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Isolation and Characterization of Plant Growth-Promotion Diazotrophic Endophytic Bacteria Associated to Sugarcane (*Saccharum officinarum* L.) Grown in Paraíba, Brazil

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HIGHLIGHTS

- The diazotrophic endophytic community found in stems were different those found in leaves of sugarcane.
- Endophytes with valuable biochemical traits have been identified in the sugarcane tissues.
- A unique symbiotic consortium between endophytes were identified in the sugarcane tissues.

Abstract: Sugarcane is an important Brazilian commodity, being usually cultivated in soils with low natural fertility. This study aimed to isolate diazotrophic endophytes from sugarcane tissues and evaluate the morphological and physiological characteristics of their colonies as well as their plant growth-promoting (PGP) traits in select diazotrophic endophytic bacteria. Fifty-six bacterial isolates were identified in the sugarcane tissues, and these isolates presented distinct morphological and physiological traits. A total of thirty-five bacterial isolates were biochemically evaluated. Overall, *Bacillus* was the dominant genus. Isolates of *Methylobacterium* spp. and *Brevibacillus agri* were present only in leaves, while *Herbaspirillum*

seropedicae occurred only in stems. Except to IPA-CF45A, all isolates were nitrogenase positive. All endophytes exhibit production of indol 3-acetic acid. Over 50% of endophytes solubilize phosphate, release N-acyl homoserine lactones, and present the activity of 1-aminocyclopropane-1-carboxylic acid deaminase, catalase, lipase and protease. The network analysis showed that isolates belonged to *Burkholderia*, *Herbaspirillum*, and *Methylobacterium* interact with *Bacillus*. Bacterial endophytes exhibited distinct morphological, physiological, and PGP traits that are useful for sustainable agriculture, highlighting the isolates IPA-CC33, IPA-CF65, IPA-CC9 and IPA-CF27. Further studies on the effects of these diazotrophic endophytes and their potential for providing microbial inoculants for improving sugarcane fields will provide valuable information to maintain the sustainability and environment quality.

Keywords: Nitrogen-fixing bacteria; phytohormones; phosphate; indol 3-acetic acid; catalase; protease.

INTRODUCTION

Sugarcane (*Saccharum* spp.) is economically important in several countries, mainly those located in Latin America. Nowadays, Brazil is the world's largest sugarcane producer, presenting an estimated area of 8.5 million hectares and an average productivity of about 75.8 Mg ha^{-1} [1]. The Northeastern region of Brazil is particularly important for sugarcane production, and Pernambuco, Paraíba, and Alagoas are the most productive states, representing about 80% of all sugarcane produced in 2018/2019 [2]. Although important, sugarcane is usually cultivated in soils with low fertility that limit its productivity and, at the same time, require the intensive use of chemical fertilizers, mainly nitrogen [3].

Nitrogen is the most required nutrient by sugarcane, and chemical fertilization is necessary to provide nitrogen to this plant species and, therefore, to increase its productivity [3]. Under Brazilian-field conditions, sugarcane absorbs 26% of the total nitrogen applied through of chemical fertilization and the remaining N is lost [4]. Thus, nitrogen can be supplied to sugarcane by the biological nitrogen fixation (BNF), and this process can significantly increase sugarcane yield [5, 6]. Nitrogen-fixing bacteria carry out the BNF process endophytically in sugarcane tissues and then can supply a considerable proportion of nitrogen to these plants and, therefore, promote plant growth and crop yield. According to Bordonal and coauthors [3], BNF represents an important source of N to sugarcane and contributes with more than 40 kg N ha^{-1} $year^{-1}$.

The endophytic bacteria colonize different plant organs, including roots, stems, leaves, flowers, and fruits, and have been recognized as efficient plant growth-promoters [7]. As they act endophytically, these bacteria are more efficient than those found in the rhizosphere because they are always in contact with plant tissues and, therefore, can be more beneficial [8]. Schultz and coauthors [6] have reported that BNF, performed by diazotrophic endophytes, contributes significantly to the total of nitrogen found in sugarcane. Beyond nitrogen-fixation, these bacteria display important plant growth-promoting (PGP) traits, such as production of phytohormones (auxins, cytokinins, and gibberellins) and *N*-acyl homoserine lactones, as well as the protection against phytopathogens, and amelioration of abiotic stress [8, 9].

There is a high diversity of PGPB associated with sugarcane; however, the knowledge of this diversity and their PGP traits is limited. Commercial sugarcane plants cultivated in Thailand exhibited an endophytic bacteria community with important PGP traits, including 1-aminocyclopropane-1-decarboxylate (ACC) deaminase, indole-3-acetic (IAA) acid, nitrogen fixation, phosphate solubilization, and siderophore production [10]. In sugarcane plants grown in nitrogen-free conditions, all bacterial endophytes displayed nitrogenase activity and three of them were able to achieve high nitrogen contents in sugarcane [5]. Diazotrophic endophytic bacteria associated to sugarcane tissues identified by Leite and coauthors [11] solubilized phosphate and produced IAA and exopolysaccharides. IAA-producing bacteria with antifungal properties were identified in endophytic bacteria community found in sugarcane tissues [12]. These studies reinforce that these bacterial endophytes can effectively stimulate the plant growth in different soils.

