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Endophytic cultivable bacterial community obtained from the *Paullinia cupana* seed in Amazonas and Bahia regions and its antagonistic effects against *Colletotrichum gloeosporioides*

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ABSTRACT

Guarana (*Paullinia cupana* var. *sorbilis*) is a plant from the Amazonas region with socio-economic importance. However, guarana production has been increasingly affected by unfavorable conditions resulting from anthracnose, caused by the *Colletotrichum* fungal genus, which primarily affects mainly the Amazonas region. The aim of the present study was to isolate bacterial endophytes from the seeds of guarana plants obtained from Amazonas region and the Northeast state of Bahia, a region where this disease is not a problem for guarana plantations. The number of bacterial Colony Forming Units (CFU/g seeds) was 2.4×10^4 from the Bahia and 2.9×10^4 from the Amazonas region. One hundred and two isolated bacteria were evaluated *in vitro* against the phytopathogenic strain *Colletotrichum gloeosporioides* L1. These isolates were also analyzed for the enzymatic production of amylase, cellulase, protease, pectinase, lipase and esterase. Approximately 15% of isolates, showing high antagonistic activity, and the production of at least one enzyme were identified through the partial sequencing of 16S rDNA. The genus *Bacillus* was the most frequently observed, followed by *Paenibacillus*, *Ochrobactrum*, *Microbacterium* and *Stenotrophomonas*. Proteolytic activity was observed in 24 isolates followed by amylolytic, pectinolytic and cellulolytic activities. No esterase and lipase production was detected. Most of the isolates, showing antagonistic effects against *C. gloeosporioides* and high enzymatic activities, were isolated from the anthracnose-affected region. A biocontrol method using the endophytes from guarana seeds could be applied in the future, as these bacteria are vertically transferred to guarana seedlings.

1. Introduction

Endophytic microorganisms are primarily bacteria and fungi that live within plants for at least part of their life cycle without causing apparent harm to the host. In many cases, the microbes protect the plant hosts against diseases and insect pests and promote plant growth [1,2]. Endophytic bacteria and fungi have been isolated from practically all organs and tissues from plant species studied. However, information regarding endophytes from plant seeds remains limited [3,4].

Guarana (*Paullinia cupana* var. *sorbilis*) is a native plant from the central amazon basin, belonging to the family Sapindaceae. The genus *Paullinia*, with approximately 200 species, is restricted to this region with few exceptions on the tropical and subtropical America [5]. The guarana seed extract has many pharmacological activities, but it is mainly consumed as a soft drink generated from industries that produce nonalcoholic beverages. Guarana is commercially cultivated only in Brazil, particularly in the states of Amazonas (AM) and Bahia (BA), the main producers. However, the state of Amazonas, although the native region of this plant, is no longer the

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commercial lead producer of guarana seeds; indeed, the state of Bahia is currently the major producer. This change likely reflects the fact that the Amazonas region has been increasingly affected by anthracnose, a disease caused by fungi from the genus *Colletotrichum*, which does not lead to economic losses in guarana cultures from the Bahia region. The use of chemical products is not recommended for the treatment of this disease, as guarana is primarily used for medicines and soft drinks. An alternative to control this disease is to detect endophytic bacteria that could be used as biological controllers of this pathogenic agent.

Endophytic bacteria isolated from soybean seeds were relatively able to control the growth of phytopathogenic fungi primarily from the *Fusarium* genus [6]. In addition, endophytic bacteria isolated from rice seeds were antagonized fungal pathogens [7]. Similar behavior was observed with some bacteria from peanuts seeds [8]. Moreover, several other beneficial endophytes from seeds conferred plant growth-promoters, enzyme production and resistance to insects, diseases and heavy metals among others [4,9].

In the present study, endophytic bacteria were isolated from the seeds of guarana (*Paullinia cupana* var. *sorbilis*) crops from the Amazonas and Bahia states in Brazil. The identification of the isolates was based on the comparison of 16S rRNA sequences. *In vitro* antagonistic activity of the bacteria against the anthracnose agent was evaluated and the enzymatic production of these bacteria, showing the potential to inhibit this pathogen. These bacteria might be used to transfer valuable properties from seeds to seedlings collaborating to control this disease.

2. Materials and methods

2.1. Plant material

Guarana seeds were kindly provided from Dr. Rosineide Souza at the National Institute of Amazonian Research (INPA), Manaus, Amazonas (S 3° 05′ 38.1″; W 59° 59′ 17.5″) and Dr. Phellippe Marbach and Jackeline Andrade, from Federal University of Bahia Reconcavo (UFRB), Cruz das Almas, Bahia (S 12° 39′ 27.6″; W 39° 05′ 17.8″).

