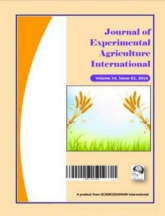

*Journal of Experimental Agriculture International*

***21(3): 1-15, 2018; Article no.JEAI.39963***
***ISSN: 2457-0591***
*(Past name: American Journal of Experimental Agriculture, Past ISSN: 2231-0606)*

# Potentially Diazotrophic Endophytic Bacteria Associated to Sugarcane are Effective in Plant Growth-promotion

**Maria Camila de Barros Silva Leite[1], Arthur Prudêncio de Araújo Pereira[2], Adijailton José de Souza[2], Pedro Avelino Maia de Andrade[2], Marisângela Viana Barbosa[3], Fernando Dini Andreote[2], Fernando José Freire[4*] and Júlia Kuklinsky Sobral[1]**

*[1]Unit Academic of Garanhuns, Federal Rural University of Pernambuco, Brazil.*
*[2]Luiz de Queiroz College of Agriculture, University of São Paulo, Brazil.*
*[3]Department of Agronomy, Federal University of Lavras, Brazil.*
*[4]Department of Agronomy, Federal Rural University of Pernambuco, Brazil.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors MCBSL, FJF and JKS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors APAP, AJS, MVB and FDA managed the analyses of the study. Author PAMA managed the literature searches. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/JEAI/2018/39963
Editor(s):
(1) Moreira Martine Ramon Felipe, Associate Professor, Departamento de Enxeñaría Química, Universidade de Santiago de Compostela, Spain.
Reviewers:
(1) Anonymous, Tamil Nadu Agricultural University, India.
(2) G. Modi Krunal, ASPEE Shakilam Biotech Institute, Navsari Agricultural University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/23525

***Original Research Article***

***Received 16th December 2017***
***Accepted 27th February 2018***
***Published 10th March 2018***

## ABSTRACT

The bioprospection of microorganisms with the potential to promote sugarcane growth has been increasingly sought, mainly to minimize the insertion of inputs that increase the costs of the production chain. The objective of this work was to isolate, identify and select endophytic bacteria from sugarcane with potential ability for plant growth promotion. Also to evaluate the influence of different levels of salinity and pH values on plant growth-promotion and to analyze the genetic diversity of the isolates by the partial sequencing of the 16S rRNA gene. Samples of stems and

**Corresponding author: E-mail: fernando.freire@ufrpe.br;*

roots of the varieties RB 92579, RB 867515 and RB 863129 were used for collecting the bacteria associated with sugarcane. The isolation was performed in LGI-P culture medium. The isolates were evaluated for biological nitrogen fixation (BNF), indole acetic acid (IAA) production, inorganic phosphate solubilization and exopolysaccharide (EPS) production. Bacterial growth and phosphate solubilization were evaluated at different salt concentrations and pH values of the culture medium. After isolation and identification were obtained 24 isolates, been that 54% grew in nitrogen-free medium. IAA was produced in 50% and 12.5% of the isolates in medium with and without the addition of L-tryptophan, respectively. In addition, 67% of the isolates were positive for phosphate solubilization and 75% produced exopolysaccharides. The presence of salt and different pH values of the culture medium influenced the growth of the isolates and the salt concentration influenced phosphate solubilization. Seven isolates showed similarity with *Burkholderia gladioli* and one with *Burkholderia heleia*, besides many genera only of *Burkholderia* sp., suggesting low genetic diversity. *Burkholderia heleia* had a lower growth rate than *Burkholderia gladioli*. It was a bacterium very sensitive to high amounts of salt when the pH was higher. The root bacterial isolate G27 obtained from RB 867515 presented the highest PSI in the lowest saline concentrations and the stem isolate G15 of the same variety was able to solubilize phosphate in high salt concentration. We conclude that sugarcane interacts with potentially diazotrophic endophytic bacteria involved with plant growth-promotion and can be used as inoculants in management programs of this crop, however, it is necessary to observe soil salinity levels and monitor pH to maximize plant growth-promotion characteristics of these bacteria.

*Keywords: Indole acetic acid; exopolysaccharides; biological nitrogen fixation; phosphate solubilization.*

## 1. INTRODUCTION

Sugarcane is one of the most important Brazilian crop production, due to the global demand of its by-products such as sugar and ethanol [1]. However, the growth and performance of sugarcane in the field have been heavily impacted by many abiotic and biotic characteristics, including the pH variations, salinity, drought stress, monospecific cultivation and the great dependence of mineral fertilizers and zenobiotics, affecting the environmental sustainability, soil health and plant yield. However, the sugarcane production is changing so far, in order to obtain a sustainable crop production.

Many researchers have highlighted the importance of maintenance of the life of the soil, principally related to microbiological parameters, including the bacteria diversity [2]. As far as the plant, its selects microbes from soil to act with it in a mutualistic association. Many studies also have raised insights into the bioprospection of plant-associated microorganisms, in order to apply known potential microbes as seed, roots or leaves inoculants. Those methodologies are in accordance to the reduction of the agrochemicals inputs influencing the crop sustainability [3].

The plant is colonized by a high diversity of bacteria, and those bacteria associated with the plant can be characterized as epiphytes and endophytes [4]. According to Hardoim [4], endophytic bacteria are those microbes able to colonize and live part of its life inside the plant tissues, in a mutualistic interaction. The plant (host) favor the microorganisms with shelter and free carbon compounds avaibility and the bacteria, in turn, stimulate the plant growth by the production of phytohormones.

Some indicators of plant growth-promotion by bacteria are indole acetic acid (IAA) production and phosphate solubilization [5-8]; biological nitrogen fixation (BNF), production of enzymes, pathogens biocontrol, and tolerance to abiotic stress [9-11]. Furthermore, among the plant associated microorganisms, the endophytic bacteria are considered as a largely untapped resource for the discovery of isolates with plant growth-promotion traits [4].

For example, *Burkholderia sp.* is a genus classified in the Betaproteobacteria sub-class, it is a group of bacteria known as to be associated with most agricultural plant. This group has been characterized living as endophytes colonizing the sugarcane tissues such as roots, stalks, and leaves [12]. In addition, they have shown the ability for plant growth-promotion and health, through BNF, production of phytohormones and antifungal compounds and induce systemic resistance to some stressed abiotic conditions [13,14].

Kuklinsky-Sobral [15] found that there is a high diversity of *Burkholderia* sp. associated with plants exhibiting the potential for plant growth promotion. Moreover, some works demonstrated that the inoculation of *Burkholderia* species in crops, such as maize and sorghum was directly correlated to increases in both root and shoot dry weights, appealing for the possibility of characterizing as inoculants using bacteria from this group [12,15].

We hypothesise that the community colonizing the inner tissues of sugarcane may represent a source of bacteria enable to promote plant growth, characterized by the ability to produce exopolysaccharides (EPS), fix atmospheric nitrogen, solubilize phosphate and produce IAA, even when cultivated in adverse conditions. In this context, the aim of this work was to isolate, identify and select endophytic bacteria from sugarcane and evaluated some characteristics of these microorganisms involved with the plant growth-promotion; We also have analyzed the influence of different levels of salinity and pH values on plant growth-promotion and to analyze the genetic diversity of the isolates by the partial sequencing the 16S rRNA gene.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

The source of our experiments were three sugarcane varieties (RB 92579, RB 867515 and RB 863129) collected at four months after plantation, in two production units in the state of Pernambuco, Brazil: Carpina Sugarcane Experiment Station (EECAC) and Plant Power Petribu S/A. After sampling, the stems and roots were separated, stored in plastic bags, kept under refrigeration, and taken to the Laboratory of Genetics and Microbial Biotechnology (LGMB).

