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Diversity of plant growth-promoting bacteria associated with sugarcane

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ABSTRACT. The sugarcane (*Saccharum* spp) presents economic importance, mainly for tropical regions, being an important Brazilian commodity. However, this crop is strongly dependent on fertilizers, mainly nitrogen (N). This study assessed the plant growth-promoting bacteria (PGPB) associated with sugarcane that could be used as a

potential inoculant to the crop. We evaluated the genetic diversity of PGPB in the plant tissue of sugarcane varieties (RB 867515, RB 1011, and RB 92579). The primer BOX-A1R was used to differentiate the similar isolated and further sequencing 16S rRNA ribosomal gene. The 16S rRNA gene showed the presence of seven different genera distributed into four groups, the genus *Bacillus*, followed by *Paenibacillus* (20%), *Burkholderia* (14%), *Herbaspirillum* (6%), *Pseudomonas* (6%), *Methylobacterium* (6%), and *Brevibacillus* (3%). The molecular characterization of endophytic isolates from sugarcane revealed a diversity of bacteria colonizing this plant, with a possible biotechnological potential to be used as inoculant and biofertilizers.

Key words: Endophytic bacteria; BOX-PCR; Genetic diversity; Biological nitrogen fixation; 16S rRNA gene

INTRODUCTION

Sugarcane (*Saccharum* spp) is an important crop cultivated in several countries from Latin America and the Caribbean. This crop has great importance for Brazilian economy as a commodity for exportation, but also as a major inducer of technological development (Pisa et al., 2011). Nowadays, Brazil is the leader in sugarcane production with assets of 10 million hectares and an average yield around 74 Mg/ha (IBGE, 2016).

This crop depends on soil nutrients, mainly nitrogen (N), and previous studies have shown that the biological nitrogen fixation (BNF) may contribute significantly for the total N required by the crop (Taulé et al., 2012). BNF is carried out by a group of bacteria commonly known as plant growth-promoting bacteria (PGPB), which can be found in several ecosystems, and establish symbiosis or association with plants. These bacteria contribute to plant growth through the N supply, and also by some specific mechanisms, such as the production of hormones, phosphate solubilization, and antagonism to pathogens (Moreira et al., 2010). The mechanisms used by PGPB during the promotion of plant growth are well known, and include plant nutrient-acquisition strategies, such as through BNF and the excretion of hormones that modulate the plant growth (Pandya et al., 2015).

There is a high diversity of PGPB associated with the rhizosphere and shoot of sugarcane, and the knowledge of the diversity of PGPB associated with sugarcane is an important step to prospect bacteria responsible for promoting BNF and plant growth, and consequently, improving the crop productivity (Pisa et al., 2011). On the hand, the number of species associated with the rhizosphere of sugarcane and identified as PGPB has increased considerably in recent years due to the development of molecular tools (Chauhan et al., 2015). On the other hand, there is little information about PGPB associated with leaves and stalk of sugarcane in tropical regions. Therefore, this study was carried out to isolate and characterize promising PGPBs with potential as N fixers and growth promoters in sugarcane. We used the BOX-PCR technique to genotype the bacteria, as this method is recognized as suitable for differentiating phylogenetically bacterial isolates (Menna et al., 2009).

MATERIAL AND METHODS

Plant material and isolation of PGPB

For the isolation of PGPBs, varieties of sugarcane RB 867515, RB 1011, and RB 92579 (collected from commercial crops before flowering) were used. Leaves and stalks were used for the isolation of PGPBs. Thus, the leaves were immersed in 70% alcohol for 30 min and then in a 5% sodium hypochlorite solution for 30 min. The leaves were washed afterwards with distilled water to remove the sodium hypochlorite and dried on absorbent paper. The stalks were washed with distilled water, immersed in 70% alcohol for 30 min, and re-washed with distilled water and dried on absorbent paper.

For the isolation, leaves and stalks (10 g) were disinfected separately with 5% NaCl and placed in penicillin-type tubes containing specific media for *Azospirillum* spp, *Herbaspirillum* spp, *Gluconacetobacter diazotrophicus*, and *Burkholderia* spp, as suggested by Döbereiner et al. (1995), with three replicates. The isolates were grown during 5 days at 30°C, and after the formation of the film, the isolates were transferred to solid media.