2.2. Isolation of endophytic bacteria from seeds

The endophytic bacteria were isolated from surface disinfected seeds for the elimination of epiphytic microorganisms. The surface disinfection of seeds was performed in the following steps: washing in distilled water followed by ethanol 70% (1 min), sodium hypochlorite 3% (v/v) (3 min), ethanol 70% (1 min) and two washings with autoclaved distilled water [10]. After surface disinfection, the seeds were triturated in sterile phosphate-buffered saline (PBS) (NaCl 8.0 g L^{-1}; Na_2HPO_4 1.44 g L^{-1}; KH_2PO_4 0.24 g L^{-1}; KCl 0.20 g L^{-1}; distilled water and pH 7.4) and maintained at 28 °C in a rotary shaker at 150 rpm. The dilution series were subsequently plated in Petri dishes (9 cm diameter) containing 10% Tryptone Soybean Agar (TSA) medium (Himedia) supplemented with 50 μg mL^{-1} of benomyl fungicide to prevent fungal growth. The effectiveness of disinfection was verified after plating aliquots of the final distilled water wash into 10% TSA [3]. The plates were incubated at 28 °C for 7 days, and the number of colony forming units (CFU) was determined to estimate the number of cultivable endophytic bacteria per gram of seeds.

Some colonies were randomly selected, purified through serial dilutions and plated onto TSA medium. Following the purification, the isolated colonies were suspended in tubes containing 2 mL of Tryptone Soybean Broth (TSB). After the bacterial growth, glycerol (20%) was added and the microtubes stored at −80 °C. According to origin of seeds, the isolates were labeled as “AM” for isolates from Amazonas state and “BA” for isolates from Bahia state.

2.3. DNA extraction of endophytic bacteria

The selected bacterial isolates were grown in Luria-Bertani Broth (LB) (1% w/v Tryptone, 0.5% Yeast extract and 1% NaCl). Each isolate was incubated for 48 h at 28 °C under agitation (150 rpm). The bacterial cultures with $OD_{600} = 0.1$ (optical density measured at 600 nm, corresponding to approximately 10^8 UFC/ml) were centrifuged at 10,000 ×*g* for 2 min, and 500 μL of extraction buffer (Tris-HCl 10 mM and EDTA 1 mM, at pH 8.0), 80 μL of 10% SDS (Sodium Dodecyl Sulfate) and 0.5 g silica (0.1 mm) was added to the pellet. The samples were homogenized through inversion, centrifuged under the same reported conditions, transferred to clean tubes and subsequently mixed with 500 μL of phenol. The solutions were homogenized again and centrifuged. The supernatants were transferred to new clean tubes, and 500 μL of chloroform was added to each sample. The solution was homogenized again and transferred to tubes containing 500 μL of phenol/chloroform/isopropyl acid (5:4:1), followed by homogenization and centrifugation at 10,000 ×*g* for 7 min and the subsequent addition of 4 μL of 5 M NaCl and 400 μL of isopropanol. The mixture was maintained for 5 min at −20 °C, followed by centrifugation (10,000 ×*g*). The resulting DNA was washed with 70% ethanol, dried at 40 °C for 30 min and eluted in 50 μL of autoclaved deionized water and quantified in 1% agarose gel.

2.4. Amplification and sequencing of 16S rRNA

Bacterial isolates were identified through the partial sequencing of 16S rDNA. For the PCR reactions the primers R1387 (5′-CGGTGTGTACAAGGCCCGGGAACG-3′) and PO27F (5′ GAGAGTTTGATCCTGGCTCAG-3′) were used [11]. The PCR reactions were performed in a 50 μL final volume containing 3.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 M of each primer, 2.5U Taq DNA polymerase (Sinapse Biotecnologia, São Paulo, Brazil), 10X Buffer and 10 ng/μL template DNA. The 16S rDNA gene was amplified using the following reaction conditions for the thermocycler (GeneAmp PCR System 9700, Applied Biosystems): initial denaturation for 10 min at 95 °C followed by 35 cycles at 94 °C for 30 s, annealing at 62.5 °C for 1 min and primer extension at 72 °C for 1 min. The amplicons were analyzed on agarose gels (1% w/v) together with 1 Kb DNA molecular weight markers (Fermentas, St. Leon-Rot, German).

The PCR products were purified using polyethylene glycol solution (PEG 8000) [12] and sequenced at the Biotechnology Laboratory, University of São Paulo (ESALQ/USP).

The sequence qualities were evaluated using the Bioedit v.7.2.2 software [13]. After the initial and final regions of the sequences were cut, the samples were submitted to chimera analysis using the DECIPHER's Find Chimeras [14].

