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## Bacteria associated with sugarcane in Northeastern Brazil

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Sugarcane has a high demand in nitrogen, increasing costs and causing damages to the environment. It is necessary to find alternatives to reduce nitrogen fertilizers use. Diazotrophic bacteria have capacity to promote growth in grass with potential to fix N<sub>2</sub> and solubilize inorganic phosphate. This study aimed to evaluate bacterial community associated with different sugarcane varieties in Northeastern Brazil, select bacteria with plant growth-promoting characteristics, and identify endophytic and epiphytic bacterial lineages in sugarcane. Endophytic bacteria of leaves and roots and epiphytic bacteria of rhizoplane were isolated from three sugarcane commercial varieties and selected for their capacity to fix N<sub>2</sub> and solubilize inorganic phosphate. Bacterial strains from different morphological groups were isolated and a sample of 27 strains with potential for the simultaneous development of these characteristics were selected and identified. The bacterial community that interacted with sugarcane was more associated with rhizoplane and roots regions than with leaves, and had a high potential to fix N<sub>2</sub> and solubilize inorganic phosphate. Bacterial lineages were mainly from genera *Pantoea* sp. and *Burkholderia* sp., but there were also genera *Enterobacter* sp., *Klebsiella* sp. and *Pseudomonas* sp. and two lineages at the species level: *Pantoea stewartii* and *Burkholderia cenocepacia*.

**Key words:** Plant/bacteria interaction, plant growth-promoting bacteria, N<sub>2</sub> fixing bacteria, bacteria identification.

### INTRODUCTION

In Northeastern Brazil, sugarcane cultivation is traditional and its management is performed in the conventional

way, using a large amount of chemical inputs, especially nitrogen and phosphate fertilizers, increasing production

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costs and contaminating the environment by hydric resources eutrophication. Studies associating microorganisms with plant growth promotion are very important for sugarcane cultivation in this region and represent technological innovation.

There are various bacterial genera associated with plants that have the capacity to fix  $N_2$  biologically, among which *Azoarcus* sp., *Azospirillum* sp., *Azotobacter* sp., *Beijerinckia* sp., *Burkholderia* sp., *Enterobacter* sp., *Erwinia* sp., *Herbaspirillum* sp., *Gluconoacetobacter* sp., *Klebsiella* sp., *Pseudomonas* sp. and *Pantoea* sp. (Carvalho et al., 2014).

Biological  $N_2$  fixation (BNF) has been one of the mechanisms explored in the interaction between plant and microorganisms in many studies and in different crops, such as wheat (Didonet et al., 1996), rice (Verma et al., 2001), maize, sorghum and wheat (Roncador-Maccari et al., 2003), soybean (Souza et al., 2008) and common bean (Brito et al., 2011). In sugarcane, the studies have focused on varieties cultivated in Southeast region of the country and have found important bacterial groups that perform BNF (Rennie et al., 1982; Oliveira et al., 2002; Loiret et al., 2004; Perin et al., 2004; Reis et al., 2004; Luvizotto et al., 2010; Costa et al., 2014).

Resende et al. (2006) reported that biological  $N_2$  fixation (BNF) performed by bacteria associated with sugarcane can be responsible for up to 60% of the total N accumulated in the plants. This means that BNF can contribute to reducing the use of N fertilizers, especially for the production of sugarcane in the first crop cycle (Polidoro et al., 2006; Resende et al., 2006).

Moreover, microorganisms perform essential functions for increasing P availability in the soil to plants, through mechanisms that affect structure, chemistry, biochemistry and physiology of the root environment. These mechanisms include capacity to solubilize insoluble inorganic phosphates, increasing P soluble content in the soil solution and, consequently, its availability to plants (Goldstein et al., 1999; Chen et al., 2006).

Microorganisms use mechanisms like production and release of low-molecular-weight organic acids, which solubilize P precipitated forms such as Fe and Al phosphates in acid soils and Ca phosphates in alkaline soils, acting as sources of protons or chelating accompanying element of the phosphate ion (Marra et al., 2012).

