



Isolation and prospection of diazotrophic rhizobacteria associated with sugarcane under organic management

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ABSTRACT

Microorganisms associated with organic management are essential in nutrient transformation and release for plant use. The present study aimed to isolate, identify and characterize plant growth promoting diazotrophic rhizobacteria associated with sugarcane under organic management. Rhizospheres of organic sugarcane varieties IAC 911099 and CTC4 were sampled and inoculated onto nitrogen free NFb and Burk media. The isolated microorganisms were screened *in vitro* concerning their ability to produce plant growth promoting factors. Eighty-one bacteria were isolated; 45.6% were positive for the *nifH* gene and produced at least one of the evaluated plant growth promotion factors. The production of indole-3-acetic acid was observed in 46% of the isolates, while phosphate solubilization was observed in 86.5%. No isolates were hydrogen cyanide producers, while 81% were ammonia producers, 19% produced cellulases and 2.7%, chitinases. Microorganisms belonging to the *Burkholderia* genus were able to inhibit *Fusarium moniliforme* growth *in vitro*. Plant growth promoting microorganisms associated with organic sugarcane, especially belonging to *Burkholderia*, *Sphingobium*, *Rhizobium* and *Enterobacter* genera, can be environmentally friendly alternatives to improve sugarcane production.

Key words: organic agriculture, plant growth promoting rhizobacteria, *nifH*, *Burkholderia*, *Sphingobium*, *Rhizobium*.

INTRODUCTION

The use of petroleum-derived fertilizers and chemical pesticides is a common practice in agriculture. In view of the current scenario, the use

of these inputs is indispensable, making cultivation expensive and, associated with the expansion of the agricultural areas, causing a series of environmental impacts (Goldemberg et al. 2008, Severiano et al. 2009, Schultz et al. 2014). In this sense, a growing interest in cheaper and less environmentally

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impacting alternatives that reduce or replace the use of these implements in traditional agriculture has been observed (Crowder and Reganold 2015, Kanchiswamy et al. 2015).

The use of plant growth promoting rhizobacteria (PGPR) is one of the available alternatives to reduce the use of synthetic fertilizers and products from non-renewable sources, presenting low production/implantation costs (Sadeghi et al. 2012, Szilagy-Zecchin et al. 2014). PGPR correspond to bacteria that inhabit the rhizosphere, the soil zone immediately adjacent to the root, capable of promoting plant growth by one or more mechanisms (Noumavo et al. 2016). Their use is commonly adopted in traditional agriculture and empirically in organic agriculture, since this type of agriculture is extremely dependent on nutrient transformations mediated by natural soil microbiota (Pariona-Llanos et al. 2010, Bhardwaj et al. 2014, Järvan and Edesi 2015).

PGPR can promote plant growth by increasing plant nutrient availability through nitrogen fixation, mineral solubilization and iron adsorption (Kukla et al. 2014). They can also produce phytoestrogens, such as indole-acetic acid (IAA), gibberilic acid, cytokinins and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which stimulate root development and plant tissue growth (Sgroy et al. 2009). In addition, they play a fundamental role as biopesticides, through the production of metabolites that exhibit antibiotic action, and can also participate in the degradation of organic pollutants (Ahemad and Kibret 2014).

Due to the biological importance of nitrogen in biomolecule constitution and the high costs and environmental impacts caused by the production of synthetic nitrogen fertilizers, nitrogen-fixing bacteria (diazotrophic bacteria) are targets of interest (Karagöz et al. 2012). These microorganisms are able to convert molecular nitrogen (N_2) into ammonia (NH_3), a nitrogenous form absorbed by plants. This conversion is mostly mediated by the

iron-molybdenum (FeMo) nitrogenase enzyme, which is encoded by the *nif* complex genes (*nifH*, *nifD*, *nifK*). Because they are highly conserved throughout species, genes from the *nif* complex can be used as indirect evidence of the presence of nitrogenase and as molecular markers for diazotrophic organism screening (Dai et al. 2014, Ji et al. 2014, Kumar 2014, MacKellar et al. 2016).

Diabotrophic PGPR associated with sugarcane present high taxonomic diversity, with several noteworthy genera, such as *Herbaspirillum*, *Pantoea*, *Burkholderia*, *Azospirillum* and *Gluconacetobacter* (Lin et al. 2012, Gopalakrishnan et al. 2015, Tam and Diep 2015). In this scenario, several studies have been conducted to isolate diazotrophic bacteria associated with sugarcane under traditional management and to characterize plant growth promoting factors produced by these organisms (Luvizotto et al. 2010, Lima et al. 2011, Beneduzi et al. 2013, Kruasuwana and Thamchaipenet 2016).

However, research on diazotrophic PGPR associated with sugarcane under organic management is scarce. This group is of great interest, since one of the major challenges of organic agriculture is soil nitrogen limitation, as synthetic nitrogen compounds are abolished (Wongphatcharachai et al. 2015). Thus, PGPR presenting multiple plant growth promotion factors can become a viable alternative in biofertilization, phytoestimulation and biocontrol practices in organic sugarcane plantations.

In this context, the present study aimed to isolate and identify diazotrophic rhizobacteria associated with sugarcane under organic management from two sugarcane processing plants located in the state of Goiás, Brazil, and to evaluate plant growth promotion factor production.