### 2.2 Isolation of Endophytic Bacteria

At the laboratory, the samples were washed in running water for the removal of soil particles. Then, the roots were submitted to a superficial disinfection approach according to Araújo [16]. The samples were aseptically cut into small fragments and grounded in a *Phosphate Buffered Saline* (PBS) buffer solution [16]. Therefore, the material was incubated under (120 rpm) at 28°C for 40 minutes. After this step, 100 µL of this suspension was inoculated into test tubes containing semi-solid LGI-P medium free of nitrogen and semi-selective for *Gluconacetobacter diazotrophicus*, and incubated at 28°C [17]. We chose a specific medium for diazotrophic bacteria because we wanted to select specific endophytes with high performance in the biological fixation of N in sugarcane.

The stem samples were cut into 8 to 12 cm segments, washed in 70% ethanol to remove the epidermis. Approximately 2.0 g of the stem samples were aseptically cut and grounded in PBS. After this step, 100 µL of this solution was inoculated into tubes containing LGI-P semi-solid medium free nitrogen [17]. All the procedures of bacterial isolation were performed in triplicates.

After ten days, the film formed in the medium were replicated into petri dishes containing solid LGI-P medium and incubated at 28°C for 10 days. Pure colonies were obtained by the depletion technique in solid LGI-P medium, added with yeast extract. The isolated colonies were stored in the solid P-potato medium and conditioned at 4°C, and stored at – 20°C in a liquid LGI-P medium containing yeast extract plus 20% glycerol [17].

### 2.3 Plant Growth-promoting Bacteria

#### 2.3.1 BNF

The selection of nitrogen-fixing bacteria was performed by inoculating pure colonies in semi-solid LGI-P medium free of nitrogen and incubated at 28°C for 10 days [17]. The experiments were carried out in triplicates and the positive result was characterized by the formation of a horizontal orange halo inside the culture medium after the peal three consecutive times [7].

#### 2.3.2 IAA production

For IAA production, 24 isolates were evaluated, it was measured at an optical density (OD) 530 nm, using a spectrophotometry by colorimetry and the characterization was obtained at a 530 nm of OD, according to Crozier [18] and Pereira [7]. Isolated colonies were inoculated in liquid 10% TSA (*Trypcase Soy Agar*) medium, supplemented and not supplemented with 5.0 mM L-tryptophan. The samples were incubated under shaking (120 rpm) at 28°C in the absence of light for 24 h. After this step, the bacterial culture was added to the Salkowski reagent (2% $FeCl_3$ 0.5 mol $L^{-1}$ in 35% $HClO_4$), in a ratio of 1:1,

incubated for 30 min at 28°C in the absence of light. As a positive control, we used the strain EN303 (*Pseudomonas oryzihabitans*) [15]. The experiments were carried out in triplicates and the positive results were compared by the formation of a pink coloration in the medium.

#### 2.3.3 Phosphate solubilization

Pure colonies of 24 isolates were inoculated into solid culture medium containing a phosphate insoluble source ($CaHPO_4$). Plates were incubated at 28°C for 72 h and the presence of a clear halo around the colony indicated phosphate solubilization. The phosphate solubilization ability were measured by a phosphate solubilization index (PSI), in accordance to Berraquero [19] and Santos [20]. The experiments were performed in triplicates.

To evaluate the influence of salinity on phosphate solubilization, nine isolates were inoculated in solid culture medium containing a phosphate insoluble source ($CaHPO_4$), plus three concentrations of NaCl: 1.5, 10 and 25 g $L^{-1}$. After inoculation, the plates were incubated at 28°C for 7 days. PSI was evaluated on the first, second, third and seventh day after inoculation.

#### 2.3.4 EPS production

EPS production was evaluated in 24 isolates using, solid LGI-P medium plus yeast extract, which is nutrient medium rich in sucrose, conducive to the production of EPS by bacteria. The isolates were streaked and incubated at 28°C for 10 days. The production of large, mucous and elevated colonies indicated EPS production. The flattened and dry colonies were characterized the test as negative.

### 2.4 Salinity and pH Influence on Bacterial Growth

Pure colonies of two isolates (*Burkholderia gladioli* and *Burkholderia heleia*) were inoculated in 10% TSA liquid medium and incubated in a shaker (120 rpm) for 24 h at room temperature. Afterward, the bacteria growth was tested under three different saline conditions: 1.5 g $L^{-1}$(control), 10 g $L^{-1}$ and 25 g $L^{-1}$ of NaCl, and in two pH conditions: 5.5 and 7.3 (control). After inoculation, the tubes were incubated at 28°C under agitation (120 rpm). The bacterial growth was measured through the OD for 6 days, using a spectrophotometer (600 nm). The test was performed in triplicate. We chose to evaluate environmental adversities such as salinity and pH in only two isolates because we did molecular identification of these isolates.

### 2.5 DNA Isolation and 16S rRNA Gene Sequencing

Eight isolates able to fix atmospheric nitrogen, produce IAA, solubilize phosphate and produce EP were selected for partial sequence of the 16S rRNA gene sequenced. The DNA isolation was performed according to Araújo [16]. To ensure the quality of the DNA, it was run in 1.5% agarose gel electrophoresis. The samples were stained in ethidium bromide solution and visualized under UV light, yielding approximately 50ηg.

Amplification of the V6 region of the 16S rRNA gene was performed with the primers 027F (5 'GAGAGTTTGATCCTGGCTCAG 3') and 1387R (5 'CGCTGTGTACAACGCCCGGGAACG 3'), using 2.5 mM dNTPs, 25 mM $MgCl^{2+}$, 1X Taq Buffer; 0.5U Taq DNA (Fermentas, Burlington, Canada) polymerase and 100 mM of each primer in a final volume of 50 μL. The reaction was performed in an Veriti® thermal cycler (Applied Biosystems, Waltham, USA), in the following reaction conditions: an initial denaturation step at 94°C for 30 sec, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 1 minute, extension at 72°C for 1 min, and a final extension step at 72°C for 10 minutes.

The PCR products were run on an agarose gel (1.2%) stained with a solution of GelRed™ (1000x) and photodocumentated under ultraviolet light. The yield of the reaction was compared with a GeneRuler DNA Ladder (1 kb). Therefore, the PCR products were purified with the PureLink® PCR Purification Kit (Invitrogen). After purification, the amount of DNA was quantified in a Qubit equipament, yielding approximately 30 ng $\mu L^{-1}$. These samples were subjected to automated sequencing using the Sanger method [21]. The reverse primer 1387R (5 'CGGTGTGTACAACGCCCGGGAACG 3') being used as a basis for the sequencing reaction in the Genetic Analyzer apparatus ABI Prism377 (Applied Biosystems).