Association between diazotrophic bacteria and sugarcane involves singular mechanisms, which are still little understood (Carvalho et al., 2014). The main sugarcane productive region in Brazil is Southeast region, where only some genera of endophytic bacteria were isolated in this crop and a few were studied, such as: *Enterobacter* sp., *Erwinia* sp., *Klebsiella* sp. (Rennie et al., 1982), *Herbaspirillum* sp. (Oliveira et al., 2002) and *Burkholderia* sp. (Reis et al., 2004; Luvizotto et al., 2010). Some species were also found and described, such as: *Bacillus polymixa* (Rennie et al., 1982), *Pantoea agglomerans*

(Loiret et al., 2004) and *Gluconoacetobacter diazotrophicus* (Perin et al., 2004).

Some studies in South region (Magnani et al., 2010; Beneduzi et al., 2013) identified bacteria in sugarcane from Parana state and Rio Grande do Sul State, but sugarcane is not expressive crop in this region.

For Northeast region, there are no published studies that have isolated and identified genera of endophytic bacteria in sugarcane. The study of Resende et al. (2006) performed a N balance in the soil/plant system in an experiment in Pernambuco state and concluded that N entry through BNF was considerable and consistent with the low responses to N application in fertilizer form and also with low efficiency of use of N fertilizer.

Some studies have recently been conducted in Northeast region (Lira-Cadete et al., 2012; Santos et al., 2012; Silva et al., 2012; Barros et al., 2014); however, they only emphasize bacteria association with sugarcane in aspects related to BNF, production of growth phytohormones and solubilization of inorganic phosphate. It is important that these studies continue to be conducted, but they should also associate these benefits of plant growth with the identification of endophytic bacterial genera in sugarcane in Brazil Northeast region.

Knowledge on the bacterial community associated with sugarcane and study on their beneficial effects can increment agricultural yield, reduce chemical inputs use, reduce production costs and minimize contamination of the production environment.

Therefore, this study aimed to evaluate cultivable bacterial community associated with different varieties of sugarcane in the first crop cycle at four months of age in Northeastern Brazil, select bacteria with plant growth-promoting characteristics and identify endophytic and epiphytic bacterial lineages in different plant/bacteria association regions.

## MATERIALS AND METHODS

### Site description

Bacterial community study was conducted in plant samples obtained from first-cycle commercial plantations of three sugarcane varieties at Sugarcane Experimental Station of Carpina (EECAC), which is located at 7°51'04" S and 35°14'27" W, in sugarcane region of Zona da Mata in Pernambuco state, Northeast Brazil. Soil cultivated with the varieties was classified as dystrocohesive Yellow Argisol, according to Santos et al. (2013), corresponding to Ultisol (Soil Survey Staff, 1998). This soil is common in Northeast Brazil, predominantly in Pernambuco, and normally used for sugarcane cultivation.

Experimental area was prepared with a disk harrow, a leveling harrow and a two-row furrower. The leveling harrow also served to incorporate 1.5 mg ha<sup>-1</sup> of dolomitic limestone with relative neutralizing value (RNV) of 95%, 30 days before planting in the areas cultivated with RB 867515 and RB 863129 varieties. Acidity correction was not necessary in the area cultivated with the variety RB 92579. Fertilization at planting corresponded to the application of 60, 120 and 90 kg ha<sup>-1</sup> of N, P<sub>2</sub>O<sub>5</sub> and K<sub>2</sub>O, respectively, at the bottom of the planting furrows. The fertilizer sources used were

ammonium sulfate, triple superphosphate and potassium chloride, respectively. The amounts of correctives and fertilizers at planting were based on the Manual of Recommendation of Fertilization for Pernambuco State (Cavalcanti et al., 2008). The area cultivated with RB 867515 variety received pre-emergence herbicide at the dose of 2.5 L ha<sup>-1</sup>, because it was vulnerable to emergence, growth and development of spontaneous plants.

### Leaves, roots and soil sampling

Sugarcane plant varieties used for sampling were RB 863129, RB 92579 and RB 867515. These are commercial varieties used in the Brazil Northeast region, and Simões Neto and Melo (2005) reported that RB 863129 has early maturation and can be used in any production environment, while RB 92579 and RB 867515 have medium and late maturation, respectively. These authors also point out that RB 867515 can be used in soil with low fertility, due to its high efficiency in nutrients use. Endophytic bacteria of leaves and roots and epiphytic bacteria of the rhizoplane (region where the soil is adhered to the roots) were isolated from these three commercial varieties of sugarcane. At for months of age, ten plants of each sugarcane variety were selected, totaling 30 samples. Plants with higher physiological vigor were selected for a better representation of the variety. Each plant, its root system and the soil from the rhizosphere were placed in 100 L plastic bags, which were identified and taken to Laboratory of Genetics and Microbial Biotechnology (LGBM), at Academic Unit of Garanhuns (UAG/UFRPE), for the procedures of isolation, plant growth promotion tests and identification.

