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Diazotrophic bacteria associated to sugarcane varieties cropped at Northeast Region of Brazil

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ABSTRACT

The objective of this work was to study the diversity of diazotrophic bacteria associated to 10 varieties of sugarcane cultivated in two states of the Northeast of Brazil and to identify the isolates by morphological and molecular methods. Diversity was studied by the application of traditional growth-depending microbiological methods: Gram-staining, comparison of colony morphology and growth on several culture media. Phylogenetic taxonomy was performed using 16S rRNA gene sequencing data. Almost all isolates obtained in semi-solid nitrogen free media were classified as Gram-negative and seven colony morphological groups were distinguished after growth on Potato medium. Among the 10 varieties evaluated, RB72454 resulted in higher number of isolates and diversity. The great majority of isolates from most of the genotypes tested showed similar characteristic to the genus *Gluconacetobacter* based on its peculiar colony morphology and growth characteristics in semi-solid media. Comparison of the 16S rRNA sequence of each isolate to the Genbank showed that they are closely related to *Gluconacetobacter*, *Burkholderia*, *Kosakonia*, *Klebsiella* and *Stenotrophomonas*.

Key words: biological nitrogen fixation, ecology of diazotrophic bacteria, sugarcane

Bactérias diazotróficas associadas a variedades de cana-de-açúcar cultivadas na região Nordeste do Brasil

RESUMO

O objetivo deste trabalho foi estudar a diversidade de bactérias diazotróficas associadas a 10 variedades de cana cultivadas em dois estados do Nordeste do Brasil e identificar os isolados por métodos morfológicos e moleculares. A diversidade foi estudada através da aplicação de métodos microbiológicos tradicionais dependentes de crescimento: coloração de Gram, comparação da morfologia de colônias e crescimento em vários meios de cultura. Taxonomia filogenética foi realizada utilizando dados de sequenciamento de gene 16S rRNA. Quase todos os isolados obtidos em meios semissólidos livres de nitrogênio foram classificados como Gram-negativos e sete grupos baseados na morfologia de colônias foram distinguidos após crescimento em meio batata. Entre as 10 variedades avaliadas, RB72454 resultou em maior diversidade e número de isolados. A grande maioria dos isolados dos genótipos testados apresentaram característica similar ao gênero *Gluconacetobacter* com base em seu peculiar crescimento em meios semissólidos e morfologia da colônia. A comparação da sequência do 16S rRNA de cada isolado com sequências do GenBank mostrou que eles estão intimamente relacionados com *Gluconacetobacter*, *Burkholderia*, *Kosakonia*, *Klebsiella* e *Stenotrophomonas*.

Palavras-chave: fixação biológica de nitrogênio, ecologia de bactérias diazotróficas, cana-de-açúcar

Introduction

Production of sugar, alcohol and energy in Brazil is associated with large scale sugarcane plantation. Nowadays, more than 9 million hectares are cultivated with different varieties adapted to soil and climate condition in almost all regions of the country. Northeast region is the oldest one cropped with sugarcane that was brought by the Portuguese as stems pieces at the time they colonized Brazil. Since then, new cultivars were developed and crop productivity increased almost 30% in the last 30 years, but the lowest response of sugarcane to nitrogen fertilizer remained critical, especially in the first year of cultivation (Urquiaga et al., 2012).

Sugarcane has been reported to associate with several bacteria considered beneficial to their development. Among them, most of nitrogen-fixing bacteria or diazotrophs were discovered due to the advent of semi-solid media without addition of nitrogen (Baldani et al., 2014). Recent studies using methods independent of cultivation also demonstrated a great diversity of sugarcane natural population of diazotrophs, indication that the contribution of biological nitrogen fixation (BNF) plays an important role in this plant. It was found that *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Burkholderia tropica*, *B. silvatlantica*, *Stenotrophomonas*, *Pantoea* and many others are part of the diazotrophic bacteria diversity found distributed in the roots, stems and leaves of sugarcane (Fischer et al., 2012; Taulé et al., 2012; Magnani et al., 2013).

Most of these diazotrophic bacteria, whether or not endophytically associated with the plant, produce substances that promote growth such as plant-growth regulators, siderophores, phosphate solubilization and/or act as agent of biological control (Spaepen et al., 2007). Several studies report that diazotrophic bacteria promote a significant development in sugarcane (Oliveira et al., 2006; Schultz et al., 2012; Schultz et al., 2014). Urquiaga et al. (2012) estimated the contribution of biological nitrogen fixation (BNF) in nine varieties using two different methods and found that at least 40 kg N ha⁻¹ per year derived from this biological process, although the contribution rate differed according to the tested genotype. These studies showed how plant genotype response oscillates towards the association with diazotrophic bacteria, indicating that further studies are needed for better understanding of the plant-bacteria interaction.