Sequences were analyzed using the CLC Genomics Workbench software (CLCbio, QIAGEN Company). The quality of the sequences was evaluated by the Quality Trimming tool with sequences with Phred quality

less than 20 and size smaller than 200 base pairs discarded from the subsequent analyses. Sequences with good quality were taxonomically affiliated through the RDP Classifier, and were compared by BLASTn (Basic Local Alignment Search Tool) against the NCBI (National Center for Biotechnology Information - https://www.ncbi.nlm.nih.gov/).

## 2.6 Statistical Analysis

The production data for IAA and PSI were submitted for analysis of variance and the F test ($p<0.05$) was performed as a function of inoculation treatments. In the variables where the main effects were significant, the Scott Knott averages test ($p<0.05$) was applied.

# 3. RESULTS

## 3.1 Indicators of Bacterial Isolates Plant Growth-promotion

### 3.1.1 BNF

The analytical procedures allowed obtaining 24 bacterial isolates, 17 from the stem and 7 from the roots. Of this total, 50% were positive for BNF, presenting a horizontal halo within the culture medium (Table 1). In the roots, only 14.2% were potential nitrogen fixers, while in the stem were 64.7%.

### 3.1.2 IAA production

As for the IAA production, 47.5% of the isolates were positive in 10% TSA medium plus L-tryptophan and 2.5% when in medium without the addition of L-tryptophan (Table 1).

The IAA production with the addition of L-tryptophan ranged from 2.7 to 90.3 μg $mL^{-1}$ (Fig. 1).The isolates that demonstrated the highest production were G25 (90.3 μg $mL^{-1}$) and G15 (26.3 μg $mL^{-1}$) (Fig. 2A). Considering the IAA production without the addition of the L-tryptophan, an independently of the main route of IAA synthesis, we observed that only three isolates were able to produce IAA, and the isolate G25 (20.2 μg $mL^{-1}$) produce a higher amount of IAA, showing to be a potential producer of IAA (Fig. 2B). Root isolates with L-tryptophan (G11, G23, G26 and G28) (Fig. 2A) and without L-tryptophan (G11 and G23) (Fig. 2B) produced less IAA than stem isolates. According to the results, the isolate G25 produced 77.6% lesser without L-tryptophan in the medium. The isolate G15 are unable to produce IAA without L-tryptophan.

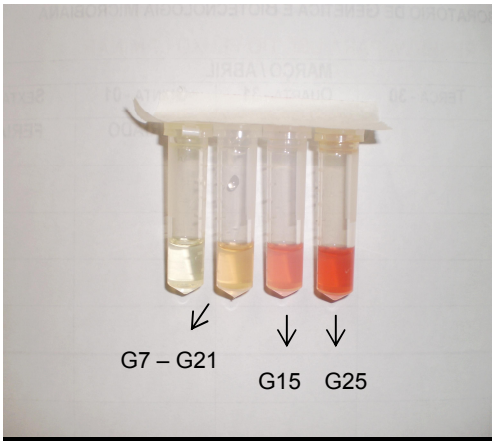


**Fig. 1. Indole acetic acid (IAA) production by endophytic bacterial isolates from sugarcane with addition of L-tryptophan showing the best performance of G25 and G15 isolates**

### 3.1.3 Phosphate solubilization

Over the potential to solubilize phosphate, 67% (16 isolates) were positive for phosphate solubilization, presenting a clear halo around the colony (Table 1). Isolates of the variety RB 867515 were the least soluble phosphate, regardless of whether the origin was from the stem or the root. The root bacterial isolate G27 had a higher PSI when compared to the other isolates (Fig. 3).

### 3.1.4 EPS producing

The great majority of bacterial isolates produced EPS (75%) (Table 1). As for the sugarcane variety, approximately 22, 61 and 17% of the positive isolates were of the varieties RB 92579, RB 867515 and RB 863129, respectively; and about 61% of the positive isolates for EPS production were from the stem. However, all isolates from roots produced EPS (Table 1).

### 3.1.5 Adverse environmental conditions (salinity and pH)

We monitored the bacterial growth of *Burkholderia gladioli* (Fig. 4A and B) and *Burkholderia heleia* (Fig. 5A and B) for 6 days every 24 h through the measurement of the optical density (600 nm) when cultivated in adverse environmental conditions of salinity and pH.

Based on the positive and maximum results obtained, the growth of *Burkholderia gladioli* when cultivated at pH 7.3 showed a lower growth rate in the concentration of 25 g $L^{-1}$ of NaCl. However, after 24 h of culture, the growth of the bacteria increased and at the end of the evaluation period (144 h) did not differ from the other salinity levels (Fig. 4A). When pH was buffered at 5.5, the bacterium grew at the highest salt level since the beginning of the culture and at 48 h, different salinity levels did not interfere with bacterial growth (Fig. 4B).

The concentrations of 1.5 and 10 g $L^{-1}$ of NaCl did not interfere with the ability of the bacteria to solubilize phosphate. However, the concentration of 25 g $L^{-1}$ of NaCl salt was inhibitory, except for the isolate G15 because it was able to solubilize phosphate in high salt concentration (Fig. 6). In this isolate, the salt stimulated the phosphate solubilization. The root bacterial isolate G27 presented the highest PSI in the lowest saline concentrations of the culture medium (Fig. 6).

*Burkholderia heleia* had a considerably lower growth rate than *Burkholderia gladioli*, since its growth peak was approximately 0.30, whereas the *Burkholderia gladioli* peak was about 0.46 (Figs. 4 and 5). In addition, the highest salt level (25 g $L^{-1}$ of NaCl) and at pH 7.3 inhibited its growth, demonstrating that *Burkholderia heleia* is very sensitive to high amounts of salt and high pH (Fig. 5A). However, when pH decreased, salinity stimulated the growth of this bacterium (Fig. 5B).

## 3.2 Identification of Bacterial Isolates

Eight isolates that showed common characteristics of plant growth-promotion, such as: BNF, IAA production, phosphate solubilization and EPS production were identified by the partial sequencing of the 16S rRNA gene. According to BLASTn, seven isolates showed similarity for *Burkholderia gladioli* and one isolate for *Burkholderia heleia* (Table 2).

**Table 1. Origin of bacterial isolates from sugarcane, plant tissue and characteristics involved with plant growth-promotion**

| Isolates | Variety | Plant tissue | BNF[1] | IAA[2] | Phosphate solubilization | EPS[3] |
|---|---|---|---|---|---|---|
| G1 | RB 92579 | Stem | + | - | + | + |
| G2 | RB 92579 | Stem | + | - | + | + |
| G4 | RB 867515 | Stem | + | - | + | + |
| G6 | RB 92579 | Stem | - | - | + | - |
| G7 | RB 92579 | Stem | + | + | + | - |
| G8 | RB 867515 | Stem | + | - | + | + |
| G9 | RB 863129 | Stem | - | - | + | + |
| G10 | RB 867515 | Stem | + | - | + | + |
| G11 | RB 867515 | Root | - | + | - | + |
| G12 | RB 867515 | Stem | - | - | - | + |
| G15 | RB 867515 | Stem | - | + | + | + |
| G16 | RB 867515 | Root | - | - | - | + |
| G17 | RB 92579 | Stem | - | + | + | + |
| G18 | RB 867515 | Stem | + | + | + | - |
| G19 | RB 92579 | Stem | + | + | + | - |
| G20 | RB 863129 | Stem | + | + | + | - |
| G21 | RB 863129 | Stem | + | + | + | - |
| G23 | RB 867515 | Root | - | + | - | + |
| G24 | RB 92579 | Root | - | - | - | + |
| G25 | RB 863129 | Stem | - | + | - | + |
| G26 | RB 867515 | Root | - | + | - | + |
| G27 | RB 867515 | Root | - | - | + | + |
| G28 | RB 863129 | Root | + | + | - | + |
| G29 | RB 867515 | Stem | + | - | + | + |