2.5. Identification of isolates

The bacterial identification was performed comparing the sequences of Type Strains deposited in GenBank (NCBI – National Center of Biotechnology Information) using BLASTn with filtering for sequences from type material. The 16S rDNA PCR products and similar sequences rescued from GenBank were aligned with MUSCLE [15] using the software MEGA 6.05 [16].

The dendogram was generated using the Neighbor-Joining method [17], using p-distance for nucleotides with “the pairwise gap deletion” option and bootstrap with 10,000 repetitions. *Wautersiella falsenii* (accession number JX100832.1) was used as the outgroup. The bacterial sequences were submitted to GenBank (accession numbers KT692645 – KT692659).

2.6. Bacterial antagonistic activity against the anthracnose agent

The bacterial isolates were evaluated *in vitro* using a parity method against the phytopathogenic fungal *Colletotrichum gloeosporioides* L1, the causal agent of guarana anthracnose [18]. This phytopathogen, isolated from a typical anthracnose lesion, was kindly provided from Dr. Pedro Queiroz Costa Neto, Federal University of Manaus, Brazil.

A preliminarily assay was performed to select bacteria with antagonistic activity. The bacteria were inoculated two-by-two in the extremities of Petri dishes (9 cm diameter) containing Potato Dextrose Agar medium (PDA) (Difco Laboratories, Detroit, USA). The phytopathogenic fungus was inoculated with a 0.5 cm agar disc in the center of the dishes. The plates were incubated for 7 days at 28 °C.

Only bacteria that inhibited fungal growth were evaluated for antagonistic activity. Each selected bacterial culture was inoculated onto solid LB medium and after 48 h transferred to 5 mL of sterilized water and the absorbance of each bacterial suspension was measured and adjusted to $OD_{600} = 0.1$. Approximately 10 μL of the bacterial suspension was inoculated on one extremity of the plates, and in the same day, the fungus (0.5 cm diameter disc) was inoculated in the center of the plate. The experiments were performed in triplicate. After incubation for 7 days at 28 °C the percentage inhibition (IA%) was calculated using formula (1) according to the scheme described in Fig. 1.

$$IA\% = 100 - \left(\frac{X1}{\left[\frac{X2+X3+X4}{3} \right]} \right) \times 100 \quad (1)$$

2.7. Enzymatic activity

All selected bacteria were evaluated for enzymatic activity using qualitative and semi-quantitative methods. The following enzymes were analyzed: amylase, esterase, lipase, cellulase, protease and pectinase. For all enzymatic assays, each isolate was previously grown on LB solid medium for 2–7 days at 28 °C.

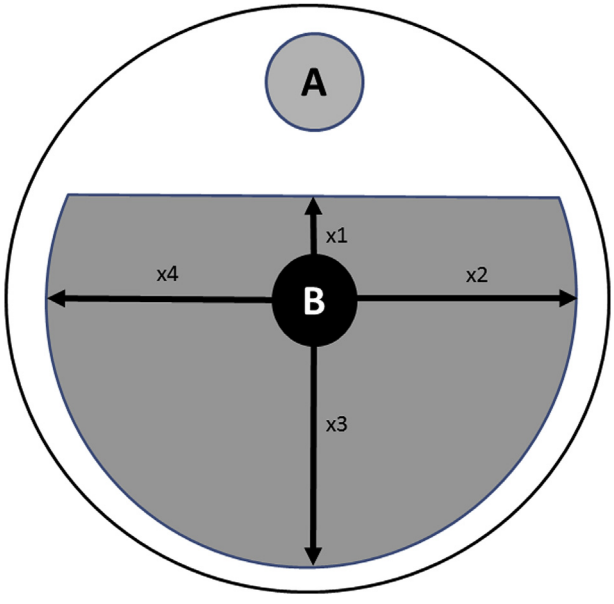

Fig. 1. Schematic showing the evaluation of the antagonism index. A) endophytic bacterial inoculum; B) *Colletotrichum gloeosporioides* L1 inoculum; ×1, ×2, ×3 and ×4 is the distance of radial growth measured in mm.

For qualitative analyses, the isolates were inoculated in specific medium for each enzyme on Petri dishes (15 cm diameter), with 96 bacteria inoculated per plate. The enzymatic activity was detected based on halos surrounding the bacterial colonies.

For the semi-quantitative analysis, each bacterial suspension, adjusted to $OD_{600} = 0.1$, was inoculated onto specific media and incubated at 28 °C. The enzymatic index (EI) was measured after 72 h of incubation and expressed as the ratio between the halo diameter and the bacterial colony diameter [19].