### Bacteria isolation

#### Endophytic bacteria isolation

The procedure was performed in four plants of each variety, using 12 samples of leaf mass and 12 samples of roots. Endophytic bacteria were isolated in cubes of tissues weighing from 1 to 3 g of fresh matter of leaves and roots. Leaves and roots were washed in running water.

#### Epiphytic bacteria isolation

The soil of the rhizosphere was used for epiphytic bacteria isolation. These bacteria were isolated using a phosphate-buffered saline (PBS) solution in 500 mL Erlenmeyer flask for each root sample (12 samples). Each Erlenmeyer received 50 mL of PBS buffer solution and glass beads to facilitate soil removal from rhizoplane. Epiphytic bacteria from rhizoplane, and endophytic bacteria from leaves and roots were isolated according to Kuklinsky-Sobral et al. (2004) and Mendes et al. (2007). TSA (Trypticase Soy Agar) medium at 10% supplemented with the fungicide Cercobyn 700 (50 µg mL<sup>-1</sup>) was used for samples incubation. The samples were maintained at 28°C during incubation and evaluated after 3, 8 and 14 days. Bacterial population (colony-forming units – CFU) per gram of fresh plant tissue (CFU/g FPT) was estimated by counting colonies cultivated in 10% TSA medium. Characteristic colonies of each morphological type were subcultured from isolation plates, purified and maintained in liquid 10% TSA supplemented with 20% of glycerol at -20°C for formation and organization of a collection of bacterial cultures associated with sugarcane varieties cultivated in Brazil Northeast region.

### Selection N<sub>2</sub>-fixing bacteria

The bacteria were inoculated in semi-solid NFb medium [5 g L<sup>-1</sup>

malate; 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>; 0.2 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.1 g L<sup>-1</sup> NaCl; 0.01 g L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O; 4 mL L<sup>-1</sup> Fe.EDTA (1.64% solution); 2 mL L<sup>-1</sup> bromothymol blue (0.5%); 2 mL L<sup>-1</sup> solution of micronutrients (0.2 g L<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O; 0.235 g L<sup>-1</sup> MnSO<sub>4</sub>·H<sub>2</sub>O; 0.28 g L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>; 0.008 g L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O); 1.75 g L<sup>-1</sup> agar], buffered at pH 6.8 and maintained incubated at 28 °C for 8 days (Dobereiner et al., 1995).

The tests were performed in triplicate and the positive result for N fixation was characterized by the presence of a growth halo inside the vials, which grew until the surface of the culture medium. The bacteria were subcultured five times more in new semi-solid NFb media for the confirmation of atmospheric N<sub>2</sub> fixer characteristic.

### Selection inorganic phosphate-solubilizing bacteria

Inorganic phosphate-solubilizing bacteria were selected according to the procedure of Rodriguez et al. (2000), with a few modifications. This methodology recommends the use of 0.7 g L<sup>-1</sup> of tri-calcium phosphate [Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>]. Since it was not possible to obtain this compound, 4.0 g L<sup>-1</sup> of mono-calcium phosphate (CaHPO<sub>4</sub>) were used. The solubility of Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> is five times higher than that of CaHPO<sub>4</sub>; thus, it was used an amount five times higher of the latter.

Bacteria were inoculated in solid culture medium (10 g L<sup>-1</sup> glucose; 5 g L<sup>-1</sup> NH<sub>4</sub>Cl; 1 g L<sup>-1</sup> NaCl; 1 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O; 15 g L<sup>-1</sup> agar), containing insoluble phosphate (4 g L<sup>-1</sup> CaHPO<sub>4</sub>) buffered at pH 7.2 and maintained incubated at 28°C for 72 h. The experiments were performed in triplicate and the presence of a clear halo around the colony indicated phosphate solubilization.

### Lineages identification

The bacterial lineages were cultivated in 5 mL of TSA for 24 h at 28 °C under agitation at 166 g. Then, 4 mL of the culture were centrifuged for 5 min at 15,500 g and the cells were re-suspended in 500 µL of TE (10 mM Tris-HCl buffered at pH 8.0), centrifuged and re-suspended again in 500 µL of TE, with the adding of 0.5 g of glass beads (0.1 mm diameter) and 15 µL of 20% sodium dodecyl sulfate (SDS).