*[1]Biological nitrogen fixation; [2]indole acetic acid; [3]exopolysaccharide; (-) isolated with negative test result; (+) isolated with positive test result*

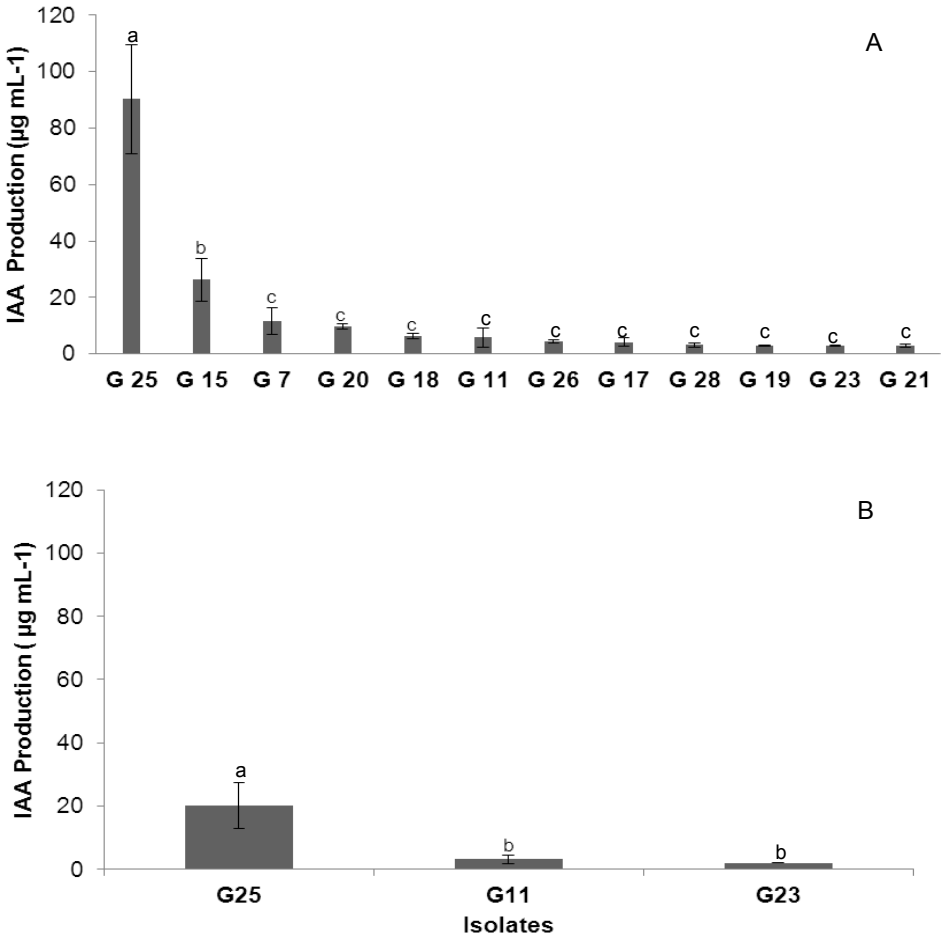


**Fig. 2. Indole acetic acid (IAA) production by endophytic bacterial isolates from sugarcane with (A) and without (B) addition of L-tryptophan. Averages followed by the same letter do not differ by Scott-Knott test (P<0.05)**

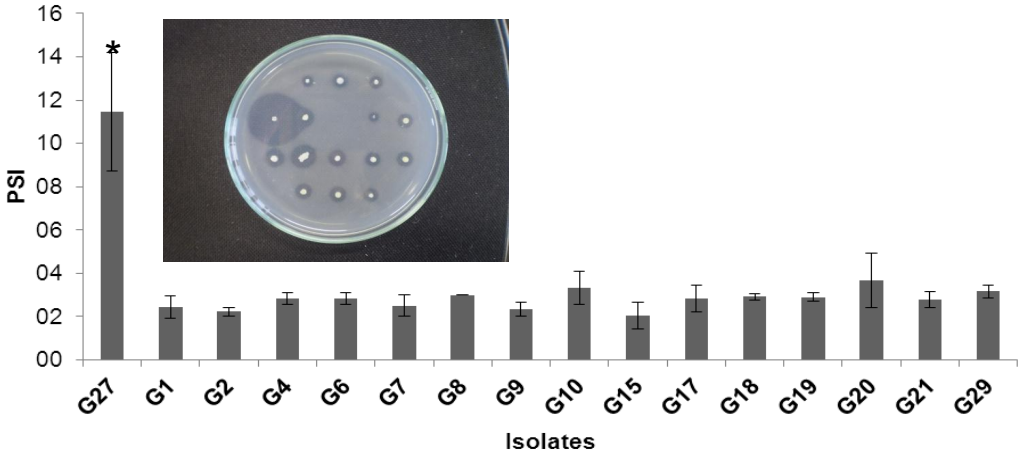


**Fig. 3. Phosphate solubilization index (PSI) of endophytic bacterial isolates from sugarcane. * Significant by Scott-Knott test (P<0.05)**

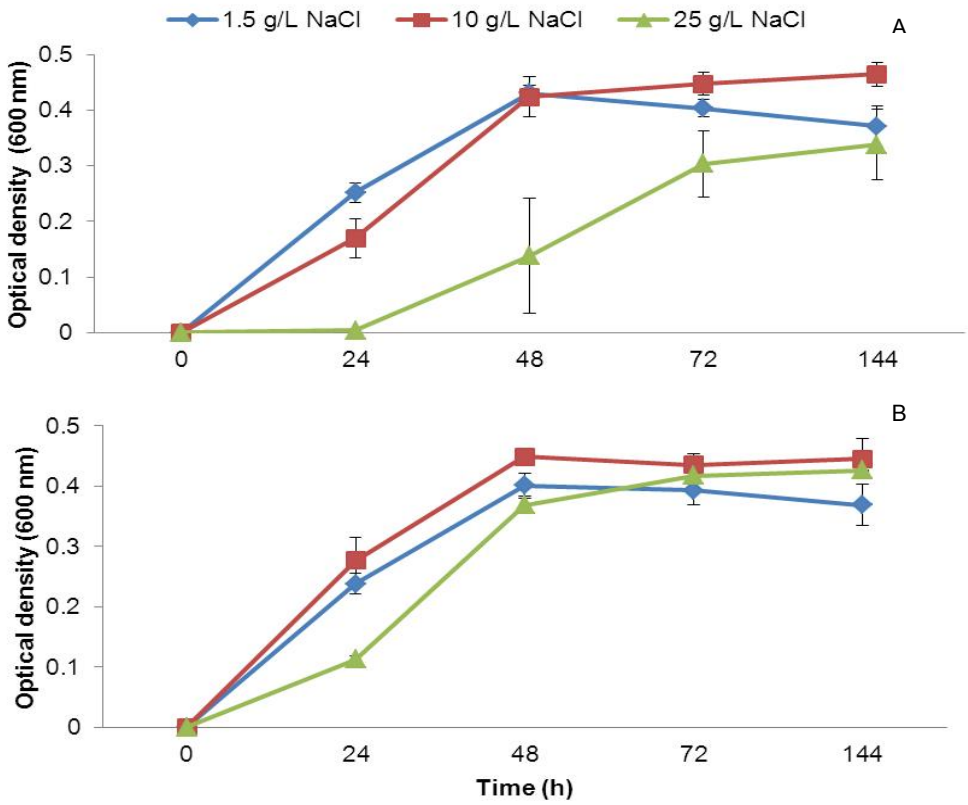


**Fig. 4. Growth of *Burkholderia gladioli* in liquid medium plus 1.5; 10; and 25 g $L^{-1}$ NaCl at pH 7,3 (A) and 5,5 (B). The error bar indicates the standard deviation of the average of three replicates**