MATERIALS AND METHODS

MICROORGANISM OR BACTERIA ISOLATION

The microorganism/microbial isolations were conducted from rhizospheric soil obtained from

two sugarcane varieties (IAC 911099 and CTC4), grown under organic management at farms belonging to two sugarcane processing plants in the cities of Goiatuba (19° 00' 023" S; 049° 40' 319" W) and Goianésia (15° 20' 241" S; 048° 54' 253" W), Goiás, Brazil. Samplings were carried out from October/2015 to January/2016, and each variety was obtained from four distinct farms, with three replications. Farms with sugarcane specimens planted in different years (2009, 2012, 2013 and 2014) and in different phenological stages were chosen, in order to obtain the greatest possible sample heterogeneity.

Sugarcane roots with adhered rhizospheric soil were conditioned in sterile plastic bags and stored at 4 °C until processing the following day. The rhizospheric soil was considered as the soil intimately connected to the roots at a distance of up to 5 mm, carefully removed with the aid of a sterilized spatula (Santos et al. 2012).

For isolation of potentially diazotrophic bacteria, 1.0 g of rhizospheric soil was incubated in 100 mL of phosphate buffer (0.8% NaCl, 0.02% KCl, 0.14% Na₂HPO₄, 0.024% KH₂PO₄, pH 7.4), under stirring at 130 rpm at 30 °C, for one hour. The obtained suspension was serially diluted and concentrations of 10⁻³, 10⁻⁴ and 10⁻⁵ were inoculated, in triplicate, in nitrogen-free semi-solid NFb (Dobereiner et al. 1976) and Burk's (Park et al. 2005) media.

The media were then incubated at 30 °C for 14 days. After growth, in the form of a diffuse film below the surface of the semi-solid medium, the microorganisms were peeled to new semi-solid media. After the growth in semi-solid media (three rounds), the microorganisms were transferred to the corresponding solid media. Microorganism isolation and purification occurred by depletion in solid media. Microorganisms whose colonies displayed the same morphology isolated from the same farm were discarded. After isolation, all colonies were transferred to nutrient agar plates

to verify possible bacterial morphotypes isolated simultaneously in the two media. After obtaining pure colonies, the samples were stored in 20% glycerol (m/v) at -20 °C.

DNA EXTRACTION

DNA extraction was performed from microorganism growth in Luria-Bertani broth (LB) for 24 hours at 30 °C. MoBio's UltraClean® Microbial DNA Isolation Kit was used for DNA extraction, according to the manufacturer's recommendations.

nifH GENE AMPLIFICATION

The gene encoding the nitrogenase reductase subunit of the enzyme nitrogenase complex (*nifH*) was amplified to confirm diazotrophic potential. The PCR reaction of the *nifH* gene was conducted using the oligonucleotide primers PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA), which generate a product with approximately 360 base pairs (Poly et al. 2001).

The reaction was prepared to a final volume of 10 µl, consisting of 1 x Taq polymerase buffer, 2.3 mM MgCl₂, 0.25 mM of each dNTP, 1.0 µM of each primer, 0.2 U of Taq DNA polymerase and 1.0 µl of template DNA (50 ng). Amplification was conducted in a thermal cycler by the following cycling steps: initial denaturation at 94 °C for 5 minutes; followed by 35 denaturation cycles at 94 °C for 45 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 30 seconds and a final extension step at 72 °C for 1 minute. The reaction products had their integrity confirmed on 1.2% (m/v) agarose gels. The nitrogen fixing strain *Azospirillum brasilense* was used as a positive control.

DIAZOTROPHIC RHIZOBACTERIA IDENTIFICATION

All bacteria whose *nifH* gene can be amplified were identified from the partial sequencing of the 16S

rRNA gene. The reaction was prepared to a final volume of 50 μL , containing 1 x Taq polymerase buffer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 0.2 mM of each primer, 2.5 U of Taq DNA polymerase and 1 μL of template DNA (50 ng). The 16S region was amplified using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3') (Lane 1991). DNA was amplified in a thermal cycler, under the following conditions: initial denaturation at 95 °C for 3 minutes; followed by 30 denaturation cycles at 94 °C for 1 minute, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds and a final extension step at 72 °C for 10 minutes.

The amplified products had their integrity confirmed on 1.2% agarose gels and purified with isopropanol and ethanol using the method described by Sambrook and Russell (2001). Sequencing was conducted on the ABI 3130xl platform (Applied Biosystems), using the BigDye terminator cycle sequencing kit and the oligonucleotide primers 27 F, 530F (5'-TGA CTG ACT GAG TGC CAG CMG CCG CGG-3') and 519R (5'-GTN TTA CNG CGG CKG CTG -3') (Lane 1991).

The reads obtained from the sequencing of each microorganism were evaluated regarding quality. Subsequently, the sequences (reads) were joined in a single sequence (contig). These steps were performed using the CodonCode Aligner software, version 6.0.2. Then, the sequences were compared to those deposited at the NCBI (National Center for Biotechnology Information) 16S rRNA library using the BLAST tool (Altschul et al. 1990). The identified sequences were deposited at the NCBI 16S rRNA database under accession numbers MG429815 to MG429819; MG459255 to MG459472 and MG459472.