Cells were agitated in a homogenizer for 30 s at 3,875 g. Then, 500 µL of phenol were added to the lysed cells, homogenized through inversion and centrifuged for 5 min at 15,500 g. Aqueous phase was extracted once with chloroform-phenol (1:1) and once with chloroform. Later, DNA was precipitated with 1/10 volume of 5 mol L<sup>-1</sup> NaCl and 6/10 volume of isopropanol, and collected through centrifugation (10 min).

DNA precipitate was washed with 70% ethanol, dried at 37°C and re-suspended in 50 µL of TE. Total DNA was analyzed through electrophoresis in agarose gel (0.8% m/v) in 1x TAE buffer (40 mM Tris-acetate; 1 mM EDTA) and colored with ethidium bromide (0.5 µg mL<sup>-1</sup>), according to the procedures described by Sambrook et al. (1989).

16S rDNA was amplified through PCR technique using the universal primers for bacteria P027F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1378R (5'-CGGTGTGTACAAGGCCCGGAACG-3'), according to Heuer et al. (1997). The reactions were performed in 50 µL final volume containing 0.5 ng of total DNA (1.0 µL); 0.2 µM of each primer (0.1 µL); 0.2 mM of each dNTPs (4.0 µL); 3.75 mM MgCl<sub>2</sub> (7.5 µL); 0.05 U of enzyme Taq DNA polymerase (*Fermentas*) (0.5 µL); 5.0 µL of 10x buffer Taq (750 mM Tris-HCl pH 8,8, 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 0,1% Tween). Final volume was completed with autoclaved pure water.

Amplification reaction was performed in a thermocycler programmed for initial denaturation at 94°C for 4 min, 25 cycles denaturation at 94°C for 30 s, annealing temperature at 63°C for 1 min and extension of primers at 72°C for 1 min, followed by final

**Table 1.** Total population density of endophytic bacterial community in leaves and roots and epiphytic bacterial community in the rhizoplane of different 4-month-old sugarcane varieties in the first crop cycle.

Variety	Plant/bacteria association region			Average
	Leaf	Root	Rhizoplane	
CFU g <sup>-1</sup> FPT (Log <sub>10</sub> )				
RB 92579	3.02	6.09	8.65	6.08 <sup>a</sup>
RB 867515	3.92	5.43	8.39	5.43 <sup>b</sup>
RB 863129	4.18	6.88	8.66	6.88 <sup>a</sup>
Average	3.92 <sup>C</sup>	6.08 <sup>B</sup>	8.64 <sup>A</sup>	
Variation coefficient (%)			11.08	
Overall average			6.13	

Averages followed by same letter, lowercase in the column and uppercase in the row, do not differ by Scott-Knott test ( $p > 0.05$ ).

extension at 72°C for 7 min. After amplification, 10 µL of PCR reaction were evaluated through electrophoresis in agarose gel (1.2% m/v) in 1x TAE buffer and colored with ethidium bromide (0.5 µg mL<sup>-1</sup>).

PCR products were purified (*PCR purification Power Clean-Up kit, MoBio Laboratories*) and subjected to sequencing with primer 1378R, in an automated sequencer. Sequences were analyzed using BLASTn against database from NCBI (National Center for Biotechnology Information website [<http://www.ncbi.nlm.nih.gov>]).

### Statistical analyses

Total population density data of bacterial community were subjected to analysis of variance and effects of variety, plant/bacteria association region and interaction were evaluated by F test ( $p < 0.05$ ). When main effects and/or interaction were significant, Scott Knott test ( $p < 0.05$ ) was applied. For relative frequency of bacterial strains number per variety and plant/bacteria association region, Chi-square test ( $p < 0.01$ ) was applied.

## RESULTS

Total population density of bacterial community associated with sugarcane varied from 10<sup>3</sup> to 10<sup>8</sup> CFU g<sup>-1</sup> FPT (Table 1). And there was preferential colonization of endophytic and epiphytic bacteria in the root zone for the sugarcane varieties evaluated.