**Table 2. The similarity of sugarcane endophytic isolates with bacterial species found by BLASTn against the NCBI database**

| Isolates | GenBank identification | Bacterial species | Similarity (%) |
|---|---|---|---|
| G1 | HM231304.1 | *Burkholderia gladioli* | 98 |
| G2 | GU936678.1 | *Burkholderia gladioli* | 99 |
| G9 | GU936678.1 | *Burkholderia gladioli* | 97 |
| G10 | GU936678.1 | *Burkholderia gladioli* | 100 |
| G18 | GU936678.1 | *Burkholderia gladioli* | 100 |
| G19 | GU936677.1 | *Burkholderia gladioli* | 99 |
| G28 | AB537486.1 | *Burkholderia heleia* | 98 |
| G29 | GU936678.1 | *Burkholderia gladioli* | 99 |

The eight isolates belong to the Betaproteobacteria class, order *Burkholderiales*, family *Burkholderiaceae*, and the differentiation of the isolates occurred until the level of genus (*Burkholderia*) (Fig. 7).

## 4. DISCUSSION

Our study revealed a higher concentration of plant growth-promoting bacteria in the sugarcane stem than in the roots. The study of Mendes [10] with TSB medium (non-selective) reported that the bacterial population density in the stem of sugarcane was lower than in the roots, being this profile observed by other authors in different host plants [10,15,22,23]. Gomes [24] observed higher population density of *Gluconoacetobacter diazotrophicus* in sugarcane roots of varieties SP70-1143 and SP79-2312. We also found a high population density of bacteria in the roots, but with characteristics of plant growth-promotion smaller than in the stem.

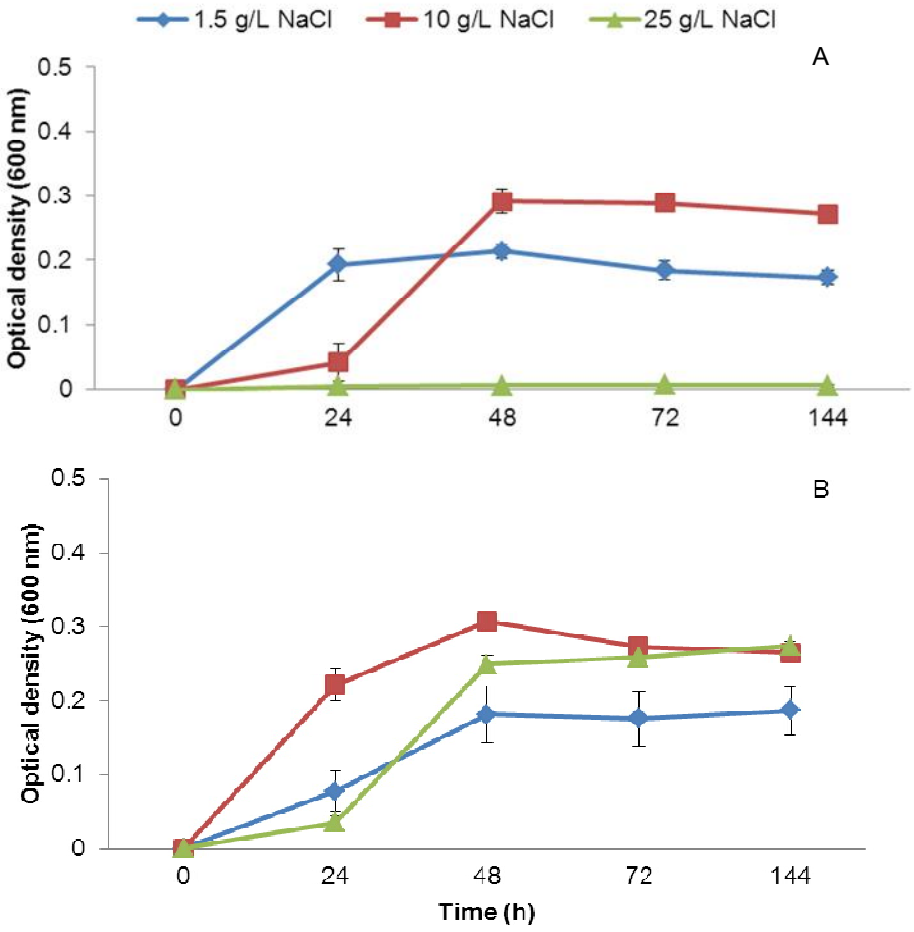


**Fig. 5. Growth of *Burkholderia heleia* in liquid medium plus 1.5; 10; and 25 g $L^{-1}$ NaCl at pH 7,3 (A) and 5,5 (B). The error bar indicates the standard deviation of the average of three replicates**

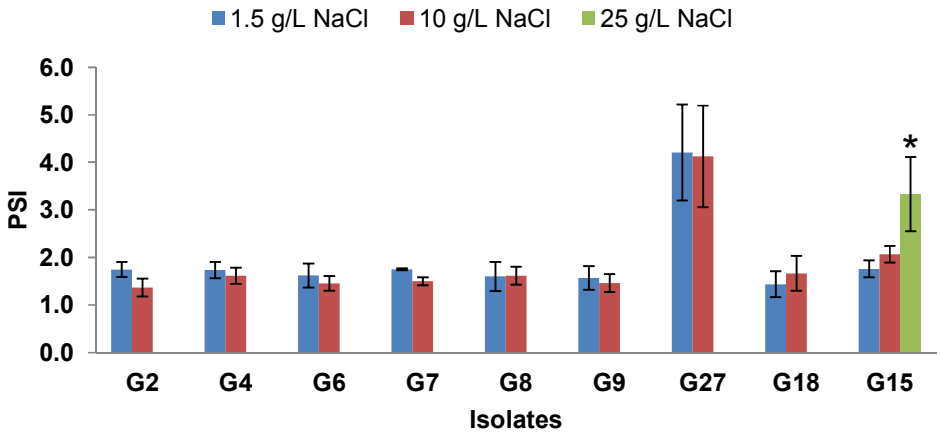


**Fig. 6. Phosphate solubilization index (PSI) of endophytic bacterial isolates from sugarcane, after 7 days of culture in rich medium in insoluble phosphate plus 1.5; 10; and 25 g $L^{-1}$ of NaCl. * Significant by Scott-Knott test (P<0.05)**