In vitro SCREENING FOR PLANT GROWTH PROMOTING TRAITS

Microorganisms positive for the *nifH* gene were screened, *in vitro*, regarding their IAA, phosphate

solubilization, ammonia, hydrogen cyanide (HCN), cellulase and chitinase production capacity and antagonism to *Fusarium moniliforme*. All evaluations were carried out in triplicate and the results are expressed as the means of the replicates.

IAA PRODUCTION

Rhizobacteria ability to produce IAA was quantitatively determined by the method described by Gordon and Weber (1951), with modifications. The microorganisms were adjusted to a concentration of 10^8 cells mL^{-1} ($\text{OD}_{550\text{nm}}=0.1$) and inoculated in 10% trypticasein soy broth (TSB), supplemented with 5mM L-tryptophan. Growth aliquots were removed after 24, 48 and 72 hours and centrifuged for 12 minutes at 10,000 xg. The Salkowisk reagent (35% perchloric acid and 1 ml of 0.5M FeCl_3) was added to the culture supernatants at a 1:1 (v:v) ratio and the solutions were then incubated at room temperature, in the dark, for 30 minutes. After incubation, absorbances were determined on a spectrophotometer at 530 nm. IAA concentrations were determined by comparison to a standard curve constructed from a commercial IAA solution (0 $\mu\text{g mL}^{-1}$; 1.0 $\mu\text{g mL}^{-1}$; 5.0 $\mu\text{g mL}^{-1}$; 10 $\mu\text{g mL}^{-1}$; 25 $\mu\text{g mL}^{-1}$; 50 $\mu\text{g mL}^{-1}$; 75 $\mu\text{g mL}^{-1}$; 100 $\mu\text{g mL}^{-1}$; 150 $\mu\text{g mL}^{-1}$; 200 $\mu\text{g mL}^{-1}$). Results were expressed as $\mu\text{g mL}^{-1}$.

PHOSPHATE SOLUBILIZATION

Phosphate solubilization was quantitatively determined according to Nautiyal (1999). The microorganisms were adjusted to a concentration of 10^8 cells mL^{-1} ($\text{OD}_{550\text{nm}}=0.1$) and inoculated, in triplicate, in NBRIP broth (Nautiyal 1999). The samples were incubated for 7 days at 30 °C, under agitation at 130 rpm. After incubation, 1,000 μL aliquots were centrifuged at 10,000 xg for 5 minutes. Soluble phosphorus content was determined by the addition of the molybdate-vandate reagent (5.0% ammonium molybdate, 0.25% ammonium

vanadate) to the supernatants at the following ratio: 200 µl supernatant, 200 µl reagent and 600 µl distilled water. Solution absorbances were determined on a spectrophotometer at 420 nm. Soluble phosphate concentrations expressed as µg mL⁻¹ were obtained by comparison to a standard curve constructed from a stock KH₂PO₄ (0.0875%) solution and different soluble phosphate concentrations (0 µg mL⁻¹; 1.0 µg mL⁻¹; 5.0 µg mL⁻¹; 8.0 µg mL⁻¹; 10 µg mL⁻¹; 12 µg mL⁻¹; 15 µg mL⁻¹; 18 µg mL⁻¹; 20 µg mL⁻¹; 50 µg mL⁻¹; 75 µg mL⁻¹).

AMMONIA AND HCN PRODUCTION

For ammonia production evaluation, microorganisms were inoculated in peptone water and incubated at 30 °C for 7 days. After centrifugation at 10,000 xg for 10 minutes, the Nessler reagent (10% HgI₂, 7.0% KI and 50% aqueous NaOH 32% solution) was added to the supernatants at a 2:1 (v/v) ratio (Cappuccino and Sherman 1996). The development of a brown color was considered indicative of ammonia production (Dey et al. 2004).

HCN production was determined according to the technique described by Cattelan (1999), with modifications. Isolates were initially grown for 24 hours in 10% tripticasein soybean (TSA) agar, supplemented with 0.4% glycine. After microorganism growth, filter papers (Whatman # 1) embedded in a picric acid solution (0.5% picric acid and 2.0% Na₂CO₃) were deposited on the plate lids. The plates were then again incubated for 48 hours. Positive HCN production was considered when the filters became brownish (Walpolá and Yoon 2013).

CHITINASE AND CELULASE PRODUCTION

The ability of the isolates to produce chitinases was verified according to Cattelan (1999). Briefly, microorganisms were cultured for 14 days at 30 °C in nitrogen-free medium (MNL) supplemented

with 8.0 g L⁻¹ of colloidal chitin, 0.78 g L⁻¹ NH₄NO₃ and 15 g L⁻¹ agar. The addition of chitin generates turbidity in the medium. A clear halo around the colony is observed when microorganism display the ability to degrade the chitin.

Cellulase production evaluation was carried out by culturing the microorganisms in minimal medium containing carboxymethylcellulose (CMC) (Stamford et al. 1998). After growth at 30 °C for 48 hours, the plates were covered with a 0.3% Lugol solution. The presence of light halos around the colony were indicative of cellulase production (Kasana et al. 2008). For all evaluated enzymes, the enzymatic index (EI) was determined, obtained by dividing the size of the halo by the size of the colony.