Population density of epiphytic bacteria was significantly higher (2.2 times) than that of endophytic bacteria in the leaves and significantly higher (1.4 times) than that of endophytic bacteria in the roots (Table 1). Endophytic bacteria population density was significantly higher (1.6 times) in the roots compared with the leaves (Table 1). The root environment, whether of endophytic or epiphytic bacteria, was more favorable for the bacterial community associated with sugarcane.

Total population densities of the bacterial community associated with the sugarcane varieties RB 92579 and RB 863129 were similar, and higher compared with RB 867515 (Table 1). Herbicide application was

necessary in the area cultivated with RB 867515, which may have compromised the bacterial colonization in this variety.

In these three sugarcane varieties evaluated were isolated 142 strains, being 88 strains of endophytic bacteria from leaves and roots and 54 strains of epiphytic bacteria from rhizoplane. Different morphological groups appeared and strains representing each group were selected.

The 142 isolated bacterial strains were evaluated regarding their potential to fix N<sub>2</sub> and to solubilize inorganic phosphate. According to the results, 79 strains were able to grow in NFb medium (Table 2), which represented 56% of the group of the evaluated bacterial strains. Therefore, the sugarcane varieties showed potential to fix N<sub>2</sub>.

Between 142 bacterial strains evaluated for their potential to solubilize inorganic phosphate, a solubilization halo was observed in 51 strains, which represented 36% of the group of evaluated bacterial strains. From these 51 strains, more than half (53%) were found in the rhizoplane of the varieties (Table 2), as also observed in the strains with potential to fix N<sub>2</sub>.

On the other hand, bacterial strains with potential to solubilize inorganic phosphate were found in all sugarcane varieties, with no difference between regions of the plants (Table 2).

When the strains in leaves, roots and rhizoplane were evaluated in each variety, only RB 867515 did not show higher number of bacterial strains with potential to solubilize inorganic phosphate in the rhizoplane (Table 2).

Depending on region of bacterial colonization, varieties showed different amounts of strains with these characteristics. RB 863129 variety differed from the others in rhizoplane region and showed the highest number of strains with potential to fix N<sub>2</sub> and solubilize inorganic phosphate (Table 3).

In bacterial strains evaluation per region from each

**Table 2.** Number and relative frequency of endophytic and epiphytic bacterial strains with capacity to fix N<sub>2</sub> or solubilize inorganic phosphate in different regions of three 4-month-old sugarcane varieties in the first crop cycle.

Factor			Factor		
Region	Variety	Relative frequency unit (%)	Variety	Region	Relative frequency unit (%)
<b>Nitrogen-fixing strains</b>					
Leaf	RB 92579	4 (25)	RB 92579	Leaf	4 (16)
	RB 867515	7 (44)		Root	5 (20)
	RB 863129	5 (31)		Rhizoplane	16 (64)
$\chi^2$		0.000 <sup>n.s</sup>	$\chi^2$		1.385 <sup>n.s</sup>
Root	RB 92579	5 (29)	RB 867515	Leaf	7 (32)
	RB 867515	3 (18)		Root	3 (14)
	RB 863129	9 (53)		Rhizoplane	12 (54)
$\chi^2$		0.059 <sup>n.s</sup>	$\chi^2$		0.000 <sup>n.s</sup>
Rhizoplane	RB 92579	16 (35)	RB 863129	Leaf	5 (16)
	RB 867515	12 (26)		Root	9 (28)
	RB 863129	18 (39)		Rhizoplane	18 (56)
$\chi^2$		4.840*	$\chi^2$		4.840*
<b>Inorganic phosphate-solubilizing strains</b>					
Leaf	RB 92579	4 (33)	RB 92579	Leaf	4 (25)
	RB 867515	7 (59)		Root	5 (31)
	RB 863129	1 (8)		Rhizoplane	7 (44)
$\chi^2$		1.000 <sup>n.s</sup>	$\chi^2$		0.529 <sup>n.s</sup>
Root	RB 92579	5 (42)	RB 867515	Leaf	7 (47)
	RB 867515	2 (16)		Root	2 (13)
	RB 863129	5 (42)		Rhizoplane	6 (40)
$\chi^2$		0.111 <sup>n.s</sup>	$\chi^2$		2.579 <sup>o</sup>
Rhizoplane	RB 92579	7 (26)	RB 863129	Leaf	1 (5)
	RB 867515	6 (22)		Root	5 (25)
	RB 863129	14 (52)		Rhizoplane	14 (70)
$\chi^2$		1.087 <sup>n.s</sup>	$\chi^2$		1.087 <sup>n.s</sup>

$\chi^2$  Calculated; <sup>n.s</sup>Not significant; <sup>o</sup>Significant (p>0.10); \*Significant (p>0.05).

variety, RB 863129 had the strains concentrated in the rhizoplane, which differed from leaves and roots. For the other varieties, there was no difference in the number of strains between leaves, roots and rhizoplane (Table 3).