DNA extraction

For the extraction of genomic DNA, the isolates were grown in 5 mL liquid culture medium (DYGS; Rodrigues Neto et al., 1986) and incubated at 30°C in a shaker at 200 rpm for 48 h. After this period, an aliquot of 1.0 mL of bacterial suspension was transferred to 1.5-mL microtubes and centrifuged for 3 min at 13,000 *g*. The supernatant was discarded, and the precipitate was used for DNA extraction, using the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer's instructions. The integrity of DNA was assessed by agarose gel electrophoresis at 0.8%.

Analysis of "fingerprinting" for the amplification of the BOX element

Samples of genomic DNA were quantified in Nanodrop® (Thermo Scientific) and diluted with 30 ng/µL sterile Milli-Q water and stored at -20°C. For amplification of the BOX element, the BOX-A1R oligonucleotide (5'-CTA CGG GGC CAA GAC GAC GCT G-3') was synthesized by Invitrogen (Life Technologies). The reaction of amplification with a final volume of 20 µL was as follows: 10X 10% Buffer; 0.2 mM dNTPs; 2 mM $MgCl_2$, 2 µM 0.3 U Taq polymerase; Template DNA (20 ng/µL). Amplification conditions were adjusted according to Freitas et al. (2007): an initial cycle of denaturation at 95°C for 9 min, 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (5 min at 72°C), and a final extension cycle at 72°C for 10 min. All reactions were performed in the Applied Biosystems 2720 Thermal Cycler.

The fragments were separated by electrophoresis containing 0.5X TBE buffer at 100 V for 40 min on 1.2% agarose gels and stained with SybrGold (Sigma) and photographed using the LPIX-HE Loccus. The profile of bands was transformed into a two-dimensional binary matrix, where 0 indicates absence, and 1 indicates the presence of bands. The clustering was performed by the NTSYS-pc 2.1 program using the UPGMA algorithm (unweighted pair-group method with arithmetic mean).

The matrix was performed by the similarity of the figures (SIMQUAL) as reported,

and the algorithm was the SAHN (sequential agglomerative hierarchical nested) cluster analysis, for the preparation of the phylogenetic tree and the formation of the graphic. The isolates that showed different band patterns were considered for 16S rRNA sequencing.

Amplification and sequencing of the 16S rRNA gene

The ribosomal 16S rRNA gene was amplified with the following primers: FD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and RD1 (5'-AAG GAG GTG ATC CAG CC-3') (Weisburg et al., 1991). The reaction was performed in a final volume of 50 μL containing: 10X 10% Buffer; 0.2 mM dNTPs; 0.2 μM primer; 2.0 mM $MgCl_2$, 0.3 U Taq DNA polymerase (5 U/μL); Template DNA (20 ng/μL). The reaction was started with 3 min denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 57°C for 50 s, extension at 72°C for 60 s, and a final extension at 72°C for 7 min. A 5.0-μL aliquot of the PCR product was analyzed by 1.2% agarose gel electrophoresis at 100 V for 25 min, stained with 1.5 μL SybrGold (Sigma), and 3.0 μL running buffer (bromophenol blue) using 1-kb Plus DNA ladder as a standard molecular marker (Promega). Gels were documented through the LabImage 1D software (Loccus) and photographed using LPIX-HE.

In the preparation of the PCR products for sequencing, the reaction purification process was performed as follows: 25.0 μL of the product sample, 2.0 μL 7.5 M ammonium acetate, 52.0 μL 100% ethanol, and centrifuged at 10,000 *g* for 45 min at 20°C, discarding the supernatant and pouring the tube on paper towel for 10 min. Then, 150 μL cold 70% ethanol was added, centrifuged again for 10 min at 10,000 *g*, the ethanol was discarded and poured on paper towels for 12 h. The pellet containing DNA was resuspended in 30.0 μL sterile ultrapure water and stored at -20°C. The sequencing of the 16S rRNA gene was provided byMacrogen Inc. (China) and STAB VIDA (Portugal).