Here we present a survey of the diversity and variability of diazotrophic bacterial community associated with 10 varieties of sugarcane generally cropped at two states in the Northeast part of Brazil, Pernambuco and Alagoas, using different semi-solid N-free medium as a pre-requisite to isolate diazotrophs.

Material and Methods

Counting and isolation from sugarcane varieties

Isolates were obtained from 10 varieties of sugarcane grown in two states of the Northeast region of Brazil, Alagoas and Pernambuco. Five out of ten varieties were cropped at both sites SP81-3250, RB92579, RB867515, RB72454 and VAT90212, 2 only at Alagoas (SP79-1011, RB931530) and three only at

Pernambuco (SP83-2847, RB863129 and RB931011). Stems of each variety were divided into sections containing one bud each (setts), and then they were heat treated at 52°C for 30 min in order to reduce the population of pathogens as described by Benda & Ricaud (1978). Then each heat treated sett were pre-germinated at the Embrapa Agrobiology greenhouse in plastic boxes containing 12 kg of a substrate prepared with a mixture of sand and sterile vermiculite in 2:1 (v/v) according to Reis et al. (1999). Each box was planted with 8 setts of the sugarcane varieties. After 21 days after planting (DAP), two replicates samples of germinated plants of each variety were collected and divided into shoot and roots (1 g each part). Samples were washed, soaked in saline solution for 1 min and diluted (10⁻² to 10⁻⁶) for bacterial counting by MPN. Briefly, 0.1 mL from each dilution was inoculated in 5 ml of semi-solid N-free medium, namely JNFb, LGI (Baldani et al., 2014) and LGI-P (Reis et al., 1994), in some cases samples were also used to inoculate JMV (Baldani, et al., 2014) and inoculation to each medium was performed in triplicates (Baldani et al., 2014). The inoculated flasks were incubated in the dark for 5-7 days at 30 °C and development of bacterial paper-like film in the surface region of each replicate was recorded. The bacterial counts were performed using the most probable number method (MPN) using McCrady's probability table (Baldani et al., 2014).

Isolates were purified as follows: the last two tubes of the serial dilution which showed positive growth were inoculated into new flasks containing the same semi-solid medium. After the characteristic film formation diazotrophic bacteria were streaked onto solid medium of the same composition of previous growth media and incubated in the dark at 30 °C for 5 days. Single colonies were inoculated into flasks containing the respective semi-solid medium to confirm the formation of the characteristic paper-like film. Then, the isolates were streaked onto plates containing DYGS medium (Baldani et al., 2014) for final purification.

Morphological analysis and grouping

For characterization of colony morphology, each isolate was stroked onto Potato solid medium and between the 5th and 7th day of growth their colonies were classified using parameters adapted from Yano et al. (1993). The parameters were: shape (circular or irregular border), colony size (pitting - smaller than 1 mm, large > 1 mm and measured in mm), elevation (flat, lenticular, convex, pulvinate, umbonate or umbilicated), margin (entire or lobate, toothed, filamentous or whole), texture (smooth or rough) and consistency (viscous, dry, slightly gummy, gummy), appearance (glistening, translucent, transparent, opaque or bright), smell (absent or present), pigmentation (yellow or cream, describe).

Based on colony morphology features the similarities between the isolates were estimated using the coefficient of similarity - SM (single match) as described by Rohlf (2000). The isolates were grouped by the method of average distances UPGMA (Unweighted Pair Group Method Arithmetic Average) (Sneath & Sokal, 1962). Based on the similarity analysis, 40 representatives of the groups were selected for further evaluation. Gram staining were performed as described by Yano et al. (1993), growth on N-free semi-solid media were

evaluated after 2, 4 or 7 days of inoculation and those that were able to form paper-like film were considered positive for nitrogen fixation.