The amylase activity was measured using M9 (Sigma-Aldrich) medium, substituting glucose with 1% soluble starch and 0.5% yeast extract. After bacterial growth, 10 mL of iodine solution was added to each plate, showing clear haloes around the colonies. As a positive control, 10 μL of α-amylase (Sigma-Aldrich, St. Louis, MO) was used.

To cellulolytic activity the isolates were grown on M9 medium substituting glucose with 1% carboxymethylcellulose (CMC) (w/v) and adding 0.6% yeast extract [20]. After bacterial growth, 10 mL of Congo red dye (1%) was added and the plates were washed with NaCl (5 M). The presence of a colorless halo around the colony was indicative of enzymatic activity. As a positive control 10 μL of cellulase (Sigma-Aldrich) was used.

To evaluate the lipolytic and esterase activities, Sierra medium was used [21]. Previously sterilized Tween 80 (final concentration 1% w/v) was added onto the culture medium. The presence of halos was considered indicative of enzymatic activities.

To determine the proteolytic activity, a culture medium containing skimmed milk was used [22]. The formation of halos was considered indicative of enzymatic activity. As a positive control, 10 μL of protease (Sigma-Aldrich) was used.

The pectinolytic activity was determined after growing the bacterial isolates on M9 medium substituting glucose with 1% (w/v) pectin and adding 0.5% yeast extract. After bacterial growth, 10 mL of Lugol's solution was added, followed by water washing. The presence of haloes around the colonies was indicative of pectinolytic activity. For pectin-lyase the pH was adjusted to 8.0. As a positive control 10 μL of pectinase (Sigma-Aldrich) was used.

Only semi-quantitatively evaluated isolates producing enzymatic activity were analyzed using the formula: IE = halo diameter/colony diameter.

2.8. Statistical analysis

All assays were performed in triplicate. The data were submitted to ANOVA analysis using the statistical software SISVAR v.5.3 [23]. The mean values were compared using the Scott-Knott test at 5% significance.

3. Results

3.1. Isolation and identification of endophytic bacteria

The seeds from guarana plants in the Amazonas (AM) and Bahia (BA) respectively showed 2.9×10^4 and 2.4×10^4 colony-forming units (CFU) per gram of seed. We randomly selected 102 colonies, 48 colonies from Amazonas (AM) and 54 colonies from Bahia (BA). After antagonistic and enzymatic activity assays, 34 isolates produced one or more enzymes or/and showed antagonistic activity against the plant pathogenic fungus. Among these, 15 bacteria showed antagonistic and/or enzymatic activities, and these microbes were selected for molecular identification (Table 1). The genera *Paenibacillus*, *Bacillus*, *Ochrobactrum*, *Microbacterium* and *Stenotrophomonas* were identified.

Table 1
Identification of bacteria isolated from guarana seeds obtained from the Amazonas (AM) and Bahia (BA).

Isolates	Identification	Best alignment with type strains on NCBI	Identity (%)	Access Number
1B.7(AM)	*Paenibacillus* sp.	*P. rhizosphaerae* (NR_043166.1)	99%	KT692645
1B.10(AM)	*Bacillus* sp.	*B. amyloliquefaciens* (NR_117946.1)	100%	KT692646
1B.11B(AM)	*Bacillus* sp.	*B. amyloliquefaciens* (NR_117946.1)	99%	KT692647
1B.12A(AM)	*Bacillus* sp.	*B. amyloliquefaciens* (NR_117946.1)	100%	KT692648
1B.12B(AM)	*Bacillus* sp.	*B. amyloliquefaciens* (NR_117946.1)	99%	KT692649
1B.12C(AM)	*Bacillus* sp.	*B. amyloliquefaciens* (NR_117946.1)	100%	KT692650
3B.7A(AM)	*Bacillus* sp.	*B. amyloliquefaciens* (NR_117946.1)	99%	KT692651
3B.11B(AM)	*Ochrobactrum* sp.	*O. pseudogrignonense* (NR_042589.1)	100%	KT692652
3B.17B(AM)	*Microbacterium* sp.	*M. invictum* (NR_042708.1)	100%	KT692653
3B.20(AM)	*Ochrobactrum* sp.	*O. pseudogrignonense* (NR_042589.1)	99%	KT692654
5.1B.1B(AM)	*Bacillus* sp.	*B. vanillea* (KF986320.1)	100%	KT692655
6B.4B(AM)	*Ochrobactrum* sp.	*O. pseudogrignonense* (NR_042589.1)	99%	KT692656
D1.2(BA)	*Bacillus aryabhattai*	*B. aryabhattai* (NR_118442.1)	100%	KT692657
D3.1B(BA)	*Bacillus safensis*	*B. safensis* (NR_113945.1)	100%	KT692658
D3.6A(BA)	*Stenotrophomonas maltophilia*	*S. maltophilia* (LN681567.1)	100%	KT692659