Analysis of the sequences

The sequences were aligned using the SeqMan Pro 7.1.0 software Lasergene package (DNASTAR) and BioEdit 7.0.9, and subsequently cut ends after using the ClustalW alignment program. After the alignment, a matrix was generated using the MEGA V. 5.1 program, and the nucleotide sequences were analyzed by the standard genetic code. The phylogenetic tree was constructed by the method of neighbor joining (NJ), using the bootstrap phylogeny test, with 1000 repetitions, pairwise deletion. The sequences obtained were submitted to a comparative analysis in the NCBI database using the BLASTn (basic local alignment search tool) GenBank, for the identification of target sequences.

RESULTS AND DISCUSSION

A total of 36 isolates were selected by BOX-PCR analysis, and they were used as template for PCR amplification (16S rRNA). This amplification produced single fragments with 1500 bp, which was expected for this gene. The alignment of sequences separated the isolates into seven groups according to the 16S rRNA ribosomal gene (Figure 1).

Group 1 presented four isolates with similarity to the order Bacillales (IPA-CC6, IPA-CC25, IPA-CC29, and IPA-CC23). In this group, the isolates IPA-CC6 and IPA-CC25 showed 99% identity with *Bacillus subtilis* strain AN5 and 98% with *Paenibacillus* sp B38-1. The

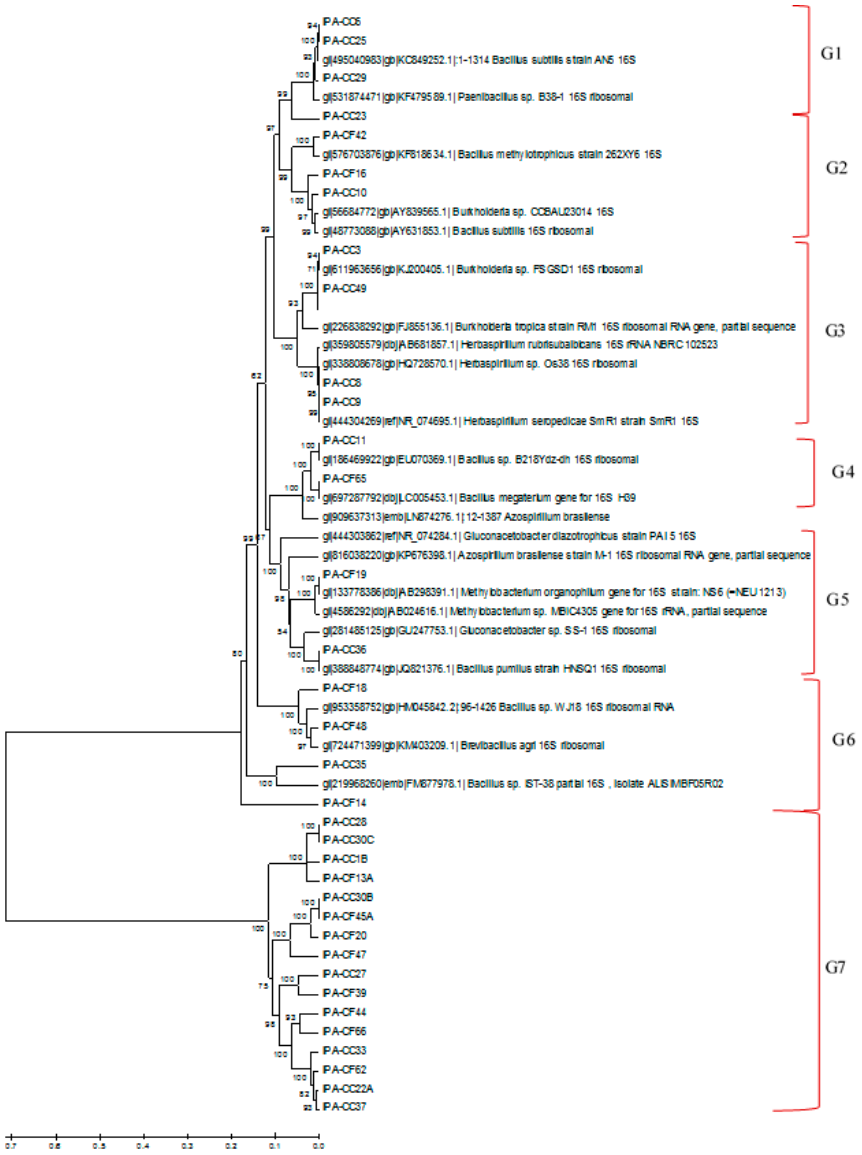

Figure 1. Phylogenetic tree constructed from the sequences of the 16S rRNA isolated from sugarcane, compared to sequences already deposited in databases, using the neighbor-joining method, with distances calculated by the Jukes-Cantor method. The values for each branch represent the percentages of 1000 bootstrap replicates. Bootstrap values below 55% are unresolved.