Molecular characterization

The DNA was extracted from bacterial cells using the QIAamp® DNA MiniKit (QIAGEN) according to the recommendations of the manufacturer. The 16S rRNA gene was amplified by PCR using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') in combination with 1492R (5'-TACGG(C/T)TACCTTGTTACGACTT-3') or a combination of two primers, 27f and Amp2R (5'-ACMC AAGGAGGTGATCCARC-3') (Heuer et al., 1997; Furushita et al., 2003) used for those that did not show amplification of this gene with the first ones. PCR was performed using the kit GoTaq® DNA polymerase (Promega) in volumes of 50 µL containing 50 ng of DNA according to the manufacturer's recommendations. Amplicons were obtained in a thermocycler (PTC 100 - MJ Research) with the following amplification program: denaturation step at 94 °C⁻² min), 30 cycles of 93 °C⁻¹ min, 60 °C⁻¹ min and 72 °C⁻² min and a final extension step at 72 °C⁻⁴ min. PCR products were analyzed by electrophoresis in 2 % agarose gel and purified using the kit Wizard® SV Gel and PCR Clean-Up System (Promega). The sequencing reaction was performed from 100 ng of purified products obtained by the PCR reaction, 5.0 µM of each inicializer with 5 pmoles µL⁻¹, 4 µL of Big Dye Terminator v3.1 (DYEnamic ET, Amersham Biosciences) and ultrapure water (ultraPURETM, Invitrogen Co.) for a final volume of 10 µL. The reaction was carried out starting from 95 °C for 25 sec, followed by 95°C for 25 sec, involving denaturation at 95°C for 25 seconds, annealing at 58°C for 15 seconds and extension at 60°C for 1 min repeated 24 cycles. After the sequencing reaction samples were precipitated using 1.0 µL of ammonium acetate 7.5 M and 27.5 µL of ethanol absolute 70% and incubated at 4°C for one night. After this incubation period, samples were centrifugated at 4,000 rpm for 45 min at 4°C. The supernatant was discarded and the precipitate washed with 100 µL of ethanol 70%, centrifuged again at 4,000 rpm for 10 min at 4°C. Samples were dried under air, resuspended in 7.5 µL of sequencing buffer 5X (Applied Biosystems Cat 4336697) and sequenced using MegaBace1000 platform (General Electric). In order to obtain the sequences, the software was used PHRED/PHRAP at the Linux operational system at Embrapa Agrobiologia data center.

The sequences obtained were compared with the sequences deposited at National Center for Biotechnology Information - NCBI using the blastn tool (<http://www.ncbi.nlm.nih.gov>).

Results and Discussion

We evaluated the presence of diazotrophic bacteria in 10 varieties of sugarcane grown in two states of the Northeast region of Brazil, Alagoas and Pernambuco. The bacterial counts (number of cells g⁻¹ of fresh weight) obtained from the sugarcane varieties tested were highly variable between sites and culture media used. They ranged from not detected (below

100-1000 cells g⁻¹ of fresh weight) to 1.40 x 10⁶ cells g⁻¹ of fresh weight depending on the medium used (Table 1).

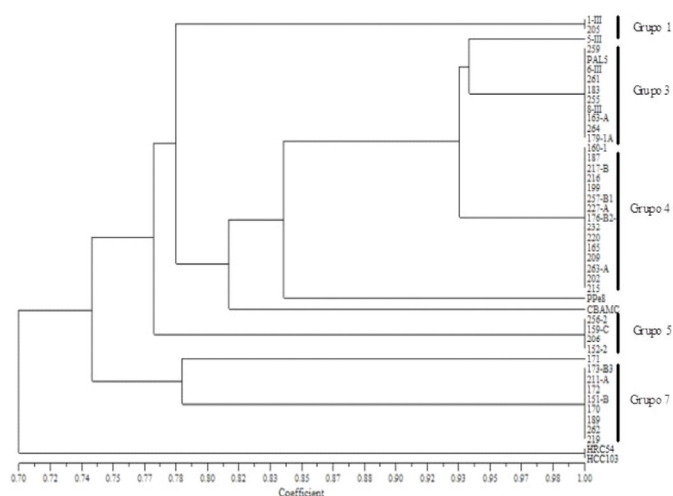
Counts were higher in JNFB and LGI semi-solid media and in general, the population in the roots, when detected, was in the range of 10⁴-10⁶ cells g⁻¹, higher than in the shoots of germinated seedlings. The variability in bacterial counts is expected since the distribution of bacteria in the plant is not homogeneous. Due to samples of plants with differences in growth ages and life cycle, climate and soil conditions, it was not possible to relate the factor (or factors) responsible for population variation. The strategy of using the pre-germinated individualized sugarcane setts for isolation of nitrogen-fixing bacteria has several advantages in different aspects. First, it allows the transport of plant varieties from its site to long distances where the isolation process will be done without loss of plant material and its native bacterial diversity. Second, the original stalks that will be used for germination have high amount of sugar and during the first days after planting these sugars are in the range of 600-800 mg g⁻¹ dry weight as observed for SP71-1406 by Melo et al. (1995), keeping this amount constant up to 21 days after planting. In contrast the amount of reducing sugars increases during this period, from approximately 5 mg g⁻¹ dry weight up to 10 mg g⁻¹ dry weight at the 21st day and during the germination these carbon sources are available to be used by the native population, favouring enrichment of their numbers. Third, the use of heat treatment can reduce the population of undesirable bacteria and external contaminants, allowing only those that are inside the setts to be benefited. The germination associated to the increased availability of carbon sources makes native population of microorganisms become actively growing within two to three weeks.