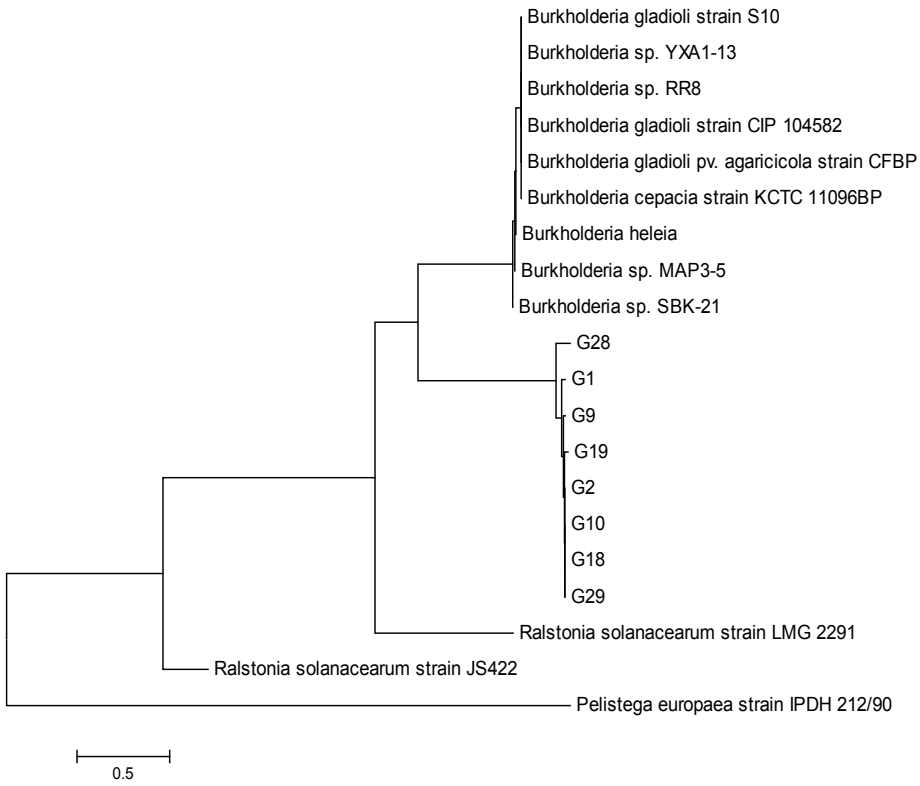


**Fig. 7. Phylogenetic tree of the 16S rRNA sequences of the bacterial endophytic isolates G1, G2, G9, G10, G18, G19, G28 and G29 obtained in the root and stem of the sugarcane varieties RB 92579, RB 867515 and RB 86 3129. The sequence of *Pelistega europaea* was used to root the phylogenetic tree as an external group**

The most important element for the increase of agricultural production is nitrogen, and the identification of bacterial isolates with respect to the element is of extreme importance for the reduction of the nitrogen fertilizers usage [7]. Our study identified isolates with potential for this purpose since they were able to grow in a nitrogen-free culture medium.

In addition to the potential for BNF of the bacteria isolated in this study, many of them also produced IAA. The study of Rodrigues [25] with mutants of *Gluconoacetobacter diazotrophicus* revealed IAA production mainly in the presence of tryptophan because your addition increased significantly the production of this auxin. However, according to Kuss [26] 90% the IAA synthesized by diazotrophic bacteria of the genus *Azospirillum* is derived from a tryptophan-independent pathway.

The variability existing in relation to biosynthetic pathway of IAA production by bacteria also extends the concentrations that the bacteria are capable of producing. According to Hernández-Rodríguez [27] about 29 μg $mL^{-1}$ of IAA was produced by *Burkholderia cepacia* in corn and rice seedlings, and in the study by Collavino [28] *Burkholderia* spp. produced only about 2.3 μg $mL^{-1}$ of IAA in vitro. The highest value found in this study was 90.29 μg $mL^{-1}$ of IAA in vitro of the G25 isolate, which shows the potential this isolate to be inoculated in sugarcane plants. According to Madhaiyan [29], *Azospirillum brasilense* produced IAA at the concentration of 71.38 μg $mL^{-1}$ and in the presence of L-tryptophan, which again emphasizes the potential of the G25 isolate, which produced a high concentration of IAA in medium with or without L-tryptophan.

According to Bastián [30], the bacterial species *Gluconoacetobacter diazotrophicus* produces different amounts of IAA when grown in culture medium containing different concentrations of sucrose, and its highest production happen in

culture medium containing 100 g $L^{-1}$ of sucrose; thus, the bacterial isolates studied here developed high IAA production capacity due to the selective isolation in LGI-P medium with 100 g $L^{-1}$ of sucrose.

The great majority of the bacteria in our study were able to solubilize phosphate. According to Shiomi [31], the inorganic phosphate solubilizing bacteria have been receiving the attention of the researchers, mainly due to the possibility of their use in programs of interaction with $N_2$-fixing bacteria.

According to Postma [32] 90% of the isolates of the genus *Burkholderia* were able to solubilize phosphate, evidencing the potential of this genus in this characteristic of plant growth-promotion [32]. In a study by Collavino [28], 33% of strains that solubilized phosphate belonged to the order *Burkholderiales*. Kuklinsky-Sobral [15] working with soybean found inorganic phosphate solubilizing strains belonging to the genus *Burkholderia*.

The variety RB 867515 presented the highest number of exopolysaccharide-producing isolates, suggesting a strong relationship between bacteria and the host plant, being influenced by several factors such as the host genotype and the microorganism itself [15,33]. Ben Ammar [34] working in of culture medium contained sucrose and with lower values of pH, reported that there may be the production of the enzyme levansucrase, which produces EPS. These same conditions were used in our work. However, further studies are needed with these isolates to identify which exopolysaccharide is produced and how much these isolates produce.

According to Ferreira [35], EPS plays an important role in the survival of bacteria of the genus *Burkholderia* contributing to their ability to thrive in different environments. Thus, the isolates studied here have a high capacity of production of EPS, which may facilitate their development in association with sugarcane and provide a better efficiency of the inoculum.

The eight isolates identified by 16S rRNA partial sequence analysis in this study were of the genus *Burkholderia* and in work by Perin [36] a great diversity of species of the genus *Burkholderia* was found in corn and sugarcane, among them the species *Burkholderia gladioli*. According to Jha [37] bacteria of the genus, *Burkholderia* was also found in rice. Perin [36] reported that the genus *Burkholderia* includes more than 30 species, but not all are atmospheric $N_2$ fixers. In our study, it was evidenced that the species found *Burkholderia* gladioli and *Burkholderia* heleia, were nitrogen fixers.

In a study carried out by Mendes [10] also were found isolates of the genus *Burkholderia* from sugarcane. However, the authors did not relate the occurrence of the two species found in our work. Luvizotto [38] also not found *Burkholderia gladioli* and *Burkholderia heleia* in 39 isolated belonging the genus *Burkholderia*.