Based on phylogenetic analysis (Fig. 2), the identification of the genera of 12 isolates and the species of 3 isolates was possible. On the clade of *Firmicutes* phylum, containing the isolates 1B.10, 1B.11B, 1B.12A, 1B.12B, 1B.12C, 3B.7A and 5.1B.1B, all isolates were from seeds provided from the state of Amazonas (AM), presenting an intra-specific grouping with 100% of bootstrap (BP), including three strains of different species, *Bacillus vanillea*, *B acillus amyloliquefaciens* and *Bacillus subtilis*, on this clade. This similarity with different species made it difficult to identify these isolates at the species level; however, these bacteria were identified as *Bacillus* sp.

The isolate D3.1B (BA), presented 99% BP on the phylogenetic analysis and 100% of ID on the alignment with *B acillus safensis*. Similarly, the isolate D1.2 (BA) presented 100% BP on the phylogenetic analysis and 100% ID with *Bacillus aryabhattai*. Another node on the *Firmicutes* phylum included the *Paenibacillus* genus and strains with 100% BP, which were observed with the alignment of the isolate 1B.7 (AM) with three other strains of *Paenibacillus*. These results facilitated the identification of *Paenibacillus* sp.

For *Proteobacteria*, four isolates were distributed into two genera. The isolates 3B.11B (AM), 3B.20 (AM), and 6B.4B (AM) were identified as *Ochrobactrum* sp. considering grouping with 100% BP with other lineages of these genera. The isolate D3.6A (BA) presented 100% ID and BP with *Stenotrophomonas maltophilia*, confirming the identification of this isolate as *S. maltophilia*.

For the *Actinobateria* phylum, only isolate 3B.17B (AM) grouped

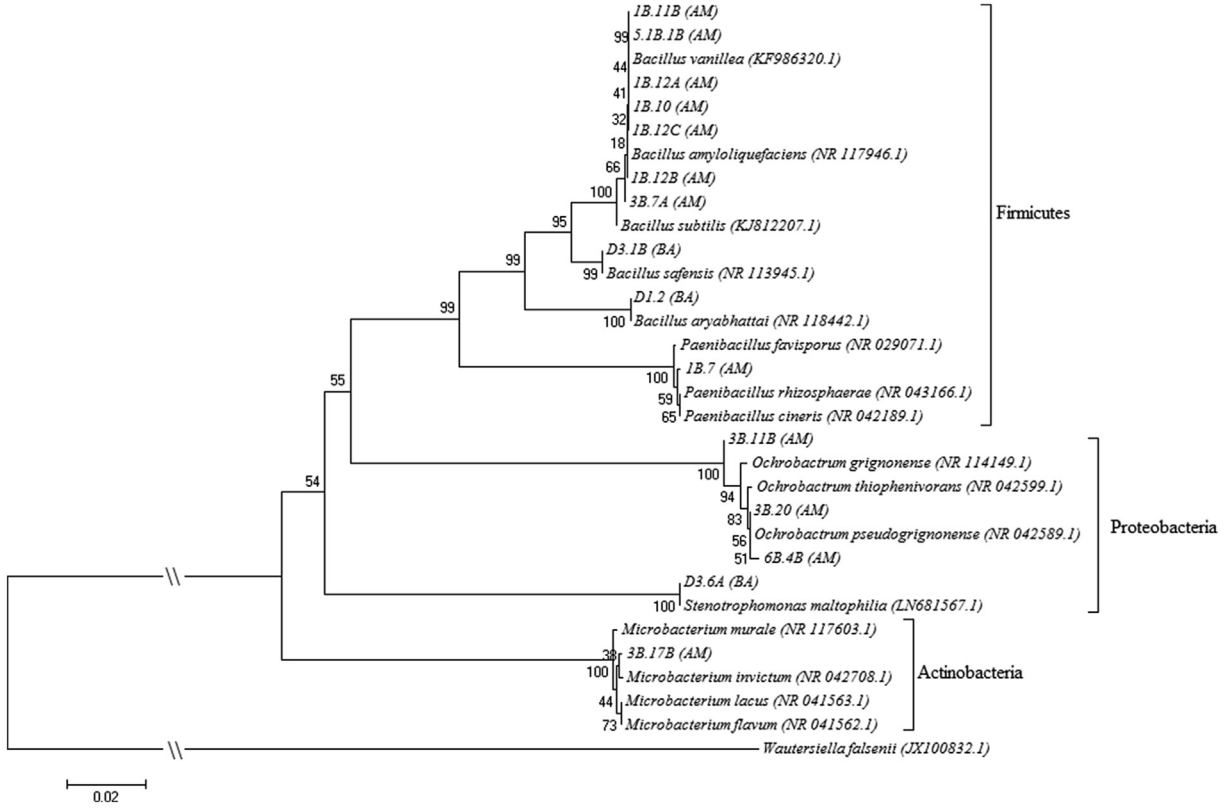

Fig. 2. Clustering of the similarity of endophytic bacteria from guarana seeds obtained from the Amazon (AM) and Bahia State (BA). The bootstraps represent the means of 1000 repetitions according to "Neighbor-joining" method using the "p-distance" to nucleotides with "the pairwise gap deletion" option.