In addition, after several transfer steps in semi-solid media we obtained a total of 260 isolates from sugarcane plants pre-germinated in greenhouse. The highest number of isolates, 200 isolates (corresponding to 77% of total), derived from root samples, while only 60 isolates were obtained from shoots (23%). In addition, the largest number of root isolates (104 isolates or 40% of total) was obtained in semi-solid JNFB medium. From Pernambuco varieties pre-germinated samples, we only could isolate bacteria from shoots of RB92579 (data not shown). Comparing the numbers of isolates obtained from each variety and part of the plant (roots and shoots) we can see that the greatest numbers were obtained from RB72454 followed by RB867515. Variety RB92579 showed a high number of isolates obtained from the root samples, but only two isolates were from shoot tissues, in accordance with the bacterial counts showed in Table 1. The JMV medium was used in part of the analysis and to samples from most of the varieties the counting numbers were lower compared to the other media used. Subsequent analyses were performed using 197 isolates, and 63 isolates from the total did not grow after storage or lost the ability to form film in semi-solid medium. Isolates were separated in seven groups based on the colony characteristics formed onto Potato medium and represented by a dendrogram obtained using the software NTSYS (Numerical Taxonomy System Using Multivariate Statistical Program), version 2.1. (Figure 1).

Table 1. Number of diazotrophic bacteria associated to sugarcane varieties cultivated at the States of Pernambuco and Alagoas and germinated under greenhouse conditions at Embrapa Agrobiologia, Rio de Janeiro State, 15 days after planting

Variety	N° of isolates	Source	N° of cells g ⁻¹ of fresh weight x 10 ⁴ ** (total sites/positive sites with isolation)**			
			LGI-Pc**	JNFb	LGI	JMV
SP81-3250	26	Roots	27,3 (5/3)	189,0 (5/4)	1132,0 (5/4)	55,0 (5/2)
	3	Shoots	9,2 (5/2)	16,0 (5/2)	226,25 (5/2)	22,7 (5/2)
RB72454	37	Roots	12,5 (2/1)	70,0 (2/1)	1,25 (2/1)	2,5 (1/1)
	23	Shoots	22,5 (2/1)	24,75 (2/2)	2,45 (2/2)	n.d.
RB92579	42	Roots	83,27 (6/6)	271,58 (6/6)	196,42 (6/6)	55,45 (2/2)
	2	Shoots	0,9 (5/2)	125,2 (5/2)	1,7 (5/2)	n.d.
VAT90212	11	Roots	551,25 (2/2)	72,25 (2/2)	701,50 (2/2)	n.d.
	1	Shoots	47,5 (2/1)	12,5 (2/1)	5,5 (2/1)	n.d.
RB867515	37	Roots	102,48 (5/5)	272,26 (5/5)	292,30 (5/5)	45,0 (1/1)
	17	Shoots	7,6 (5/4)	5,475 (5/4)	12,725 (5/4)	n.d.
RB863129	5	Roots	32,87 (3/3)	166,5 (3/3)	468,83 (3/3)	n.d.
	5	Shoots	0,8 (3/2)	5,17 (3/3)	1,27 (3/3)	n.d.
SP83-2847	1	Roots	450,0 (1/1)	250,0 (1/1)	1400,0 (1/1)	n.d.
	3	Shoots	45,0 (1/1)	40,0 (1/1)	450,0 (1/1)	n.d.
RB931011	3	Roots	30,0 (1/1)	30,0 (1/1)	450,0 (1/1)	n.d.
	1	Shoots	25,0 (1/1)	9,0 (1/1)	1400,0 (1/1)	n.d.
RB931530	10	Roots	7,5 (1/1)	15,0 (1/1)	4,5 (1/1)	45,0 (1/1)
	-	Shoots	- (1/0)	- (1/0)	- (1/0)	n.d.
SP79-1011	3	Roots	2,5 (1/1)	25,0 (1/1)	0,9 (1/1)	25,0 (1/1)
	-	Shoots	- (1/0)	- (1/0)	- (1/0)	0,9 (1/1)

*Values are means of MPN of bacteria grown per number of positive sites. **LGI-P supplemented with 5 mL of sugarcane juice. n.d. = not determined. **A comparison was performed dividing the number of locations where the sugarcane plants were collected by the number of location that present a positive isolated of the diazotrophs tested in this publication.