Among 43 strains identified with potential to fix N<sub>2</sub>, a sample of 27 lineages with higher potential to solubilize inorganic phosphate was selected and subjected to identification through the analysis of partial 16S rDNA gene sequencing. Sequences were subjected to the database of the NCBI (*National Center for Biotechnology Information* [<http://www.ncbi.nlm.nih.gov>]) and analyzed using BLASTn (*Basic Local Alignment Search Tool*), being aligned and compared with existing sequences and identified at the level of genus and species when they showed an index of similarity higher than 90% (Table 4).

In these selected lineages, eight are from genus *Pantoea* sp., eight from *Burkholderia* sp., four from *Pseudomonas* sp., three from *Enterobacter* sp., two from *Klebsiella* sp. and two lineages were identified with similarity at the species level: *Pantoea stewartii* and

*Burkholderia cenocepacia* (Table 4). Identified bacterial lineages were mainly from the genera *Pantoea* sp. and *Burkholderia* sp. Genus *Burkholderia* sp. is widely found and studied due to the great capacity of its species to produce pentachlorophenols (PCPs), fix N<sub>2</sub> and solubilize inorganic phosphate in many agricultural crops of economic interest and under various environmental conditions. It is a genus that has a considerable physiological versatility and occupies a great variety of ecological niches (Perin et al., 2006a). Perin et al. (2006b) report that the genus *Burkholderia* includes more than 30 species, but not all of them are able to fix N<sub>2</sub>. However, in the present study, all the lineages from this genus are N<sub>2</sub> fixing and most of them solubilize inorganic phosphate (Table 4).

## DISCUSSION

Variation observed in total population density of bacterial

**Table 3.** Bacterial strains with capacity to fix N<sub>2</sub> and solubilize inorganic phosphate from sugarcane varieties in the first crop cycle.

Factor			Factor		
Region	Variety	Relative frequency unit (%)	Variety	Region	Relative frequency unit (%)
<b>Nitrogen-fixing and inorganic phosphate-solubilizing strains</b>					
Leaf	RB 92579	4 (33)	RB 92579	Leaf	4 (29)
	RB 867515	7 (58)		Root	3 (21)
	RB 863129	1 (9)		Rhizoplane	7 (50)
$\chi^2$		0.333 <sup>n.s</sup>	$\chi^2$		0.077 <sup>n.s</sup>
Root	RB 92579	3 (60)	RB 867515	Leaf	7 (58)
	RB 867515	0 (0)		Root	0 (0)
	RB 863129	2 (40)		Rhizoplane	5 (42)
$\chi^2$		0.200 <sup>n.s</sup>	$\chi^2$		1.667 <sup>n.s</sup>
Rhizoplane	RB 92579	7 (27)	RB 863129	Leaf	1 (6)
	RB 867515	5 (19)		Root	2 (12)
	RB 863129	14 (54)		Rhizoplane	14 (82)
$\chi^2$		4.263*	$\chi^2$		4.263*

Variation observed in total population density of bacterial community with sugarcane was also obtained in other studies on bacteria associated with other host plants (Kuklinsky-Sobral et al., 2004; Barreto et al., 2008). Preferential colonization of endophytic and epiphytic bacteria in the root zone, regardless of type of sugarcane variety, reflected the presence of greater amount of nutrients located in the rhizosphere and rhizoplane, compared with the leaves (Table 1), because these nutrients are responsible for higher bacterial growth and development (Cocking, 2003; Medeiros et al., 2006).

Rhizoplane of the sugarcane varieties was the most populated region by bacteria. In the traditional fertilization management of this crop, all the fertilizers are locally applied at the bottom of the planting furrow and in a single application, as performed in the present study. Therefore, this type of management may have favored the bacterial colonization for making the micro-environment of the rhizoplane more fertile.