When adverse environmental factors were evaluated, their influence on bacterial growth and in characteristics of plant growth-promotion was well evident. Isolates of root and stem nodules of *Discolobium* spp. in study performed Campos [39] showed a growth of the bacteria in the concentration of 30 g $L^{-1}$ of salt. This behavior was also observed by others authors [13,39,40] in rhizobia strains of cowpea. While in this study the maximum salt concentration evaluated was 25 g $L^{-1}$, resulting in inhibition of G28 lineage growth. In the same work, Campos [39] demonstrated that the alkaline pH was more harmful to the growth of the isolates than the acidic pH. This is in agreement with the observed here because the decrease of the growth was accentuated for the treatment with 25 g $L^{-1}$ of NaCl only in pH 7.3; in at pH 5.5, the bacterial growth was similar to the other treatments.

According to work done by Medeiros [41] with rhizobia, resistance to salinity was observed up to 50 g $L^{-1}$ of NaCl in pH 6.8. In our study, the limiting salt concentration was lower, but the pH of the medium was 7.3; suggesting that the negative influence on the growth of the strains was due to the combination of the salt with the pH, because at the same concentration (25 g $L^{-1}$) but at lower pH the growth was satisfactory.

In our study the salinity of culture medium was harmful the phosphate solubilization performed by the bacteria. Ramani [42] observed that phosphate solubilization by *Bacillus sphaericus* was affected by the use of some pesticides. Leite et al. [11] also demonstrated the negative effect of NaCl under the capacity of the bacteria perform phosphate solubilization. This demonstrates that environmental characteristics, such as salinity and crop management directly influence this characteristic of plant growth-promotion.

Medeiros [43] demonstrated that nodulation in cowpea is highly sensitive to the salinity of soils, damaging the BNF. Thus, salinity effects should be more extensively studied so that the bacterial/plant interaction becomes more efficient and the plant growth-promotion is performed more efficiently, even in limiting environments such as saline soils.

## 5. CONCLUSION

The presence of salt and different pH values of the culture medium influenced the growth of the isolates and the salt concentration influenced phosphate solubilization. Seven isolates showed similarity with *Burkholderia gladioli* and one with *Burkholderia heleia*, besides many genera only of *Burkholderia* sp., suggesting low genetic diversity. *Burkholderia heleia* had a lower growth rate than *Burkholderia gladioli*. It was a bacterium very sensitive to high amounts of salt when the pH was higher. The root bacterial isolate G27 obtained from RB 867515 presented the highest PSI in the lowest saline concentrations and the stem isolate G15 of the same variety was able to solubilize phosphate in high salt concentration. We conclude that sugarcane interacts with potentially diazotrophic entophytic bacteria involved with plant growth-promotion and can be used as inoculants in management programs of this crop, however, it is necessary to observe soil salinity levels and monitor pH to maximize plant growth-promotion characteristics of these bacteria.

## ACKNOWLEDGEMENTS

This study was supported by CNPq Project Nº 552842/2007-9 “Sustainability of Northeast sugarcane production: Bioprospecting of microorganisms/plant relationships in reducing the use of nitrogen fertilizers”.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. CEPEA - Centro de Estudos Avançados em Economia Aplicada. GDP of Brazilian agribusiness chains - January to December 2016. 2016;16:1. Portuguese
(Accessed 23 February 2017)
Available:http://www.cepea.esalq.usp.br/upload/kceditor/files/Relatorio%20PIBAGRO%20Cadeias_2016(1).pdf
2. Andreote FD, Silva MCP. Microbial communities associated with plants: Learning from nature to apply it in agriculture. Curr Opin Microbiol. 2017;37: 29–34.
Available:http://dx.doi.org/10.1016/j.mib.2017.03.011
3. Bhattacharjee RB, Singh A, Mukhopadhyay SN. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: Prospects and challenges. Appl Microbiol Biotechnol. 2008;80:199–209.
Available:http://dx.doi.org/10.1007/s00253-008-1567-2
4. Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, et al. The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev. 2015;79:293–320.
Available:http://dx.doi.org/10.1128/MMBR.00050-14
5. Grant CA, Flaten DN, Tomasiewicz DJ, Sheppard SC. The importance of phosphorus in early plant development. Piracicaba: Potafos (Informações agronômicas, 95); 2001. Portuguese
6. Vassilev N, Vassileva M. Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes. Appl Microbiol Biotechnol. 2003;61:435–440.
Available:http://dx.doi.org/10.1007/s00253-003-1318-3
7. Pereira APA, Silva MCB, Oliveira JRS, Ramos APS, Freire MBGS, Freire FJ, et al. Iinfluence of salinity on the growth and yield of indole acetic acid of *Burkholderia* spp. endophytic sugarcane. Biosci J. 2012;28:112–121. Portuguese
8. Estrada-Bonilla GA, Lopes CM, Durrer A, Alves PRL, Passaglia N, Cardoso EJBN. Effect of phosphate-solubilizing bacteria on phosphorus dynamics and the bacterial community during composting of sugarcane industry waste. Syst Appl Microbiol. 2017;40:308–313.
Available:http://dx.doi.org/10.1016/j.syapm.2017.05.003
9. Gyaneshwar P, Kumar GN, Parekh LJ, Poole PS. Role of soil microorganisms in

improving P nutrition of plants. Plant Soil. 2002;245:83–93.
Available:http://dx.doi.org/10.1023/A:1020663916259

10. Mendes R, Pizzirani-Kleiner AA, Araujo WL, Raaijmakers JM. Diversity of cultivated endophytic bacteria from sugarcane: Genetic and biochemical characterization of *Burkholderia cepacia* complex isolates. Appl Environ Microbiol. 2007;73:7259–7267.
Available:http://dx.doi.org/10.1128/AEM.01222-07
11. Leite MCBS, Farias ARB, Freire FJ, Andreote FD, Kuklinsky-Sobral J, Freire MBGS. Isolation, bioprospecting and diversity of salt-tolerant bacteria associated with sugarcane in soils of Pernambuco, Brazil. Anais do II Inovagri International Meeting. 2014;3715–3725.
Available:http://dx.doi.org/10.12702/ii.inovagri.2014-a498
12. Compant S, Duffy B, Nowak J, Cle C, Barka EA. Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Appl Environ Microbiol. 2005;71:4951–4959.
Available:http://dx.doi.org/10.1128/AEM.71.9.4951
13. Nóbrega RSA, Motta JS, Lacerda AM, Moreira FMS. Tolerance of diazotrophic symbiotic bacteria to salinity. Ciênc Agrotec. 2004;28:899–905. Portuguese
Available:http://dx.doi.org/10.1590/S1413-70542004000400024
14. Amarante CVT, Ernani PR, Souza AG. Influence of liming and phosphate fertilization on the accumulation of nutrients and growth of São-João grass. Hortic Bras. 2007;25:533–537. Portuguese
Available:http://dx.doi.org/10.1590/S0102-05362007000400008
15. Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environl Microbiol. 2004;6:1244–1251.
Available:http://dx.doi.org/10.1111/j.1462-2920.2004.00658.x
16. Araújo WL, Lacava PT, Marcon J, Lima AOS, Kuklinsky-Sobral J, Pizzirani-Kleiner AA, et al. Practical guide: Isolation and characterization of endophytic micro-organisms. Piracicaba: CALO; 2010. Portuguese
17. Döbereiner J, Baldani VLD, Baldani JI. How to isolate and identify diazotrophic bacteria from non-leguminous plants Brasília: EMBRAPA; 1995. Portuguese
18. Crozier A, Arruda P, Jasmim JM, Monteiro AM, Sandberg G. Analysis of indole-3-acetic acid and related indoles in culture medium from *Azospirillum lipoferum* and *Azospirillum brasilense*. Appl Environ Microbiol. 1988;54:2833–2837.
19. Berraquero FR, Baya AM, Cormenzana AR. Establishment of indices for the study of the solubilization of phosphates by soil bacteria. ARS Pharm. 1976;17:399-406. Spanish
20. Santos IB, Lima DRM, Barbosa JG, Oliveira JTC, Freire FJ, Kuklinsky-Sobral J. Diazotrophic bacteria associated to roots of sugarcane: Inorganic phosphate solubilization and the salinity tolerance. Biosci J. 2012;28:142–149. Portuguese
21. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol. 1975;94:441–448.
Available:http://dx.doi.org/10.1016/0022-2836(75)90213-2
22. Rosenblueth M, Martínez-Romero E. Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact. 2006;19:827–837.
Available:http://dx.doi.org/10.1094/MPMI-19-0827
23. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013;31:814–821.
Available:http://dx.doi.org/10.1038/nbt.2676
24. Gomes AA, Reis VM, Baldani VLD, Goi SR. Relationship between nitrogen distribution and colonization by diazotrophic bacteria in sugarcane. Pesqui Agropecu Bras. 2005;40:1105–1113. Portuguese
Available:http://dx.doi.org/10.1590/S0100-204X2005001100008
25. Rodrigues EP, Oliveira ALM, Vidal MS, Simões-Araújo JL, Baldani JI. Obtaining