**Figure 1.** Analysis of similarities of the 40 isolates obtained from sugarcane plants collected the different locations in Northeast region of Brazil. Distribution of the isolates were obtained by the mean distance method UPGMA (Unweighted Pair Group Method) (Sneath & Sokal, 1962) using the colony morphology and appearance observed during growth on Potato agar medium

Groups 2 and 6 presented only 3 isolates each, groups 1 and 5 presented 5 each and group 7 presented 8 isolates. Noteworthy, the groups 3 and 4 presented the highest number of isolates, 91 and 82 respectively. Interestingly, isolates characterized as *G. diazotrophicus* grouped massively in group 4 and only 1 was in group 3. A total of 40 isolates representatives of all these groups were selected for further characterization.

It is frequently reported that semi-solid N-free media, such as JNFb, LGI, LGI-P and JMV, are used to enrich the diazotrophic bacterial population and to separate them from other non-diazotrophic bacteria. We assumed that the use of various growth media during the isolation process enabled that bacteria of different nutritional requirements could be isolated when their preferred carbon source and pH were provided. It certainly influences the number of individuals and favour their detection and isolation. For example, the LGI-P medium supplemented with 5 mL of sugarcane juice, allows the detection of *G. diazotrophicus* faster and in higher number than in other media (Reis et al., 1994). However, depending on frequency of transfer to new media, depth of the inoculation and consistency

of the medium, the population of diazotrophic and facultative aerobic bacteria will prevail at 5 - 7 days after inoculation. If the transfer is made directly from the original semi-solid medium used for inoculation of plant extract serial dilution, other species can co-inhabit at these conditions, as reviewed by Baldani et al. (2014). This may explain why isolates grouping together with *G. diazotrophicus* used as reference strain could be isolated from JNFB medium inoculated with serial dilution of plant extract (data not shown).

The isolates distribution according to colony morphology, growth on several semi-solid media and their identity inferred by phylogenetic analysis is shown (Table 2). The 16 S rRNA gene size range from 900 to 1400 bp.

According to growth on semi-solid media, a group of isolates developed paper-like film in all semi-solid media tested, as well as strains of *Burkholderia tropica* (PPe8), *Herbaspirillum seropedicae* (HRC 54) and *H. rubrisubalbicans* (HCC 103), showing great ability to adapt to different carbon sources and pH. Another group of 22 isolates (corresponding to 55% of

all isolates) failed to grow in the presence of malic acid as a carbon source behaving similarly to *G. diazotrophicus* PAL-5 type strain (Table 2).

The phylogenetic analysis of these isolates based on 16S rDNA sequence similarity revealed the presence of five genera of diazotrophic bacteria: *Gluconacetobacter*, *Burkholderia*, *Klebsiella*, *Stenotrophomonas* and *Kosakonia* (Table 2). Representatives of *Gluconacetobacter* and *Burkholderia* genera were isolated in high numbers from the sugarcane varieties from Northeast of Brazil. The molecular characterization of almost of all isolates previously separated into seven groups showed diversity of diazotrophic species associated with sugarcane plants, as would be expected (Table 2). Other isolates were phylogenetically related to the *Enterobacteraceae* family such as *Klebsiella* and representatives of the new described genus *Kosakonia*. The last genus was created for the description of eleven isolates previously classified as *Enterobacter* species (Brady et al., 2013). Recently, *Enterobacter sacchari* a species of diazotrophic bacteria isolated from sugarcane was renamed

Table 2. Characterization of isolates in groups based on colony morphology and growth on semi-solid medium and their taxonomic identification based on 16S rRNA blast analysis