with other sequences of the *Microbacterium* genera, and this isolate was identified as *Microbacterium* sp.

3.2. Antagonistic and enzymatic activities

The *Bacillus* genus (primarily the isolates obtained from Amazonas seeds) showed a higher antagonistic activity compared with other isolates, demonstrating an IA% between 53 and 63% of inhibition. The isolates 1B.12B (*Bacillus* sp.), 1B.2A, 1B.12C (*Bacillus* sp.) and 5.1B.1A showed an IA% greater than 60% (Fig. 3). The isolates from Bahia showed a lower IA%, and only three strains demonstrated inhibition activity under *in vitro* conditions.

The qualitative assay for enzymatic activity showed that among the 102 evaluated endophytic bacteria, proteolytic isolates were most frequently observed (23.5%), followed by amylolytic (12.7%), pectinolytic (10.8%) and cellulolytic (6.9%) isolates. No esterase and lipase activity was observed in the analyzed bacteria. The Table 2 shows the results obtained for strains that showed at least one activity for enzyme production or antagonism against *C. gloeosporioides* L1.

The distribution of amylolytic and cellulolytic activity was observed in one single group for both enzymatic activities. The highest amylase activity was observed for isolate 1B.12C (*Bacillus* sp.), with values varying from 1.19 to 2.26, and the cellulose activity varied from 1.61 to 1.78. No amylolytic, cellulolytic and pectinolytic activity for the isolates from Bahia.

For pectinolytic activity, two statistical groups were obtained, with the highest values averaging from 1.55 to 1.66. The isolate 1B.11B (*Bacillus* sp.) presents the highest EI.

The highest amount of endophytic bacteria with potential for enzyme production was detected for proteolytic activity. From this analysis, two statistically significant values of 1.23 and 2.33 were obtained. The best values were presented for isolates 3B.19 and D3.1B (*Bacillus safensis*) with an EI = 1.97, 1B.11B (*Bacillus* sp.) with EI = 2.11, 1B.12A (*Bacillus* sp.) with EI = 2.23 and 6B.4B (*Ochrobactrum* sp.) with EI = 2.33.

Regarding the enzyme tests evaluating the 15 bacteria identified, only two isolates of the genera *Ochrobactrum* sp. did not show enzymatic activity.

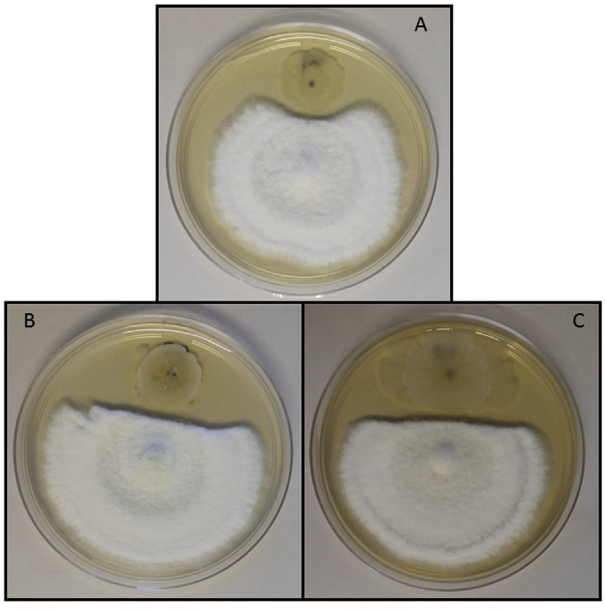

Fig. 3. Antagonistic activity of endophytic bacteria obtained from guarana seeds against the phytopathogen *C. gloeosporioides* strain. A) Isolated 1B.11A; B) 1B.2A; and C) 1B.12C.

