by setting initial bacteria density and monitoring bacterial growth at 1-h intervals for 24 h. In some cases, symbols obscure error bars; RZ2MS16:*gfp* cells expressing GFP. Magnification: 400 × ; bar, 100 µm (B).

pea or cotton) (Hallmann et al., 1999).

There are several mechanisms by which PGPR can promote plant growth (Ahemad and Kibret, 2014; Rana et al., 2011), and we investigated four PGP traits: IAA production, siderophore production, potential to biological nitrogen fixation and phosphate solubilization. Approximately 19% of the guarana isolates exhibited at least three PGP traits, and only 5% did not show any PGP traits. Almost all evaluated strains (93%) were capable of producing IAA. This high percentage of IAA producers in the rhizosphere agrees with other studies that reported more than 90% of evaluated rhizobacteria produced indolic compounds (Arruda et al., 2013; Farina et al., 2012; Passos et al., 2014; Souza et al., 2013). RZ2MS9 showed low IAA production but greatly improved plant growth, whereas RZ2MS16 showed high production of this hormone. These results support the importance of investigating multiple PGP traits. Among the putative BNF-positive strains of this study, the majority were identified as *Burkholderia* spp. Remarkably, several *Burkholderia* spp. were reported as able to convert atmospheric nitrogen to ammonia via BNF (Reis et al., 2004; Walker et al., 2015). All high phosphate-solubilizing strains (ISF > 3) in this study were identified as *Burkholderia* spp. Arruda et al. (2013) also reported *Burkholderia* spp. among most common phosphate-solubilizing bacteria in the corn rhizosphere. Interestingly, in the present study, the RZ2MS16 strain was not able to solubilize phosphate *in vitro* but was able to promote soybean and corn growth. Higher siderophore-producing strains also belonged to the *Burkholderia* genus, with the exception of the *Bacillus* strain RZ2MS9. In the present study, among the 96 evaluated strains, 25% displayed the ability to produce siderophores and, among those, 83% were identified as *Burkholderia* spp. Thus, we selected two bacterial strains, RZ2MS9 (*Bacillus* sp.) and RZ2MS16 (*B. ambifaria*) based on both their *in vitro* PGP traits and on the fact that each strain was representative of the most abundant isolated groups from the guarana rhizosphere.

RZ2MS9 and RZ2MS16 were also selected due to the fact that their draft genome sequence was recently published (Batista et al., 2016a, 2016b), providing the genetic basis for many of the mechanisms possibly involved in their beneficial plant effects. RZ2MS9 has 33 genes related to nitrogen fixation and 19 genes related to IAA production, including *ipdC*, a gene that encodes the key enzyme indole-3-pyruvate decarboxylase of the IAA biosynthetic pathway. The annotated genome also has several genes for parts of the iron- and siderophore-uptake systems, such as the uptake regulation protein (*fur*). Similarly, the RZ2MS16 draft genome sequence presents genes involved in auxin biosynthesis and genes related to nitrogen fixation by the vanadium nitrogenase system. In addition, the genome of RZ2MS16 has the gene for 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that may potentially contribute to plant growth (Glick, 2014). These additional traits will be further investigated.

Due to the cosmopolitan behavior of *Bacillus* and *Burkholder*ia genera, both are difficult to taxonomically classify; certain strains of their subgroups can be beneficial or pathogenic to plants or humans (Helgason et al., 2000; Suaréz-Moreno et al., 2012). Strains belonging to the *Burkholderia* genus are known for their ability to improve plant growth, for example, in sugarcane (Govindarajan et al., 2006; Paungfoo-Lonhienne et al., 2014), rice (Souza et al., 2013), bean (de Oliveira-Longatti et al., 2015), and sunflower (Ambrosini et al., 2012). Although *B. ambifaria,* a member of the *Burkholderia cepacia* complex