Isolate	Morphological group	Growth group ¹	Access number ²	% of identity ³	Species
205	1	C	KP969080	98	<i>Burkholderia vietnamiensis</i>
1-III	1	F	KP969079	99	<i>B. vietnamiensis</i>
5-III	2	F	KP969077	99	<i>Stenotrophomonas panacihumi</i>
183	3	A	KP969068	99	<i>Burkholderia tropica</i>
264	3	A	KP974654	99	<i>B. tropica</i>
255	3	F	KP974655	99	<i>B. tropica</i>
179-A	3	A	KP969067	97	<i>B. tropica</i>
163-A	3	G	KP974661	99	<i>Burkholderia cenocepacia</i>
259	3	B	KP969073	98	<i>Gluconacetobacter diazotrophicus</i>
8-III	3	H	KP993230	98	<i>Klebsiella pneumoniae</i>
6-III	3	B	KP993220	95	<i>Paenibacillus</i> sp
261	3	B	KP993218	94	<i>Bacillus</i> sp
257-B	4	F	KP993216	100	<i>Stenotrophomonas maltophilia</i>
173-B2	4	B	KP993228	99	<i>S. maltophilia</i>
176-B2-1	4	E	KP969070	99	<i>G. diazotrophicus</i>
232	4	B	KP969072	99	<i>G. diazotrophicus</i>
263-A	4	B	KP969074	96	<i>G. diazotrophicus</i>
202	4	B	KP969071	99	<i>G. diazotrophicus</i>
165	4	B	KP969069	98	<i>G. diazotrophicus</i>
220	4	B	KP974653	100	<i>G. diazotrophicus</i>
160-1	4	B	KP974662	99	<i>G. diazotrophicus</i>
227-A	4	B	KP993217	99	<i>G. diazotrophicus</i>
215	4	B	KP993225	97	<i>G. diazotrophicus</i>
199	4	A	KP993222	92	<i>Ochrobactrum</i> sp
217-B	4	B	KP993226	99	<i>Bacillus cereus</i>
172	4	A	KP993224	99	<i>Kosakonia radicincitans</i>
187	4	B	KP993229	99	<i>Gluconacetobacter liquefaciens</i>
216-A	4	B	KP974659	99	<i>K. pneumoniae</i>
209	4	B	KP993227	98	<i>Bacillus</i> sp
159-C	5	D	KP974657	99	<i>Ks. radicincitans</i>
206	5	D	KP969078	99	<i>S. panacihumi</i>
256-2	5	A	KP974660	99	<i>Ks. radicincitans</i>
152-2	5	A	KP993219	99	<i>Paenibacillus</i> sp
171	6	G	KP969066	99	<i>B. tropica</i>
211-A	7	B	KP993221	99	<i>Burkholderia</i> sp
219-B	7	B	KP993223	93	<i>Burkholderia</i> sp
170	7	B	KP969076	100	<i>B. cenocepacia</i>
151-A	7	B	KP969075	100	<i>B. cenocepacia</i>
262	7	B	KP974656	99	<i>B. cenocepacia</i>
189	7	B	KP974658	99	<i>K. pneumoniae</i>

Main characteristics of morphological groups 1 - 7 are detailed in Table 1. ¹Growth on semi-solid media: A) positive growth in JMV, LGI, LGI-P and JNFB; B) positive growth in JMV, LGI and LGI-P; C) positive growth in LGI and LGI-P; D) positive growth in JMV, LGI-P and JNFB; E) positive growth in LGI-P and JNFB; F) negative growth in all tested media; G) positive growth only in JNFB; H) positive growth in JMV and LGI-P. ²GenBank code of 16S rDNA sequence of the isolate. ³Percentage of similarity to sequences of the same species deposited at the GenBank.

into the new genus *Kosakonia* (Gu et al., 2014). The type strain SP1T of this species was isolated from stems of sugarcane cultivar GT11 in China and its genome was completely sequenced by Chen et al. (2014). The authors selected this strain as it could act as a plant growth-promoting bacterium to this crop. In this survey it was also possible to identify isolates phylogenetically related species of the *Kosakonia* genus, *K. radicincitans* that are also described as diazotrophs (Table 2) (Kämpfer et al., 2005).

The potential of the present strategy using traditional growth dependent parameters for screening and grouping isolates was confirmed by their phylogenetic identification based on 16S rDNA gene sequencing analyses. Isolates identified as *Burkholderia tropica* belonged mostly to the group 3, that included also one isolate identified as *Burkholderia cepacia*, but all representatives of group 7 were *B. cenocepacia*. Isolates showing colony morphology similar to *G. diazotrophicus* strains belonged to group 4, together with fermentative bacteria *G. liquefaciens*, *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia*. Interestingly, during isolation process many of the isolates characterized as *G. diazotrophicus* were purified from growth of serial dilutions originally in JNFb medium (data not shown). Although in this medium malic acid is the carbon source and *G. diazotrophicus* does not prefer tricarboxylic acids for growth, this behaviour can be explained by the presence of sugars and other nutrients derived from macerated tissues in plant extract used for serial dilution and inoculation on semi-solid during the first step of isolation and that it could provide conditions for isolation of this species. The distribution of these species in the plant tissues shows that most of them derived from root samples, corresponding to the highest population values observed during bacterial counts, and it is also reflected in the presence of bacterial diversity of species in roots (Figure 2, Table 1). On the other hand, only representatives of *B. vietnamiensis*, *B. tropica*, *K. radicincitans*, *S. panacihumi*, *Ochrobactum* sp and *G. diazotrophicus* were present in the shoots. For this reason, it is important to show that even using the germination procedure in a sterile substrate the bacterial diversity differ in relation to plant part as well.