ANTAGONISM AGAINST *F. moniliforme*

Bacteria were evaluated, *in vitro*, regarding their ability to inhibit the phytopathogenic fungus *F. moniliforme*, according to the methodology described by El-Sayed et al. (2014), with modifications. The microorganisms were initially cultured in TSA broth at 30 °C for 48 hours. Subsequently, they were inoculated into two parallel striae, 1.5 cm distant from the plate border, in potato dextrose agar. A 5 mm diameter disc with fungus growth was placed in the center of the plate, previously cultured for 7 days at 30 °C in potato dextrose agar. After 7 days, the ability of the isolates to inhibit fungal growth was evaluated by measuring the distance between the bacterial colony and the fungal colony. A plaque without the bacterial inoculum was used as a negative control.

STATISTICAL ANALYSES

The data obtained from the *in vitro* tests were submitted to an analysis of variance (ANOVA) and means were compared applying the Scott-Knott test at a 5.0% significance using the Sisvar software, version 5.6 (Ferreira 2011). When necessary, data

that did not present homogeneous variances or normal distribution were log corrected.

RESULTS

MICROORGANISM ISOLATION AND IDENTIFICATION

Eighty-one sugarcane rhizosphere bacteria were isolated from sugarcane under organic management in nitrogen-free culture media. For the purposes of isolate nomenclature, the first letter corresponds to the isolation medium (B = Burk and N = NFB), the second, to the sugarcane variety (C = CTC4 and U = IAC 911099), and the numbers refers to farm and striking order, respectively (Table I). Regarding origin, 57.3% of the bacteria were isolated from the CTC4 variety and 42.7% from the IAC 911099 variety. The *nifH* gene was amplified in 45.6% of the isolates. All bacteria whose *nifH* gene could be amplified produced more than one mechanism to promote plant growth, characterizing them as rhizobacteria carrying multiple plant growth promoting factors.

The 37 diazotrophic microorganisms identified by the partial sequencing of the 16S rRNA gene were distributed throughout 13 genera (Table I). The size of the obtained fragments varied from 473 to 1467 base pairs, with similarity between 97 and 99% (Table I).

The most common genera detected were *Burkholderia*, *Bacillus* and *Rhizobium*, with 15, 6 and 3 isolates, respectively. In addition, bacteria belonging to the *Cupriavidus* (n=2), *Dyella* (n=1), *Enterobacter* (n=2), *Erwinia* (n=1), *Flavobacterium* (n=1), *Methylobacterium* (n=1), *Mitsuaria* (n=1), *Sphingobium* (n=2), *Sphingomonas* (n=1) and *Variovorax* (n=1) genera were also detected. The classification of isolates NC92, BC26, NU45, NU92, NU32, BU97, BU32, BU92, NC93, BC91 and BU24 occurred at the species level, supported by 99% similarity values (Table I).

All microorganisms belonging to the *Bacillus* genus were isolated from the CTC4 variety and found in most of the investigated sugarcane processing farms. The *Burkholderia* genus, however, was most frequently associated with IAC 911099 variety (Table I). The microorganism *Sphingobium yanoikuyae* (BU32 and BU92) was isolated from different locations and in the same sugarcane variety, while *Rhizobium tropici* (BC91 and BU24) was isolated from different localities and varieties (Table I).

In vitro SCREENING FOR PLANT GROWTH PROMOTING TRAITS

IAA production was observed in 46% of the evaluated diazotrophic isolates. The produced IAA concentrations ranged from 0.5 to 36.15 $\mu\text{g mL}^{-1}$ in 24 hours, from 0.5 to 59.48 $\mu\text{g mL}^{-1}$ in 48 hours and 0.23 to 87.07 $\mu\text{g mL}^{-1}$ in 72 hours (Table II). At 72 hours, most isolates produced over 20 $\mu\text{g mL}^{-1}$ IAA. The highest IAA value was produced by *Sphingobium yanoikuyae* (BU32), followed by microorganisms belonging to the *Sphingobium* (BU92), *Sphingomonas* (BC44), *Enterobacter* (NC25 and NU33) and *Rhizobium* (NC24) genera.

The ability to solubilize phosphate was observed in 86.5% of the evaluated isolates, ranging from 1.82 to 53.78 $\mu\text{g mL}^{-1}$ (Table II). Microorganisms belonging to the *Burkholderia* genus were noteworthy regarding phosphate solubilization, solubilizing 53.78 $\mu\text{g mL}^{-1}$ (NC97), 50.08 $\mu\text{g mL}^{-1}$ (NU93), 50.03 $\mu\text{g mL}^{-1}$ (BU95), 45.82 $\mu\text{g mL}^{-1}$ (NU92) and 44.8 $\mu\text{g mL}^{-1}$ (BC43). It is importante to note that *Sphingobium yanoikuyae* (BU32), *Rhizobium* sp. (NC24), *Enterobacter* sp. (NC25) and *Enterobacter* sp. (NU33) as well as being the best IAA producers, were also efficient regarding phosphate solubilization (Table II).

Ammonia production was detected in 81% of the isolates, being the second most common plant growth promotion factor, after phosphate solubilization. In addition to nitrogen fixation

TABLE I
Rhizobacteria isolated from sugarcane under organic management identification based on comparisons with 16S rRNA region sequences deposited at the GenBank database.