and selection of Tn5 mutants of *Gluconacetobacter diazotrophicus* (Pal 5) with changes in production of auxins. Seropédica: UFRRJ; 2007. Portuguese

26. Kuss AV, Kuss VV, Lovato T, Flôres ML. Nitrogen fixation and indoleacetic acid production *in vitro* by endophytic diazotrophic bacteria. Pesqui Agropecu Bras. 2007;42:1459–1465. Portuguese
Available:http://dx.doi.org/10.1590/S0100-204X2007001000013
27. Hernández-Rodríguez A, Heydrich-Pérez M, Diallo B, Jaziri M, Vandeputte OM. Cell-free culture medium of *Burkholderia cepacia* improves seed germination and seedling growth in maize (*Zea mays*) and rice (*Oryza sativa*). Plant Growth Regul. 2010;60:191–197.
Available:http://dx.doi.org/10.1007/s10725-009-9433-5
28. Collavino MM, Sansberro PA, Mroginski LA, Aguilar OM. Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. Biol Fertil Soils. 2010;46:727–738.
Available:http://dx.doi.org/10.1007/s00374-010-0480-x
29. Madhaiyan MS, Kang PBG, Lee YJ, Chung JB, Sa TM. Effect of co-inoculation of methylotrophic *Methylobacterium oryzae* with *Azospirillum brasilense* and *Burkholderia pyrrocinia* on the growth and nutrient uptake of tomato, red pepper and rice. Plant Soil 2010;328:71–82.
Available:http://dx.doi.org/10.1007/s11104-009-0083-1
30. Bastián F, Cohen A, Piccoli P, Luna V, Baraldi R, Bottini R. Production of indole-3-acetic acid and gibberellins A1 and A3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically-defined culture media. Plant Growth Regul. 1998;24:7–11.
Available:http://dx.doi.org/10.1023/A:1005964031159
31. Shiomi HF, Dedori C, Vicensi M, Oliari ICR. Selection of inorganic phosphate solubilizing bacteria present in biofertilizers. Rev Bras Agroecol. 2009;4:290–293. Portuguese
32. Postma J, Nijhuis EH, Someus E. Selection of phosphorus solubilizing bacteria with biocontrol potential for growth in phosphorus rich animal bone charcoal. Appl Soil Ecol. 2010;46;464–469.
Available:http://dx.doi.org/10.1016/j.apsoil.2010.08.016
33. Boddey RM, Urquiaga S, Alves BJR, Reis V. Endophytic nitrogen fixation in sugarcane: Present knowledge and future applications. Plant Soil. 2003;252:139–149.
Available:http://dx.doi.org/10.1023/A:1024152126541
34. Ben Ammar Y, Matzubara T, Ito K, Lizuka M, Limpaseni T, Pongsawasdi P, et al. Characterization of a thermostable levansucrase from *Bacillus* sp. TH4-2 capable of producing high molecular weight levan at high temperature. J Biotechnol. 2002;99:111–119.
Available:http://dx.doi.org/10.1016/S0168-1656(02)00160-8
35. Ferreira ADS, Leitão JH, Silva IN, Pinheiros PF, Sousa SA, Ramos CG, et al. Distribution of cepacian biosynthesis genes among environmental and clinical *Burkholderia* strains and role of cepacian exopolysaccharide in resistance to stress conditions. Appl Environ Microbiol. 2010;76:441–450.
Available:http://dx.doi.org/10.1128/AEM.01828-09
36. Perin LL, Martinez-Aguilar G, Paredes-Valdez JI, Baldani P, Estrada-de los Santos P, Reis VM, et al. *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. Int J Syst Evol Microbiol. 2006;56:1931–1937.
Available:http://dx.doi.org/10.1099/ijs.0.64362-0
37. Jha B, Thakur MC, Gontia I, Albrecht V, Stoffels M, Schmid M, et al. Isolation, partial identification and application of diazotrophic rhizobacteria from traditional Indian rice cultivars. Eur J Soil Biol. 2009;45:62–72.
Available:http://dx.doi.org/10.1016/j.ejsobi.2008.06.007
38. Luvizotto DM, Marcon J, Andreote FD, Dini-Andreote F, Neves AAC, Araújo WL, et al. Genetic diversity and plant-growth related features of *Burkholderia* spp. from sugarcane roots. World J Microbiol Biotechnol. 2010;26:1829–1836.
Available:http://dx.doi.org/10.1007/s11274-010-0364-0

39. Campos LL, Martins ME, Elias Neto N, Loureiro MF. Physiological characterization of rhizobia isolated from root and stem nodules of *Discolobium* spp. Scientia Agraria Paranaensis. 2010;9:75–84. Portuguese

40. Xavier GR, Rumjanek NG, Neves MCP. Tolerance of rhizobia of cowpea to salinity and temperature *in vitro* condition. Caatinga. 2007;4:1–9. Portuguese

41. Medeiros EV, Julliany K, Silva P, Martins CM, Borges WL. Tolerance of nitrogen-fixing bacteria from Rio Grande do Norte municipalities at temperature and salinity. Bioterra. 2007;7:160–168. Portuguese

42. Ramani V. Effect of pesticides on phosphate solubilization by *Bacillus sphaericus* and *Pseudomonas cepa*cia. Pestic Biochem Physiol. 2011;99:232–236. Available:http://dx.doi.org/10.1016/j.pestbp.2011.01.001

43. Medeiros R, Santos V, Araújo A, Oliveira Filho C. Saline stress on nodulation in cowpea. Caatinga. 2008;21:202–206. Portuguese



*Peer-review history:*
*The peer review history for this paper can be accessed here:*
*http://www.sciencedomain.org/review-history/23525*