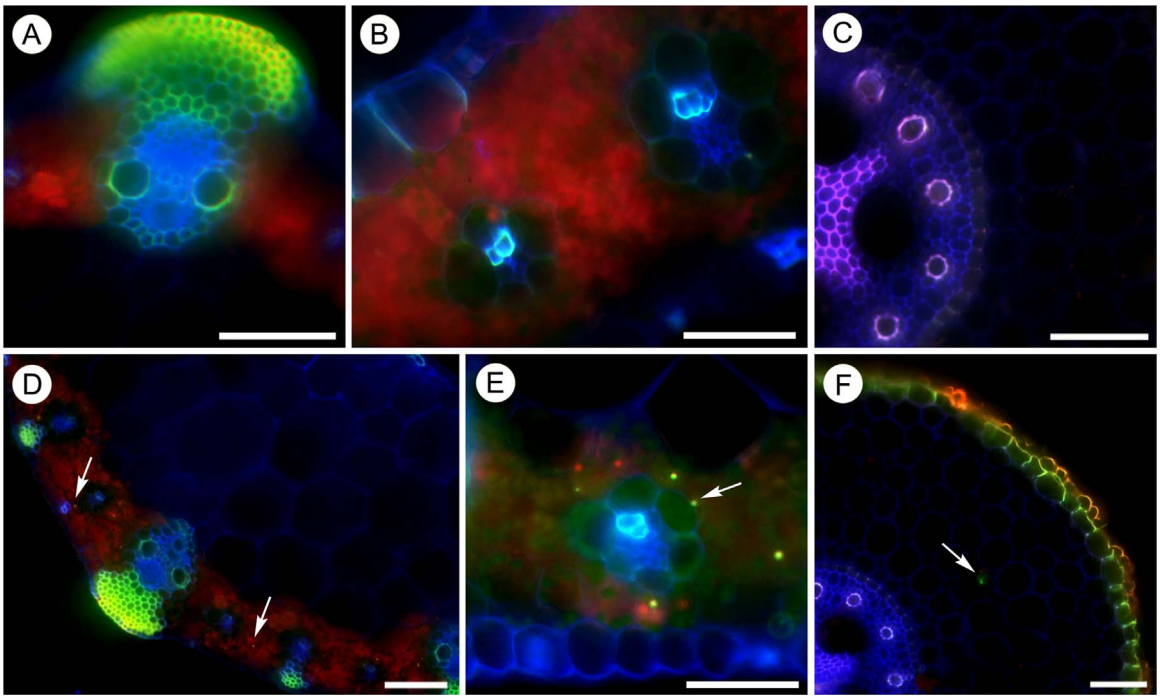


**Fig. 5.** Visualization of inoculated RZ2MS16:*gfp* within corn tissues in leaf (A, B) and root cells (C) free of bacterial colonies in the non-inoculated control plants. Corn tissues of leaf (D, E) and root (F) colonized by RZ2MS16:*gfp* at 9 DAI. The RZ2MS16:*gfp* colonies in plant tissues are indicated by arrows. Scale bars represent 50 μm (B, E) and 100 μm (A, C, D, F). The magnification used for each figure was 100 × for D and F, 200 × for A and C and 400 × for B and E.

(BCC) (Coenye et al., 2001), has been increasingly described as a PGP bacterium (Ciccillo et al., 2002; Parra-Cota et al., 2014), some strains have been recovered from cultures of respiratory tract specimens from cystic fibrosis patients (Suaréz-Moreno et al., 2012). Several genes are described as virulence determinants in the *Burkholderia cepacia* complex (Loutet and Valvano, 2010). Of 33 of those genes, only 3 (the type 3, 4 and 6 secretion systems) were detected in the *Burkholderia* RZ2MS16 draft genome sequence. In this sense, it is of fundamental importance to complete its genome sequence to have a better understanding of the virulence determinants.

Similarly, strains of the *Bacillus cereus* sensu lato group have different phenotypes, from human pathogens (*B. cereus* sensu stricto and *B. anthracis*) to biological agents involved in the control of insect larvae widely used for agricultural purposes (*B. thuringiensis*) (Okinaka and Keim, 2016). Since genes associated with many medically relevant phenotypes in this group are plasmid encoded (Rasko et al., 2005) and since the draft genome sequence of *Bacillus* RZ2MS9 does not includes plasmid genes, its analysis was not enough to predict if this strain is potentially pathogenic to man. Thus, the plasmid of this strain will be further investigated by sequencing.

Many studies have shown that inoculation with *Bacillus* species resulted in positive effects on plant growth (Kumar et al., 2012; Lim and Kim, 2009). Moreover, there are many studies that have reported strains of *Bacillus* spp. and *Burkholderia* spp. as corn and soybean

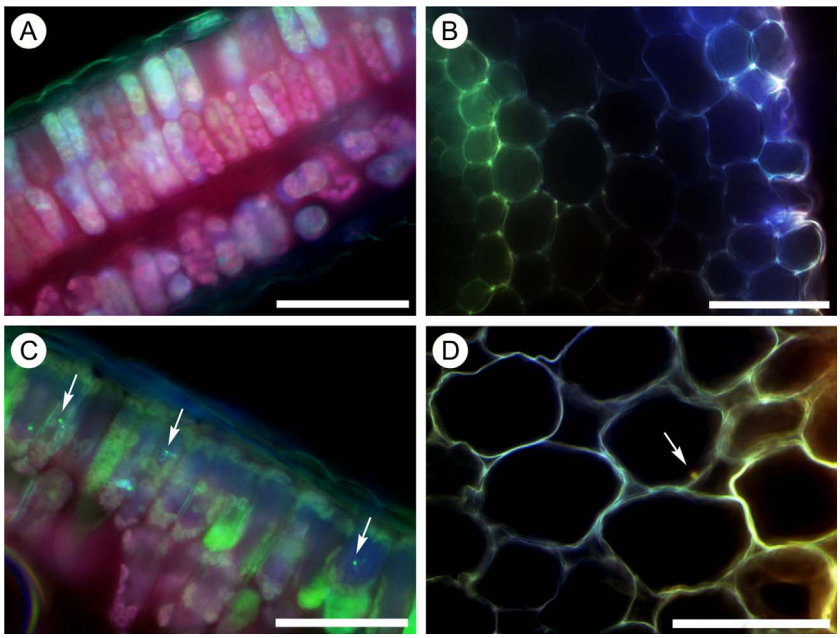


**Fig. 6.** Visualization of inoculated RZ2MS16:*gfp* within soybean tissues in leaf (A) and root (B) cells free of bacterial colonies in the non-inoculated control plants. Soybean tissues of leaf (C) and root (D) colonized by RZ2MS16:*gfp* at 9 DAI. The RZ2MS16:*gfp* colonies in plant tissues are indicated by arrows. Scale bars represent 50 μm. The magnification used for each figure was 200 × for B and 400 × for A, C and D.