Isolate identification*	Fragment size	Nearest species	Similarity	Accession number of the nearest species	Deposit number
BC211	1171	<i>Burkholderia</i> sp.	97%	NR_026462.1	MG459286
BC23	961	<i>Bacillus aryabhatai</i>	99%	NR_115953.1	MG459285
BC26	473	<i>Bacillus megaterium</i>	99%	NR_117473.1	MG459284
BC27	1443	<i>Bacillus</i> sp.	97%	NR_116873.1	MG459283
BC28	1071	<i>Bacillus</i> sp.	98%	NR_148626.1	MG459282
BC42	1255	<i>Cupriavidus</i> sp.	98%	NR_113619.1	MG459281
BC43	1246	<i>Burkholderia</i> sp.	98%	NR_041720.1	MG459280
BC44	522	<i>Sphingomonas</i> sp.	99%	NR_113637.1	MG459279
BC91	1183	<i>Rhizobium tropici</i>	99%	NR_102511.1	MG459278
BC93	723	<i>Burkholderia</i> sp.	97%	NR_102890.1	MG459277
BC95	1241	<i>Burkholderia</i> sp.	99%	NR_042632.1	MG459276
BU23	1239	<i>Burkholderia</i> sp.	98%	NR_126274.1	MG459275
BU24	1170	<i>Rhizobium tropici</i>	99%	NR_102511.1	MG459274
BU32	777	<i>Sphingobium yanoikuyae</i>	99%	NR_113730.1	MG429815
BU92	1197	<i>Sphingobium yanoikuyae</i>	99%	NR_113730.1	MG459272
BU95	1229	<i>Burkholderia</i> sp.	99%	NR_136496.1	MG459271
BU96	1266	<i>Dyella</i> sp.	97%	NR_043258.1	MG459270
BU97	1258	<i>Burkholderia vietnamiensis</i>	99%	KF114029.1	MG459269
BU98	1138	<i>Burkholderia</i> sp.	97%	NR_114491.1	MG459268
NC24	724	<i>Rhizobium</i> sp.	97%	FN645738.1	MG459267
NC25	1233	<i>Enterobacter</i> sp.	97%	NR_146667.2	MG459266
NC26	1467	<i>Flavobacterium</i> sp.	97%	NR_134727.1	MG459265
NC27	1168	<i>Bacillus</i> sp.	97%	NR_118442.1	MG459472
NC29	1118	<i>Cupriavidus</i> sp.	97%	NR_113619.1	MG459264
NC43	535	<i>Erwinia</i> sp.	97%	NR_074869.1	MG459263
NC49	1253	<i>Methylobacterium</i> sp.	99%	NR_074244.1	MG459262
NC92	631	<i>Bacillus megaterium</i>	99%	NR_117473.1	MG429816
NC93	1090	<i>Variovorax soli</i>	99%	NR_043811.1	MG459261
NC97	755	<i>Burkholderia</i> sp.	99%	NR_104975.1	MG429817
NU21	1406	<i>Burkholderia</i> sp.	99%	NR_113645.1	MG459260
NU31	1082	<i>Burkholderia</i> sp.	99%	NR_114491.1	MG459259
NU32	770	<i>Burkholderia gladioli</i>	99%	NR_113629.1	MG429818
NU33	1376	<i>Enterobacter</i> sp.	99%	KF010362.1	MG459258
NU41	769	<i>Mitsuaria</i> sp.	98%	NR_114070.1	MG429819
NU45	1279	<i>Burkholderia ambifaria</i>	99%	NR_074687.1	MG459257
NU92	1197	<i>Burkholderia cepacia</i>	99%	NR_114491.1	MG459256
NU93	1339	<i>Burkholderia</i> sp.	99%	NR_114491.1	MG459255

* Regarding isolate nomenclature: N/B corresponds to the isolation medium (NFB and Burk) and C/U correspond to the variety (C = CTC4 and U = IAC1099).

capacity, ammonia production was the only plant growth promoter observed for isolates *Methylobacterium* sp. (NC49), *Mitsuaria* sp. (NU41) and *Variovorax soli* (NC93). HCN production was not observed in any of the isolates.

Cellulase producers accounted for 19% of the isolates, with enzymatic indices ranging from 1.5 and 3.41. Most of the cellulase-producing isolates belonged to the *Bacillus* genus (BC23, BC26, BC27, BC28 and NC27), but representatives of the *Flavobacterium* (NC26) and *Sphingobium* (BU32) genera were also proven as enzyme producers (Table II). Only *Dyella* sp. (BU96) was chitinase-producing, with an enzyme index of 2.8 (Table II).

The ability to inhibit the pathogenic fungus *F. moniliforme* was observed for 21.7% of the isolates. All isolates displaying antagonism to the pathogenic fungus belonged to the *Burkholderia* genus. Isolates NC97, BU95, NU21, NU31 and NU45 exhibited the greatest distances between the fungal and bacterial colonies (Table II). It is noteworthy that not all microorganisms that presented fungus antagonism were indirect plant growth mechanisms producers (ammonia, HCN, cellulases and chitinases), responsible for pathogen attacks.