4. Discussion

Endophytic bacteria were isolated from guarana seeds from two regions (AM and BA), showing similar bacterial densities of 2.9×10^4 CFU/g for AM and 2.4×10^4 CFU/g for BA. The number of CFU/g of seeds reported in literature varies according to the plant species. For *Eucalyptus* from 12 species and hybrids analyzed, the CFU/g ranged from none in *E. grandis* to 1.83×10^2 in *E. urophylla* [3]. In rice (*Oryzae sativa*), the numbers varied from 10^2 to 10^3/g [24]. The bacterial densities in some species might also depend on the plant variety, age and other conditions. However, in general, the number and species variation is higher in other plant organs and tissues compared with seeds. In the leaves of guarana obtained from the Amazonas, the numbers varying from 10^4 to 10^5 CFU/g [25]. The isolation of endophytes in seeds is used to examine beneficial microorganisms that might be transferred early to seedlings contributing to promote plant growth, the biological control of pests and other advantages. Although the importance of endophytic bacteria from seeds has only recently been recognized [4], there are few data showing the beneficial application of these microorganisms.

The role of seed bacteria includes growth promoters, increased disease resistance, nitrogen fixation and the production of secondary metabolites as antimicrobials and exoenzymes. It is likely that these bacteria might be selected by plants via seeds to benefit the next generation [4].

In the present study, the genus *Bacillus* was the most frequently observed, followed by *Paenibacillus*, *Ochrobactrum*, *Microbacterium* and *Stenotrophomonas*. These genera have already been described as seed endophytes from other plant seeds, and the genus *Bacillus* was practically observed in all plant species seeds examined thus far. The genera *Paenibacillus*, *Ochrobactrum* and *Microbacterium* were also described in the seeds of several species, such as *Eucalyptus*, rice and soybeans [3,6,24], and *Stenotrophomonas* in coffee [26]. The role of these important seed-borne bacteria must be valuable for their hosts, as these microorganisms are preserved in seeds and are the first organisms to act in seedlings.

Considering the endophytic genera identified in guarana seeds, many *Bacillus* species promote plant growth and protect plants against diseases through antibiosis systemic resistance, competition for niches and nutrients, the secretion of exoenzymes as proteases and other plant benefits [27]. *Ochrobacterium*, such as *Ochrobactrum oryzae*, might possess the ability to fix nitrogen [28] and *Ochrobactrum anthropi* promotes increased growth in the Jerusalem artichoke [29]. *Stenotrophomonas*, such as *S. maltophilia*, control *Bipolaris sorokiniana* in tall fescue [30], *Paenibacillus,* identified in pumpkin seeds, suppresses diseases, such as powdered mildew, also increasing harvest yields [31], and finally *Microbacterium* stimulate growth in *Limonium sinense* [32] and reduce the severity of leaf rust in coffee [33].

Among the 102 bacteria isolated from guarana seeds, approximately 15% of these organisms inhibited the growth of *C. gloeosporioides*. In a similar study, 18% of isolated bacteria from the seeds of soybean (*Glycine max*) antagonized plant pathogenic fungi from the genus *Fusarium* [6]. Although *in vitro* antagonistic assays do not always generate the same positive results when applied *in vivo*, the detection of isolates with antagonistic characteristics might indicate and facilitate a rapid and easy preliminary selection with less cost than field experiments [34]. Further, selected bacteria with high IA% activity might be evaluated *in vivo.* This methodology was also used in other plants, such as potatoes (*Solanum tuberosum*) [35], with success to select *Erwinia*

Table 2
Antagonistic and enzymatic activities of endophytic bacteria isolated from guarana seeds.