Concerning differences between studied varieties, possibly the herbicide application in the RB 867515 area had promoted the lowest result in total population density in this variety area. This application was performed in the soil immediately after planting, which may have negatively affected bacterial colonization, because there were reductions in population densities of endophytic bacteria from roots and epiphytic bacteria from rhizoplane, and not in that of endophytic bacteria from leaves (Table 1), for RB 867515 variety. Malkones (2000), working with effects of different herbicide formulations on soil microorganisms activity, observed a reduction in bacterial colonization and warned for the deleterious effects of certain cultural practices on plant/microorganism association.

Bacterial strains with potential to fix N<sub>2</sub> found in leaves and roots did not differ between the sugarcane varieties.

However, in rhizoplane, there was a significant difference in number and percentage of strains found in these varieties (Table 2). 39% of bacterial strains were isolated in rhizoplane of RB 863129, suggesting that the N fertilization management of this variety may significantly differ from that of RB 92579 and RB 867515. In RB 863129 variety, rhizoplane was the region with the highest number of bacterial strains with potential to fix N<sub>2</sub> (56%), in comparison to leaves and roots. In the other varieties, there were no differences between leaves, roots and rhizoplane (Table 2).

In this work, most bacterial strains (46) with potential to fix N<sub>2</sub> were found in rhizoplane region (58%). This was a region of high bacterial colonization (Table 1) and with characteristics highly favorable for sugarcane nutrition, such as the potential to supply N (Table 2).

Beyond N, P is another important element to plant nutrition. And P fixation in weathered tropical soils is high, due to both adsorption to Fe and Al oxides and precipitation (Simões Neto et al., 2015). These processes limit P availability to plants. When a group of bacteria is able to solubilize phosphate, which occurs preferentially in the rhizoplane, it significantly favors plant phosphate nutrition, especially in sugarcane, because the usual P fertilization management corresponds to one single application at planting.

In modern agriculture, it is common to apply large amounts of P from soluble sources. In tropical soils, this P is rapidly fixed. Hence, one way of minimizing these losses is the application of inoculants containing bacteria that can solubilize this P and transform it into forms assimilable by plants, resulting in better use and crop yield (Wakelin et al., 2004; Canbolat et al., 2006; Dias et al., 2009), and contributing to sustainability.

Since endophytic bacteria colonize inside of host plant and inorganic phosphate is in the soil, it is possible to

**Table 4.** Identification of bacterial genera and species found in the NCBI by the BLAST database in different regions of three 4-month-old sugarcane varieties in the first crop cycle, index of similarity and their potential to promote plant growth.

Strains	Genus	Similarity (%)	Variety	Region	Growth promotion	
					N <sub>2</sub> -fixing	Phosphate-solubilizing
UAGC 5	<i>Pantoea stenwartii</i>	91	BR 92579	Leaf	+	+
UAGC 7	<i>Pantoea sp.</i>	99	BR 92579	Leaf	+	+
UAGC 8	<i>Pantoea sp.</i>	99	BR 92579	Leaf	+	+
UAGC 9	<i>Pantoea sp.</i>	99	BR 92579	Leaf	+	+
UAGC 16	<i>Pantoea sp.</i>	99	BR 867515	Leaf	+	+
UAGC 17	<i>Klebsiella/Enterobacter</i>	99	BR 867515	Leaf	+	+
UAGC 18	<i>Pseudomonas sp.</i>	100	BR 867515	Leaf	+	+
UAGC 19	<i>Klebsiella/Enterobacter</i>	99	BR 867515	Leaf	+	+
UAGC 26	<i>Pantoea sp.</i>	97	BR 867515	Leaf	+	+
UAGC 46	<i>Pantoea sp.</i>	100	BR 92579	Root	+	+
UAGC 62	<i>Pantoea sp.</i>	98	BR 867515	Root	+	-
UAGC 70	<i>Enterobacter sp.</i>	99	BR 863129	Root	+	-
UAGC 76	<i>Burkholderia sp.</i>	99	BR 863129	Root	+	-
UAGC 78	<i>Burkholderia sp.</i>	99	BR 863129	Root	+	-
UAGC 86	<i>Pseudomonas sp.</i>	99	BR 92579	Rhizoplane	+	+
UAGC 87	<i>Pseudomonas sp.</i>	99	BR 92579	Rhizoplane	+	-
UAGC 97	<i>Pseudomonas sp.</i>	100	BR 92579	Rhizoplane	+	-
UAGC 102	<i>Pantoea sp.</i>	99	BR 92579	Rhizoplane	+	+
UAGC 103	<i>Enterobacter sp.</i>	96	BR 867515	Rhizoplane	+	-
UAGC 105	<i>Burkholderia cenocepacia</i>	99	BR 867515	Rhizoplane	+	-
UAGC 114	<i>Burkholderia sp.</i>	99	BR 867515	Rhizoplane	+	+
UAGC 122	<i>Enterobacter sp.</i>	94	BR 863129	Rhizoplane	+	+
UAGC 125	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 127	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 130	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 131	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 140	<i>Burkholderia sp.</i>	98	BR 863129	Rhizoplane	+	+