DISCUSSION

In the present study, 81 microorganisms from sugarcane under organic management were isolated using NFb and Burk nitrogen-free semi-solid media. The bacteria were isolated based on their morphological diversity, which takes into account characteristics such as color, contour and elevation which allows to classify the bacteria differentially (Di Franco et al. 2002). This strategy was used to capture the largest number of isolates belonging to different species and to maximize resources. On the other hand, when discarding morphologically equivalent microorganisms, some species may have been underestimated, since there is no genetic

evidence that two similar colonies are of the same species (Lebaron et al. 1998).

Although the NFb medium facilitates the isolation of bacteria belonging to the *Azospirillum* genus (Kuss et al. 2007), several studies using this medium have described the isolation of other genera, such as *Gluconacetobacter*, *Herbaspirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Pantoea* and *Pseudomonas* (Ambrosini et al. 2012, Tam and Diep 2015). On the other hand, Burk's medium is not specific, being associated to a range of fixing species (Park et al. 2005). Semi-solid nitrogen free media are considered ideal for diazotrophic bacteria isolation and screening. The reduced agar concentration determines an ideal microaerophilic condition for nitrogenase action, reduced in high oxygen concentrations (Baldani et al. 2014).

The isolation and application of diazotrophic bacteria in crops under organic regimes is extremely interesting from an agricultural point of view, since the great limitation of organic crops is precisely the available nitrogen supply. Diazotrophic microorganisms could be applied as substitutes to synthetic fertilizers (Wongphatcharachai et al. 2015) or in association with animal and vegetable manure, usually used as nitrogen sources in organic crops (Hirel et al. 2011).

The *nifH* gene, being conserved throughout species, is often used as molecular marker for the screening of nitrogen-fixing bacteria (Raymond et al. 2004, Gaby and Buckley 2012) and was amplified in 45.6% of the isolates detected in this study. The fact that the *nifH* gene has not been amplified, however, does not mean that the microorganism is not fixative. The sequence of the main widely used primers, mostly constituted by degenerate bases, are designed to flank the same region or overlapping sites, restricting their reach (Poly et al. 2001, Zehr et al. 2003). In addition, not all organisms exhibit iron-molybdenum nitrogenase (*nif*), since other nitrogenases also exist, differentiated by the metal

TABLE II
In vitro production of plant growth promoting traits by diazotrophic bacteria isolated from sugarcane under organic management¹.

Isolate	IAA ($\mu\text{g mL}^{-1}$)			Phosphate ($\mu\text{g mL}^{-1}$)	Amo	Cel ²	Chit ²	Ant ³
	24h	48h	72h					
BC211	0	0	0	26.29g	+	-	-	-
BC23	0	0	0	18.11f	+	2.71a	-	-
BC26	0	0	0	13.36e	-	3.41a	-	-
BC27	0	0	0	12.59e	+	2.20a	-	-
BC28	0	0	0	3.32b	+	2.00a	-	-
BC42	0	0	0	1.82a	+	-	-	-
BC43	0	0	1.19b	44.81i	+	-	-	-
BC44	0	27.46f	48.65h	0	+	-	-	-
BC91	0	0	9.85e	10.20d	-	-	-	-
BC93	0	0	0	26.57g	-	-	-	-
BC95	4.49c	16.25e	14.04f	29.12g	+	-	-	-
BU23	0	0	0	27.67g	+	-	-	-
BU24	2.47b	3.25b	6.06d	6.66c	+	-	-	-
BU32	36.15e	43.22g	87.07h	34.29h	-	3.00a	-	-
BU92	25.84e	59.48g	63.68h	0	+	-	-	-
BU95	0	0	0	50.03i	+	-	-	6a
BU96	0	0	0	9.64d	+	-	2.8a	-
BU97	0	0	4.15c	10.57d	+	-	-	3b
BU98	0	11.55d	30.23g	26.98g	+	-	-	-
NC24	23.59e	48.73g	64.52h	33.23h	+	-	-	-
NC25	22.81e	50.52g	80.42h	38.56h	+	-	-	-
NC26	0	0	0	16.14f	+	1.50a	-	-
NC27	0	0	0	24.96g	-	2.60a	-	-
NC29	0	0	0	15.64f	+	-	-	-
NC43	0	0	23.29g	35.32h	+	-	-	-
NC49	0	0	0	0	+	-	-	-
NC92	0	0	0	6.54c	+	-	-	-
NC93	0	0	0	0	+	-	-	-
NC97	0.5a	0.5a	3.37c	53.78i	-	-	-	7a
NU21	0	0	0	38.89h	+	-	-	5a
NU31	0	0	0.23a	32.98h	+	-	-	5a
NU32	0	0	0	15.09f	-	-	-	-
NU33	28.49e	37.85f	77.41h	34.37h	+	-	-	-
NU41	0	0	0	0	+	-	-	-
NU45	0	2.25b	2.9c	15.45f	+	-	-	4a
NU92	8.92d	9.29d	9.63e	45.82i	+	-	-	3b
NU93	0	0	0	50.08i	+	-	-	2c

IAA= production of 3-indole-acetic acid; Phosphate = phosphate solubilization; Amo = Ammonia; Cel = Cellulase; Chit = Chitinase; Ant = antagonism. ¹ Means followed by the same letters do not differ statistically by Scott-Knott's test at a 5% probability. ² The expressed values correspond to the enzymatic index, obtained by dividing the diameter of the halo by that of the colony. The "-" label indicates bacterial growth without halo formation and label "+" indicates a positive test. ³ The values presented correspond to the distance between the fungal and bacterial colony. The label "-" indicates that the bacterium was not antagonistic to the fungus.

contained in their structures, such as iron-vanadium nitrogenase (Vnf) or iron nitrogenase (Anf) (Dahal et al. 2017).