isolates IPA-CC29 and IPA-CC23 had 98 and 88% identities with *B. subtillis* and *Paenibacillus* sp, respectively. Group 2 presented three isolates belonging to the orders Bacillales and Burhholderiales. The isolate IPA-CF42 showed 96% identity with *B. methylotrophicus* strain 262XY6, while both IPA-CF16 and IPA-CC10 showed 96% identity with *B. subtilis* and *Burkholderia* sp strain CCBAU23014.

Group 3 presented isolates belonging to class β-proteobacteria. The isolates IPA-CC3A and IPA-CC49 showed 98 and 99% identities with *Burkholderia* sp FSGSD1, respectively; whereas IPA-CC8 and IPA-CC9 showed 99% identity with *Herbaspirillum seropecicae* strain SMR1. Group 4 represented isolates from the order Bacillales, where IPA-CC11 had 100% identity with *Bacillus* sp B218Y-dh and IPA-CF65 showed 99% identity with *Bacillus megaterium* DSM319.

Two isolates (IPA-CF19 and IPA-CC36) belonged to Group 5, where both IPA-CF19 and IPA-CC36 showed 99% identity with *Methylobacterium organophilum* and *Bacillus pumilus* HNSQ1, respectively. Group 6 consisted of four isolates belonging to the order Bacillales. In this group, the isolates IPA-CF18 and IPA-CF48 had 92 and 97% identities with *Bacillus* sp and *Brevibacillus agri*, respectively; while IPA CC35 and IPA-CF14 presented 82 and 84% identities with *Bacillus* sp and *Paenibacillus* sp B381, respectively (Table 1).

Table 1. Identification of endophytic stalk isolates and sugarcane leaves based on the sequence identity of the partial 16S rRNA performed by the BLAST GenBank.

Isolates	Base pairs	Max. score[1]	Query cover[2]	Identity[3]	E-value[4]	Accession[5]	Most significant alignment with the NCBI GenBank
IPA-CC1B	1331	2438	100%	99%	0.0	HM045842.1	*Bacillus* sp
IPA-CC3A	1401	2569	100%	99%	0.0	HQ023278.1	*Burkholderia gladioli*
IPA-CC6	1401	2418	100%	98%	0.0	KF479589.1	*Paenibacillus* sp
IPA-CC8	1378	2531	100%	99%	0.0	NR_074695.1	*Herbaspirillum seropedicae*
IPA-CC9	1378	2536	100%	99%	0.0	NR_114142.1	*Herbaspirillum seropedicae*
IPA-CC10	1433	2418	99%	97%	0.0	AY839565.1	*Burkholderia* sp
IPA-CC11	1381	2551	100%	100%	0.0	EU070369.1	*Bacillus* sp
IPA-CF13A	1360	2132	99%	95%	0.0	HM045842.1	*Bacillus* sp
IPA-CF14	1113	649	97%	84%	0.0	KF479589.1	*Paenibacillus* sp
IPA-CF16	1408	2252	99%	96%	0.0	LM655316.1	*Bacillus* sp
IPA-CF18	1071	2037	99%	94%	0.0	HM045842.1	*Bacillus* sp
IPA-CF19	942	1733	99%	99%	0.0	AB298391.1	*Methylobacterium organophilum*
IPA-CF20	762	1216	96%	96%	0.0	KC443087.1	*Bacillus megaterium*
IPA-CC22A	1382	2327	100%	97%	0.0	KF479589.1	*Paenibacillus* sp
IPA-CC23	1003	1077	94%	88%	0.0	KF479589.1	*Paenibacillus* sp
IPA-CC25	1401	2399	93%	99%	0.0	KC849252.1	*Bacillus subtilis*
IPA-CC27	1171	1971	99%	97%	0.0	AY873982.1	*Burkholderia* sp
IPA-CC28	1377	2407	100%	98%	0.0	AM110940.1	*Bacillus subtilis*
IPA-CC29	1395	2178	100%	98%	0.0	KC849251.1	*Bacillus subtilis*
IPA-CC30B	1399	2584	100%	100%	0.0	KR259220.1	*Bacillus megaterium*
IPA-CC30C	1364	2388	99%	98%	0.0	AM110938.1	*Bacillus subtilis*
IPA-CC33	1373	2518	100%	99%	0.0	FJ588230.1	*Pseudomonas* sp
IPA-CC35	1050	889	100%	82%	0.0	FM877978.1	*Bacillus* sp
IPA-CC36	1332	2449	100%	99%	0.0	JQ821376.1	*Bacillus pumilus*
IPA-CC37	659	1092	100%	97%	0.0	KF479589.1	*Paenibacillus* sp
IPA-CF39	599	985	96%	97%	0.0	JQ659427.1	*Methylobacterium* sp
IPA-CF42	1390	2302	100%	96%	0.0	KF818634.1	*Bacillus methylotrophicus*
IPA-CF44	589	1024	100%	98%	0.0	DQ813326.1	*Burkholderia* sp
IPA-CF45A	1257	2313	100%	99%	0.0	KR259244.1	*Bacillus megaterium*
IPA-CF47	1395	2429	99%	98%	0.0	AM162328.1	*Paenibacillus* sp
IPA-CF48	531	874	98%	97%	0.0	KM403209.1	*Brevibacillus agri*
IPA-CC49	1378	2492	100%	99%	0.0	KJ200405.1	*Burkholderia* sp
IPA-CF62	1392	2390	99%	98%	0.0	KF479589.1	*Paenibacillus* sp
IPA-CF65	1385	2558	100%	100%	0.0	LC005453.1	*Bacillus megaterium*
IPA-CF66	880	1531	99%	98%	0.0	JX867250.1	*Pseudomonas* sp