+ and + : N<sub>2</sub>-fixing and phosphate-solubilizing; + and - : N<sub>2</sub>-fixing and not confirmed phosphate-solubilizing in later tests.

speculate that endophytic bacteria, during their process of plant colonization, are able to solubilize phosphate and increase its availability to the host plant (Massensini et al., 2008).

Regarding plant growth promoted by endophytic or epiphytic bacteria, it is important that these bacteria have more than one characteristic that promotes such growth. Bacterial strains that are able to fix N<sub>2</sub> and solubilize inorganic phosphate are more efficient than those that have only one of these characteristics. In the present study, from 142 isolated bacterial strains, 43 were identified with potential to fix N<sub>2</sub> and solubilize inorganic phosphate, representing 30% of the group of evaluated strains.

In general, bacterial strains were concentrated in rhizoplane, which is the region where the interaction between plant and soil is more intense. This observation suggests that localized fertilization management in sugarcane cultivation must be further intensified, creating micro-environments favorable for these microorganisms

development. Managements that promote local application of fertilizers, irrigation and minimum tillage favor the creation of micro-environments and soil/plant/microorganism relationships.

Identification of bacterial genera found in the Northeast indicated that the bacterial niches are different for each region of the country, because we found one genus (*Pseudomonas sp.*) and two lineages at the species level (*P. stewartii* and *B. cenocepacia*). Beneduzi et al. (2013), working with diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil, found the genus *Pseudomonas sp.* that yet there was not been described in the country. Review by Carvalho et al. (2014) on nitrogen signaling in plant interactions with associative and endophytic diazotrophic bacteria not related the genus *Pseudomonas sp.* associate with any crop. However, Kuklinsky-Sobral et al. (2004) identified the species *Pseudomonas citronellolis* in soybean. Genus *Pantoe sp.* was described in rice and soybean (Kuklinsky-

Sobral et al., 2004; Verma et al., 2001) and species *Pantoea aglomerans* in sugarcane (Loiret et al., 2004) different from species found this study (*P. stewartii*). Genus *Burkholderia* sp. was identified in rice (Baldani et al., 2000; Oliveira et al., 2002), maize (Riggs et al., 2001) and sugarcane (Reis et al., 2004; Luvizotto et al., 2010; Beneduzi et al., 2013). In South of Brazil, Beneduzi et al. (2013) identified two species: *Burkholderia phenazinium* and *Burkholderia cepacia*, but in our study we found a different species (*B. cenocepacia*). Evidencing that each bacterial group changes its presence in the habitat according to its demands and the environment where they are inserted.

However, even at Northeastern Region there are different soils under sugarcane growth, and the micro-organisms population may change because soil and climate conditions. It would be necessary deeper studies in other sugarcane areas to find and identify them. Moreover, after strains identification, we need to apply these micro-organisms and test them in their capacity to improve sugarcane development and productivity. This would allow a more sustainable tillage, decreasing chemical fertilizers use, and improving environmental quality of soils.

## Conclusions

In this work, bacterial community that interacted with sugarcane at four months of age in the first crop cycle in Northeast Brazil was more associated with the regions of rhizoplane and roots than with leaves. Bacterial community that interacted with sugarcane had a high potential to fix N<sub>2</sub> and solubilize inorganic phosphate in vitro. And identified bacterial lineages were mainly from *Pantoea* sp. and *Burkholderia* sp., but there were also the genera *Enterobacter* sp., *Klebsiella* sp. and *Pseudomonas* sp. and two lineages at species level: *P. stewartii* and *B. cenocepacia*.

## Conflicts of Interests

The authors have not declared any conflict of interests.

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