Other microorganisms are capable of growing in nitrogen-free media and are not fixers. It has been suggested that these organisms may incorporate ammonia or other nitrogen species at very low concentrations, or that they are capable of using fractions fixed by other organisms (Beneduzi et al. 2013, MacKellar et al. 2016). In addition, we emphasize that the amplification of the *nifH* gene has a confirmatory character, but cannot quantify the amount of fixed nitrogen, and the use of techniques such as acetylene reduction (ARA) and the incorporation of the $^{15}\text{N}_2$ isotope is recommended (Gtari et al. 2012).

The genus most detected in this study, *Burkholderia*, is widely distributed in nature and is often found associated with sugarcane under traditional management (Luvizotto et al. 2010, Castro-Gonzalez et al. 2011). From the point of view of organic agriculture, the *Burkholderiaceae* family and *nifH* genes have been reported as being more abundant in agricultural soils abundant in swine manure (Xun et al. 2018). In addition to their ability to fix nitrogen, it is also suggested that plant growth promotion performed by these organisms is associated with the production of IAA, phosphate solubilization and *in vitro* pathogen inhibition (Paungfoo-Lonhienne et al. 2014).

The genus *Bacillus*, the second most detected in the present study, has also been described in association with sugarcane under traditional management and presents potential regarding the production of enzymes of agronomic interest (Beneduzi et al. 2008, Madhaiyan et al. 2011, Pisa et al. 2011). The other genera found herein have been mostly associated with sugarcane under traditional management or cultivated soils (Nicolaisen et al. 2008, Burbano et al. 2011, Anand et al. 2012, Bers et al. 2012, Santi Ferrara et al. 2012, Fischer et al.

2012, Lin et al. 2012, Hoang et al. 2015, Solanki et al. 2017).

The presence of *Bacillus* and *Burkholderia* as more predominant groups was also observed by Xia et al. (2015), when evaluating the taxonomic distribution of maize, melon, pepper and tomato bacteria in organic systems. Oliveira (2009) using selective media for nitrogen fixers, including NFb, observed a range of microorganisms associated with sugarcane plantations under organic management in São Paulo, Brazil, with emphasis on *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Burkholderia* and *Beijerinckia* species. Those authors also detected other species, such as *Agrobacterium*, *Azospirillum*, *Bosea*, *Bradyrhizobium*, *Brucella*, *Cohnella*, *Erwinia*, *Gluconacetobacter*, *Rhizobium*, *Stenotrophomonas*, *Variovorax* and *Xanthomonas*.

The similarities and divergences of this study in relation to the study conducted by Oliveira (2009) reinforce the idea that, even if *Enterobacter*, *Burkholderia* and *Rhizobium* species are ubiquitous, factors such as plant age, variety, root exudates, management and soil chemical and physical attributes can influence the community distribution and species (Beneduzi et al. 2008, 2013, Santi Ferrara et al. 2012, Noumavo et al. 2016). Therefore, for each evaluated region, distinct distribution patterns of diazotrophic microorganisms are observed, which is interesting regarding PGPR prospecting.

Less than half of the evaluated isolates produced IAA, and a broad taxonomic variety was observed among the best producers. In addition, some individuals belonging to the same genus produced distinct IAA values and were classified into different groups, according to Scot-Knott's test at a 5.0% probability. *Enterobacter* sp. NC25 and NU33 strains produced 80.42 and 77.41 mg mL⁻¹, respectively, similar to the strains reported by Rodrigues et al. (2016) when evaluating *Enterobacter* sp. rhizospheres from traditionally managed sugarcane.

Although IAA production is commonly found among rhizobacteria (Sagar et al. 2017), different types of fertilizers impact on the number of bacteria in the soil and the diversity of bacteria producing this compound. In systems where, chemical fertilizers are withdrawn or where organic fertilizers are applied, the number and diversity of IAA-producing bacteria increases. The use of organic fertilizers improves the structure of the microbial community, in addition to selecting beneficial microorganisms (Yuan et al. 2011, Duangpaeng et al. 2012).

In addition to being the most active auxin, it is believed that IAA is physiologically active even at low concentrations, so even organisms that produce small IAA fractions may be able to contribute to plant health (Hayat et al. 2010, Vejan et al. 2016). IAA production leads to increased lateral roots, in addition to increasing root surface area and size, leading to increased water and nutrient supplies to plants (Brandl and Lindow 1998, Vessey 2003).

The ability to solubilize phosphate was the most common feature of the diazotrophic bacteria evaluated herein, with emphasis on bacteria belonging to the *Burkholderia* genus. In the case of sugarcane rhizobacteria under traditional management, Inui-Kushi et al. (2012) verified that *Burkholderia* bacteria displayed with the highest phosphate solubilization index among 10 different bacteria belonging to the same genus.

Although not a mandatory feature of the *Burkholderia* genus, several species belonging to this genus have been associated with varying phosphate solubilization and rhizosphere acidity levels (Castro-Gonzalez et al. 2011). When studying the impact of organic management on the diversity of pepper phosphate-solubilizing bacteria (*Capsicum frutescens* L. cv. Hua Rua), Surapat et al. (2013) observed that most of their phosphate-solubilizing isolates were recovered from organic systems, and that most belonged to the *Burkholderia* genus.