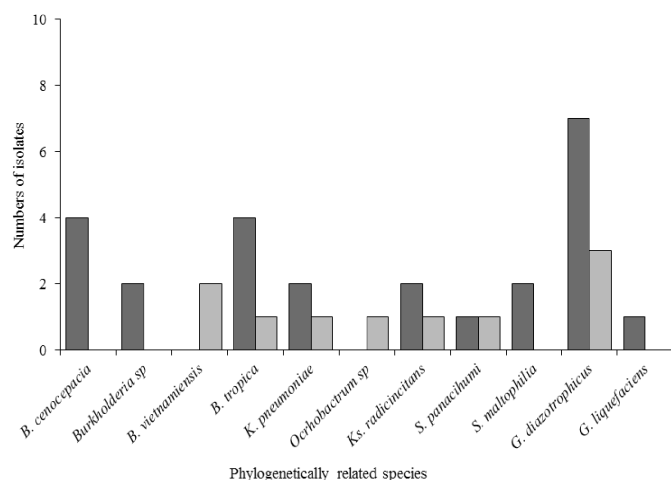


Figure 2. Species distribution in roots (black bar) and shoots (gray bar) of sugarcane varieties used as source for isolation

As plant samples were collected in two different states, Alagoas and Pernambuco, a comparison between these sites was performed comparing the 40 isolates used for the grouping and identity analysis. The Figure 3 shows that samples collected at Alagoas present higher species diversity than samples collected at Pernambuco. *G. diazotrophicus* and *B. tropica* were the two bacterial species recovered in majority and exclusively from Alagoas cropped varieties. Important to address that Alagoas was the original site of isolation and the first report of *G. diazotrophicus* in sugarcane (PAL5^T = AL means Alagoas). It was also observed that *B. tropica* was present at only one of the sites, although *B. vietnamiensis* was present at both sites. In sugarcane from Pernambuco an interesting distribution of 1/3 for each species identified (*B. vietnamiensis*, *S. panacihumi* and *Klebsiella pneumoniae*) were observed. They were also present at samples from Alagoas, but in proportion lower than other species. Representatives of *G. diazotrophicus* species were isolated from all sugarcane varieties while *B. tropica* was only obtained from RB72454 and RB867515. Many diazotrophic *Burkholderia* species were isolated and described from sugarcane samples grown in Brazil, *B. tropica* (Reis et al., 2004), *B. unamae* (Caballero et al., 2004) and *B. silvatlantica* (Perin et al., 2006).

Although, the first isolates of *B. tropica* were obtained from pre-born setts cultivated in soil originated from Pernambuco, it was not detected during this work. Instead, *B. vietnamiensis* was the species that predominated into roots and shoots samples from sugarcane cropped in this state, while *G. diazotrophicus* and *B. tropica* were predominant in samples from Alagoas. Based on this data we can assume that a site specificity of bacterial species distribution may be influencing sugarcane bacterial diversity independent of the plant genotype. In addition, representatives of other genera, such as *Kosakonia*, *Klebsiella*, *Stenotrophomonas*, and some Gram-positive or Gram-variable isolates were also found as previously reported by others, using culture dependent or independent methods (Magnani et al., 2010; Taulé et al., 2012; Fischer et al., 2012; Magnani et al., 2013). The occurrence of representatives of *Gluconacetobacter* as the most abundant and present in both roots and shoots of sugarcane varieties cropped in Alagoas could be explained by the use of LGI-P medium with addition of sugarcane juice in which the initial pH is 5.7 during

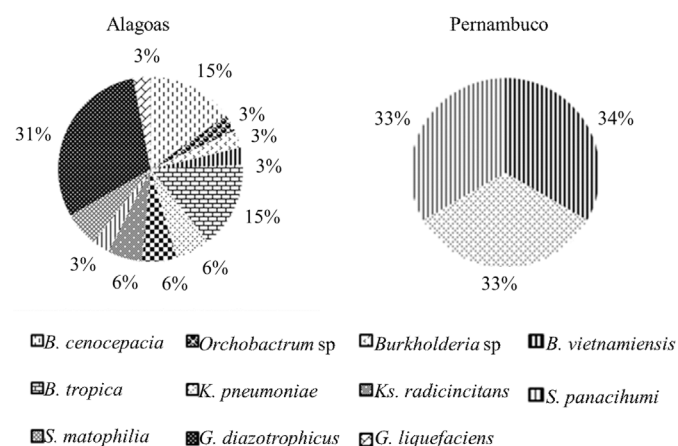


Figure 3. Species representatives observed in sugarcane samples from Alagoas and Pernambuco States of Brazil

isolation procedure. In this case, the drop of pH to 3.0 observed during growth development probably limited the growth of other species sensitive to acidic conditions. In addition, when *Gluconacetobacter* spp are grown in solid medium containing bromothymol blue orange colonies are observed after growth, facilitating the identification and purification of this sort of isolates on Potato agar medium containing sucrose 100 g L⁻¹ (Potato-P). In Potato-P, *G. diazotrophicus* form brown chocolate-like colonies, guaranteeing the identification of individuals of this species only applying traditional growth strategy. Another important factor that allows the isolation of this species more easily is the germination of sugarcane setts. Other studies have shown that the isolation from pre-sprouted stalks and addition of sugarcane juice increase the chances of obtaining high number of *Gluconacetobacter* (Reis et al., 1999).