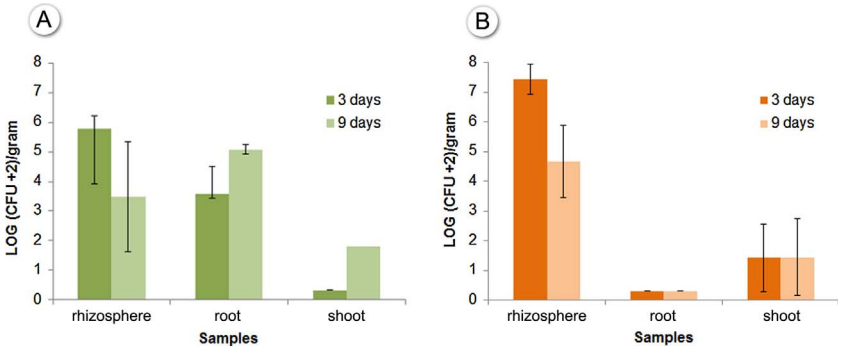


**Fig. 7.** *Burkholderia* sp. RZ2MS16:*gfp* quantification during cross-colonization of corn (A) and soybean (B). Bacterial density was measured using a cultivation-based approach at 3 and 9 DAI. The abundance data, in CFU.g of tissue$^{-1}$, were log-transformed to stabilize the variance. The results are the means of the four replicates for each sample. The bars represent the standard error of each treatment.

growth-promoters. Huang et al. (2014) reported a strain of *Bacillus pumilus* (C9) isolated from rainforest soil that promotes corn growth under greenhouse conditions. Inoculation of corn with C9 increased fresh shoot weight (22.9%) and dry shoot weight (31.5%). Wahyudi et al. (2011) also demonstrated significant growth of soybean seedlings with inoculation of *Bacillus* sp. strains isolated from its rhizosphere. Significant growth-promoting results were also obtained by the inoculation of soybean plants with *Burkholderia* sp. PER2F (Fernández et al., 2007). In general, there is great variability among the results of the most diverse crops and strains tested. Comparing with commercial products, we have similar results. *Azospirillum brasilense*, a reference strain already marketed for corn crops, can increase plant growth by approximately 30% compared to controls (Picazevicz et al., 2017), while *Bradyrhizobium* spp. strains recommended for soybean crops can provide increases in total soybean biomass of approximately 65% (Argaw, 2014), values below/compatible to those found in this study.

An inoculant that can be used for several crops is of great agricultural interest due to its greater applicability, and both selected bacterial strains, RZ2MS9 (*Bacillus* sp.) and RZ2MS16 (*Burkholderia* sp.), were not plant specific plant-growth promoters. Likewise, Quecine et al. (2012) reported sugarcane growth-promotion by *P. agglomerans* strain 33.1 isolated from *Eucalyptus grandis*. Zinniel et al. (2002) studied the host range of 29 endophytic bacteria that had been isolated from sorghum or corn; 26 were able to colonize at least one other host plant in sufficient densities, leading to the conclusion that these interactions can be largely nonspecific.

We also found that RZ2MS16:*gfp* was able to colonize soybean and corn plants and displayed endophytic behavior. Several bacteria derived from the rhizosphere not only colonize the rhizosphere and/or the rhizoplane but can also live in plant tissues, and many of them have shown plant growth-promoting effects (Compant et al., 2005; Hardoim et al., 2008). After penetration, rhizobacteria/endophytes can move towards the xylem and be transported systemically into stems and leaves (Olivares et al., 1996; Bacon and Hinton, 2006). In grapevine, it was reported that *Burkholderia phytofirmans* PsJN colonized the rhizosphere, penetrated the roots, and then migrated through all plant tissues within 94 h of bacterial application (Compant et al., 2005). Similarly, the target strain RZ2MS16:*gfp* displayed the endophytic behavior of colonization of soybean and corn because it could penetrate plant roots and move to shoots with decreasing bacterial density in comparison to rhizosphere- or root-colonizing populations.

Finally, due to the controversial classification of the evaluated strains, more studies are needed to verify the security of RZ2MS9 and RZ2MS16 as bio-fertilizers. Additionally, future investigations should include the functional genomic study of the most promising PGPRs to provide a fundamental basis to fully understanding their interaction with plant hosts.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2017.09.007

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