The most well-known phosphate solubilization mechanism involves medium acidification. Plants display the ability to acidify the rhizosphere, leading to phosphate solubilization, although this capacity is quite limited (Hamdali et al. 2008). It is suggested that even the phosphate solubilized in small amounts by bacteria still corresponds to a large part of the soluble phosphate available to plants, especially in organic crops, that are largely dependent on the natural soil microbiota and that display high amounts of organic phosphates (Pariona-Llanos et al. 2010).

Ammonia production is quite common among rhizobacteria, as observed in this study and in studies conducted by Joseph et al. (2007) and Gayathri et al. (2010). HCN production is rare, a fact corroborated by Kavamura et al. (2013) when evaluating bacteria obtained from cacti. Ammonia and HCN are volatile compounds that act as biocontrol agents. In addition to acting as pathogen responses, it is believed that ammonia can be used as a source of nitrogen supplementation by the host plant (Joseph et al. 2007, Marques et al. 2010, Passari et al. 2015). A study conducted by Marques et al. (2010) observed that ammonia production was positively correlated to nitrogen and phosphorus accumulation, root and stem elongation and biomass increases in maize.

The present study observed that most cellulase-producing isolates belonged to the *Bacillus* genus, similarly to the results reported by Zhao et al. (2015). Cellulase-producing microorganisms belonging to the *Bacillus* genus are widely distributed in the sugarcane rhizosphere. They are believed to participate in the degradation of cellulolytic debris originating from the plant itself and, consequently, in element cycling (Ratón et al. 2012). In addition to potential for pathogen inhibition, cellulase production in rhizobacteria plays an important role in the penetration of these organisms into the host plant during colonization (Pariona-Llanos et al. 2010).

The BU96 isolate from the *Dyella* genus isolated herein was the only isolate able to degrade chitin. Microorganisms belonging to this genus have been isolated from soil and described as chitin-degrading agents (Lee and Lee 2009). Although the presence of chitinolytic bacteria is ubiquitous, it is estimated that only 1% of rhizobacteria isolated from wheat, rice and maize present chitinolytic potential (Someya et al. 2011), which reinforces the low number of chitinolytics found in this study. The lytic enzymes cellulases and chitinases were selected for this screening since they are considered limiting factors for the growth of pathogenic fungi, as their lytic action is capable of causing degradation of the structural matrix of the fungal cell wall (El-Sayed et al. 2014).

Sugarcane is attacked by several pathogens such as viruses, bacteria, nematodes, insects and fungi. These organisms cause a number of productivity losses (Zhang et al. 2015). Among the main fungi species that attack sugarcane is *F. moniliforme*, which causes changes in leaf and stem morphology in non-pest-tolerant varieties (Lin et al. 2014, 2015). In this sense, rhizobacteria have been reported as biocontrol agents against pathogenic *F. moniliforme* strains (Hebbar et al. 1992, Figueroa-López et al. 2016).

Bacteria belonging to the *Burkholderia* genus are noteworthy with regard to the inhibition of the *F. moniliforme* pathogen. Several bacteria belonging to this genus isolated from different environments, such as tomato (Omar et al. 2006), barley (Simonetti et al. 2018) and soil in the Amazon region (Silva et al. 2012), are also capable of inhibiting the growth of phytopathogenic *Fusarium* strains. Some microorganisms belonging to this genus are believed to produce the antibiotic pyrrolnitrine, which is a fungus respiratory chain inhibitor (Parke and Gurian-Sherman 2001).

In vitro antagonistic test is important for the detection of potential biocontrol agents, especially in the presence of numerous isolates. However,

there is no guarantees that microorganisms producing antifungal substances *in vitro* will produce the same effects when inoculated in plants (Knudsen et al. 1997). In this sense, Shehata et al. (2016), simultaneously evaluated, *in vitro* and *in vivo*, the antifungal activity of 190 endophytic bacteria against the pathogen *Sclerotinia homoeocarpa*. These authors observed that out of 5 positive microorganisms in the *in vitro* tests, only 3 had *in vivo* activity. To produce antagonistic effects *in vivo*, microorganisms must be able to properly colonize the plant, settle in the plant tissue affected by the pathogen and survive the competition with the host's natural microbiota (Pliego et al. 2011, Deketelaere et al. 2017).

It is recommended that the inoculant candidate presents the highest number of *in vitro* plant growth promoting traits, since this would lead to a higher probability of survival in the field, due to their ability to use several substrates (Rana et al. 2011). In this sense, the isolates: *Sphingobium yanoikuyae* (BU32), *Rhizobium* sp. (NC24), *Enterobacter* sp. (NC25) and *Enterobacter* sp. (NU33) are noteworthy in this study due to their ability to produce IAA, as well as the ability to solubilize phosphate, and microorganisms belonging to the *Burkholderia* genus, namely NC97, BU95, NU31, NU21 and NU92, due to their simultaneous ability to solubilize phosphate and promote biocontrol. In addition, it is suggested that these bacteria can be applied in mixed form in greenhouse and field evaluations in subsequent studies.

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