[1]Note assigned by the algorithm and based on the number of perfect matching (match) and imperfect (mismatch) between the input sequence and any sequence database. Its high value gives an indication of the alignment of quality. [2]Percentage of the input sequence aligned with the sequences of databases. [3]Number of base pairs identified between sequences isolated of sugarcane and the related body. [4]Alignment likely real or purely random among sequences, inputs, and databases obtained. The closer to zero, the more significant is the alignment. [5]Number of the sequence of related body access.

Finally, Group 7 showed sixteen distinct isolates without similarity with PGPB species from the GenBank database. Interestingly, this group revealed an unknown diversity of bacteria associated with sugarcane and could represent species with some biotechnological potential.

In our study, the high number of isolates found in the stalks and leaves of sugarcane indicates that these parts of the plant can host a high bacterium diversity as also reported by Gomes et al. (2005) and Suman et al. (2005), which confirmed that the stalk and leaves of sugarcane present high occurrence of PGPBs. This finding is important for sugarcane because its propagation occurs through a vegetative way and, thus, other plants can disseminate these PGPBs strongly.

Despite the high similarity found between isolates using the simple matching coefficient, some isolates presented high genetic difference, considering a cutoff of 80%. This cutoff may be considered high and can differentiate the isolates phylogenetically, as reported by Antunes (2010) studying the diversity of bacteria in *Phaseolus lunatus* L., where the cutoff was 80%. We found *Bacillus* as the most important genus associated with the stalk and leaves of sugarcane. Bacilli are microbes distributed in a great range of rhizosphere from different plants (Seldin et al., 1998), and recent studies have reported *Bacillus* associated with the rhizosphere of sugarcane (Gargi et al., 2014). Thus, the discovery of *Bacillus* hosted in the shoot of sugarcane open several possibilities to promote plant growth with this genus. These bacteria present high genetic diversity and agricultural relevance (McSpadden Gardener, 2004), mainly for presenting nitrogenase activity in some species, such as *B. subtilis*, *B. pumilus*, and *B. megaterium* (Xie et al., 1998). A previous study has reported that many species of *Bacillus* can contribute significantly to the plant growth (Davolos et al., 2015). Recently, Zhang et al. (2015) inoculated *Bacillus megaterium* in rice and observed a great promotion of growth, Mn uptake, and photosynthetic efficiency.