Using the counting of each isolate it was observed the distribution of the identified species within the sugarcane varieties tested. Comparing this species distribution among the six varieties we noticed that each one harbour different species representatives, showing that plant plays a role in the observed diversity (Figure 4). Variety RB72454 was the plant genotype were almost all strains (11 from 13 representative strains) were obtained. For example, *K. pneumoniae* was present in RB72454 and the only species identified at RB863129. Isolates of *B. vietnamiensis* and *B. tropica* were obtained from RB72454 and RB867515.

Noteworthy, these are varieties of a descending progeny, since RB867515 derived from crossing of RB72454 with another genotype. *B. cenocepacia* was found in RB72454 and VAT90212. As expected, representatives of *G. diazotrophicus* were isolated in almost all varieties because of its prevalence of association with sugarcane plants, with exception to the variety RB863129 cropped only at Pernambuco. In fact, the species of *Gluconacetobacter* and *Burkholderia* were the one obtained at high number (Figure 3).

The high number of isolates obtained from a single variety RB72454 showed some important points of this survey. This variety was used during other surveys of diazotrophic bacteria counts and isolates identification in Brazil (Magnani et al., 2013) and for inoculation studies using five different

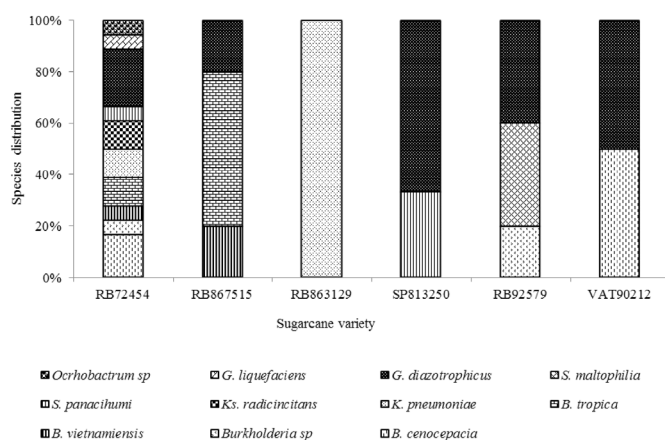


Figure 4. Species distribution among sugarcane varieties evaluated using pre-germinated sugarcane samples from Pernambuco and Alagoas States of Brazil

diazotrophs known to contribute for plant growth promotion (Schultz et al., 2012; Schultz et al., 2014). It is known to be adapted to soils of low fertility throughout the Brazilian territory. We can speculate that the association with beneficial microorganisms can be an advantage for these genotypes to grow under adverse fertility conditions. Interestingly, taking into account the distribution of species of isolates in sugarcane varieties it was observed that RB72454 is the variety where almost every single species was found. This variety has been widely used in Brazil and covered almost 50% of our cropping area 10 years ago. Its cropping was reduced all over the country since it is a variety sensitive to the smut disease (Lwin et al., 2012) and have limited bud sprouting in the ratoon crop, reducing the stand of plants after few years.

The strategy presented here used a single medium for colony characterization and group separation of all isolates. It was possible because the Potato medium is a complex medium considered rich and normally applied at the final stage of purification during isolation of diazotrophic bacteria in our Laboratory. The advantages of using a single culture medium refers to the practicality of groups formation without need for further steps, it is cheaper and easily reproduced in many laboratories independent of their infrastructure. Nonetheless, the formation of groups of isolates that share similar phenotypic characteristics whose individuals differ in terms of metabolism and phylogenetic relationship cannot be ruled out. The data presented in this work showed a strong correlation between classical microbiology, used during purification and identification of species, and phylogenetic analysis. It is a plausible strategy to screen large environmental samples prior to physiological and molecular characterization, saving time and costs.

Conclusions

Variety RB72454 harbor 92% of the isolates diversity than that observed. Although it is well established that plant genotype plays an important role in bacterial diversity our data indicate that development of new cultivars can reduce diversity and also soil adaptation.

G. diazotrophicus and *B. tropica* has an advantage during isolation procedure in comparison to the representatives of other species detected.

The other genera identified were *Klebsiella*, *Stenotrophomonas* and *Kosakonia*, all Gram negative bacteria, and also some Gram-positive or Gram-variable isolates classified as *Bacillus* and *Paenibacillus*.

The utilization of semi-solid medium is considered enrichment medium and can contribute to the isolation of diazotrophs.

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