Isolates[a]	Identification[b]	Growth pathogen inhibition (%)[c]	Enzimatic Index (EI)[c]			
			Amylase	Celullase	Protease	Pectate-lyase
1B.1(AM)	N.I.	–	–	1.69 a	–	–
1B.10(AM)	*Bacillus* sp.	57.7 a	1.79 a	–	1.27 b	–
1B.11A(AM)	N.I.	49.0 b	1.61 a	–	–	1.35 b
1B.11B(AM)	*Bacillus* sp.	57.4 a	1.53 a	–	2.11 a	1.66 a
1B.12A(AM)	*Bacillus* sp.	58.9 a	2.05 a	–	2.23 a	1.39 b
1B.12B(AM)	*Bacillus* sp.	63.8 a	1.30 a	–	1.41 b	1.60 a
1B.12C(AM)	*Bacillus* sp.	61.9 a	2.26 a	–	1.53 b	1.62 a
1B.13(AM)	N.I.	–	–	1.77 a	–	–
1B.1A(AM)	N.I.	51.6 a	1.67 a	–	1.54 b	1.30 b
1B.1B(AM)	N.I.	58.3 a	1.54 a	–	1.33 b	1.31 b
1B.2A(AM)	N.I.	62.9 a	–	–	–	–
1B.6(AM)	N.I.	–	–	1.64 a	–	–
1B.7(AM)	*Paenibacillus* sp.	–	–	1.78 a	–	–
1B.8(AM)	N.I.	–	–	1.61 a	1.68 b	–
1B.9(AM)	N.I.	–	–	1.62 a	1.60 b	–
3B.1(AM)	N.I.	–	1.69 a	–	1.45 b	1.28 b
3B.11B(AM)	*Ochrobactrum* sp.	–	–	–	–	–
3B.17B(AM)	*Microbacterium* sp.	–	–	–	–	1.65 a
3B.19(AM)	N.I.	–	–	–	1.97 a	–
3B.20(AM)	*Ochrobactrum* sp.	–	–	–	–	–
3B.7A(AM)	*Bacillus* sp.	53.8 a	1.62 a	–	1.65 b	1.55 a
3B.7B(AM)	N.I.	48.7 b	1.19 a	–	1.31 b	1.31 b
3B.9(AM)	N.I.	–	–	–	1.37 b	–
5.1B.1A(AM)	N.I.	60.7 a	1.38 a	–	1.77 b	–
5.1B.1B(AM)	*Bacillus* sp.	59.2 a	1.85 a	–	1.54 b	–
6B.4B(AM)	*Ochrobactrum* sp.	–	–	–	2.33 a	–
D1.2(BA)	*Bacillus aryabhattai*	–	–	–	1.48 b	–
D1.3(BA)	N.I.	–	–	–	1.67 b	–
D2.3B(BA)	N.I.	–	–	–	1.43 b	–
D3.1B(BA)	*Bacillus safensis*	41.9 b	–	–	1.97 a	–
D3.6A(BA)	*Stenotrophomonas maltophilia*	–	–	–	1.23 b	–
D3.6B(BA)	N.I.	–	–	–	1.33 b	–
D3.7C(BA)	N.I.	32.3 c	–	–	–	–
D5.9B(BA)	N.I.	55.4 a	–	–	1.42 b	–

[a] (AM) = Isolates from Amazon seeds; (BA) = Isolates from Bahia seeds.
[b] N.I. = isolates not identified.
[c] The IA% and EI values followed by the same letter in the columns were not distinguished by the Scott-Knott test ($p < 0.05$).

carotovora-antagonizing rhizobacteria.

Interestingly, the isolates from AM seeds showed higher antagonism indices compared with BA isolates. In the AM, the disease incidence is higher than in BA, suggesting that some types of selection might be responsible for selection against *Colletotrichum* in AM, maintaining more antagonistic bacterial isolates in seeds. In bacterial isolated from leaves of asymptomatic and symptomatic guarana plants, was observed that symptomatic plants (with anthracnose) contained higher numbers of bacteria and increased genera diversity compared with asymptomatic plants [25]. These results suggested that anthracnose restructure endophytic bacterial communities through the selection of certain isolates in the phyloplane of *P. cupana*. The understanding of this interaction will be important for the development of biocontrol strategies against *Colletotrichum.*

The percentage of enzymatic production from AM bacteria (48 evaluated bacteria) was 45.8% compared with 13.5% from the 52 isolates producing the enzymes tested from BA. The increased enzymatic activity could be locally associated with a high incidence of anthracnose, but many other factors, such as climate, soil and plant propagation, accomplished using seeds in BA and cuttings in AM, might explain the differences between the AM region where *Colletotrichum* is a severe problem and the BA region.

This is the first report showing endophytic bacteria in *P. cupana* seeds. The results indicate that the bacteria from these seeds might be applied to increase plant growth and disease resistance to anthracnose. Bacterial seeds could also be used to introduce valuable genetic traits from seeds to seedlings using DNA recombinant techniques. Guarana products are used as stimulants, and soft drinks contain 2 to 5 times higher caffeine levels than coffee seeds [5]. The consumption of these products is increasing as the production, is exported to several other world countries in addition to Brazil. Characteristics, such as taste and flavor, have been modified in coffee using selected microorganisms that inhabit the coffee seeds as epi or endophytes [36,37], demonstrating the importance of studies of microbial communities from seeds.

5. Conclusions

To our knowledge, this study is the first report of endophytic bacteria from the seed of guarana, a typical tropical plant. All bacterial genera isolated from guarana seeds have previously been described as seed endophytes conferring some beneficial effects to their hosts. The presence of these bacteria in guarana seeds likely reflects selection facilitating the vertical transmission of these microbes, transferring valuable properties to the seedlings. Interestingly, the majority of isolates showing antagonistic activities to the fungus *C. gloeosporioides* and high enzymatic activities were identified in Amazonas isolates compared with Bahia isolates, suggesting a type of selection for bacterial isolates according to region and/or anthracnose disease that should be further investigated.

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