As the second most abundant genus found in our study, *Paenibacillus* presented similarities with some isolates and this genus is described as able to hydrolyze cellulose efficiently and economically (Hu et al., 2016). Daane et al. (2002) described *Paenibacillus* as distributed in soil, water, rhizosphere, plants, food, and insect larvae feed. *Burkholderia* was another important genus found in the stalk and leaves of sugarcane, and it agrees with previous studies that reported *Burkholderia* colonizing rhizosphere of some important crops, such as wheat (Balandreau et al., 2001) and corn (Arruda et al., 2013). Also, *Burkholderia* was reported in the tissue of grapes (Compant et al., 2005) and sugarcane (Boddey et al., 2003).

Similarly with *Bacillus* and *Paenibacillus*, *Burkholderia* has direct and positive effects on plant growth through BNF, phosphate solubilization, and the production of hormones (Bacha et al., 2016). Indirectly, this genus produces antagonistic compounds for pathogens, such as hydrolytic enzymes, and antibiotics (da Costa et al., 2014).

Bacteria from the genus *Herbaspirillum* were also found associated with the sugarcane varieties. The most known species *H. seropedicae* has been found associated with sugarcane, rice, maize, and sorghum (Baldani et al., 1996). This genus presents bacteria able to promote plant growth, mainly by the process of BNF, in wheat (Neiverth et al., 2014) and sugarcane (Canuto et al., 2003).

Although in low occurrence, *Methylobacterium* was found in the tissue of sugarcane, and this result corroborates with previous studies that reported this genus associated with sugarcane (Madhaiyan et al., 2006), rice (Madhaiyan et al., 2009), sunflower, corn, and soybean (Raja et al., 2008). These bacteria promote the plant growth through several mechanisms, such as using methanol as carbon source, production of hormones, auxins, and cytokinins (Raja et al., 2008). Other mechanisms promoted by *Methylobacterium* are regulation of ethylene levels

caused by stress in plants through the production of ACC deaminase (Madhaiyan et al., 2006), BNF (Sy et al., 2001), and synthesis of cellulase and pectin (Madhaiyan et al., 2006).

Bacteria from the genus *Pseudomonas* have been widely applied to produce organic compounds and to solubilize phosphates, and, thus, have high potential as PGPBs (Amaresan et al., 2012). Several studies have found *P. fluorescens*, *P. putida*, *P. aeruginosa*, and *Pseudomonas* sp promoting the plant growth through the BNF, production of hormones, and siderophores in rice (*Oryza sativa*), rapeseed (*Brassica napus* L.), ginger (*Zingiber officinale*), corn (*Zea mays* L.), and sugarcane (*Sarcarumm* L.) (Sulochana et al., 2014).

In this study, a single isolate (IPAC-CF48) showed similarity with the genus *Brevibacillus*, as also reported by Magnani (2010), who observed this genus associated with sugarcane in Brazil. Similarly, de Los Milagros Orberá Ratón et al. (2012) identified, in the roots of sugarcane, six isolates of *Brevibacillus* with antifungal activity, production of hormones, and phosphate solubilization.

Interestingly, the high dominance of bacteria from the phylum Firmicutes (*Bacillus* and *Paenibacillus*) was found, and it disagrees with previous studies which found Proteobacteria (*Burkholderia* and *Herbaspirilllum*) as the most abundant phylum associated with sugarcane (Reis Junior et al., 2000). In contrast with a previous study (Broek and Vanderleyden, 1995), we did not find *Azospirillum* associated with the stalk and leaves of sugarcane, and the main reason would be that this genus presents occurrence in the rhizosphere. Finally, our results highlight that there could be more distinct PGPB diversity in tissues than in roots of sugarcane, and suggest a novel and important source of new bacterium species for agricultural use as plant growth promoters.

CONCLUSIONS

The stalk and leave tissues of sugarcane revealed a high and distinct PGPB diversity associated with this crop. Different genera of PGPB, such as *Bacillus*, *Paenibacillus*, *Burkholderia*, *Herbaspirillum*, *Methylobacterium*, *Pseudomonas*, and *Brevibacillus* were found, indicating high ability to colonize the tissues of sugarcane, and opening a new way to prospect new species for agricultural use. Further studies should be done regarding the evaluation of the efficiency of these isolates as plant growth promoters and to produce new inoculants for sugarcane.

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