As reported above, these bacteria are easily found in plant tissues and can be isolated to enable the evaluation of their biochemical capabilities related to BNF and plant growth. Thus, these endophytic bacteria can be used to benefit sugarcane by increasing its growth and yield. This study isolated diazotrophic endophytes from leaves and stems of sugarcane and evaluated their morphophysiological and biochemical traits. The main hypothesis is that diazotrophic endophytic bacteria associated with sugarcane could produce and release useful biochemical compounds and, thus, act as plant growth-promoters. Therefore, these selected bacteria could be used to promote the growth of sugarcane and other agronomically important crops.

MATERIAL AND METHODS

Plant material and bacterial isolation

Samples of leaves and stems of sugarcane (varieties RB 1011, RB 867515, and RB 92579) from fields located in distillery Japungú (7°10'38.85"S and 34°58'12.72"W; Santa Rita, Paraiba, Brazil) and distillery Tabú (7°30'30.19"S and 34°52'33.17" W; Alhandra, Paraiba, Brazil) were used for the isolation of endophytic diazotrophic bacteria. These samples were transferred to the Laboratory of Soil Biology from the Agronomical Institute of Pernambuco (Recife, Pernambuco, Brazil). Leaves and stems (10 g each) were macerated, separately, in 5% sterile saline solution, and plated on tubes containing semisolid broth without nitrogen: LGI (Liquid Glucose Ivo) [13], LGI-P (Liquid Glucose Ivo Pernambuco) [14], JMV (Johanna Mannitol Vera) [15], JNFb (Johanna New Fabio) [16] and NFb (New Fabio) [17]. The full composition of each nitrogen-free semisolid culture media are specified in supplementary table 1.

The tubes containing semisolid and nitrogen-free broth were incubated at 30 °C until diazotrophic bacteria exhibit typical growth, i.e., the formation of a pellicle near the surface of the medium (microaerophilic film) [18]. After re-isolation and confirmation of purity, the bacterial strains were stored in potato-P media with added sterile mineral oil. The procedure was conducted in triplicate. The potato-P media (pH 5.5) was composed by: potato filtrate (200 g L^{-1}); crystalized cane sugar (100 g L^{-1}); KOH (2.0 g L^{-1}); vitamin solution (1.0 mL L^{-1}); micronutrient solution (2.0 mL L^{-1}); two drops of bromothymol blue (5 g L^{-1} in 0.2 N KOH); and agar (15.0 g L^{-1}). The potato filtrate was obtained after sliced potatoes homogenized with distilled water were boiled for 30 min. The vitamin solution was composed by biotin (10 mg L^{-1}) and pyridoxal-HCl (20 mg L^{-1}). The micronutrient solution contains: $CuSO_4.5H_2O$ (0.04 g L^{-1}), $ZnSO_4.7H_2O$ (0.12 g L^{-1}), H_3BO_3 (1.40 g L^{-1}), $Na_2MoO_4.2H_2O$ (1.0 g L^{-1}) and $MnSO_4.H_2O$ (1.175 g L^{-1}).

Morphological and physiological characterizations

Morphological and physiological characterizations of bacterial isolates were performed after 192 hours of bacterial growth in Petri dishes containing culture medium 79 with bromothymol blue [19]. The following characteristics of bacterial colonies were evaluated: size/diameter (<1.0 mm or > 1.0 mm); shape (circular, lenticular or irregular); edge (whole, wavy or filamentous); surface (smooth, rough or filamentous); appearance (translucent, opaque or transparent); color (white, yellow, cream, transparent or pink); formation of acids and alkalis; formation and volume of mucus (absent or present, much or little); and growth time (fast, intermediate, or slow). The Gram stains of bacterial isolates were performed according to Yano and coauthors [20].

Bacterial identification

Polymerase chain reaction (PCR) amplification of repetitive bacterial DNA fingerprinting (rep-PCR) using the BOX-A1R primer was used to differentiate bacterial isolates, while ribosomal 16S rRNA gene was amplified and analyzed to identify bacterial isolates at genus or species level. Bacterial isolates were retrieved from storage by subculture on liquid DYGS (Dextrose Yeast Glucose Sucrose) culture medium [21] and incubated at 30 °C (200 rpm; 48 h). Bacterial genomic DNA was extracted from colonies using Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA) according to the manufacturer's recommendations. The extracted DNA was diluted in sterile Milli-Q water (30 ng μL^{-1}) and stored at -20 °C until further use.

Amplification was performed in an Applied Biosystems 2720 Thermal Cycler (Life Technologies, CA, USA) using the BOX-A1R oligonucleotide (5'-CTACGGGGCCAAGACGACGCTG-3') (Invitrogen, MA, USA). To rep-PCR, DNA template (20 ng μL^{-1}) was used in a 20 µL reaction mixture containing 2.0 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTPs), 0.2 µM primer, 10X 10% Buffer and 0.3 U Taq polymerase. The rep-PCR program was conducted using an initial denaturation of 9 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 5 min, with a final extension at 72 °C for 10 min. The rep-PCR products were analyzed by 1.2% agarose gel electrophoresis and visualized on LPIX HE transilluminator (Loccus Biotechnology, SP, Brazil). The resulting fingerprints were transformed into a two-dimensional binary matrix (‘1’ for the presence and ‘0’ for the absence of a band at a particular position) and analyzed by NTSYSpc 2.1 software using the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

Following rep-PCR fingerprinting analysis, ribosomal 16S rRNA genes sequencing of the bacterial isolates using the FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RD1 (5'-AAGGAGGTGATCCAGCC-3')

primers was performed. PCR reaction was carried out using a 50 µL reaction mixture containing 0.2 mM dNTPs, 10X 10% Buffer, 0.2 µM of each primer, 2.0 mM $MgCl_2$, 0.3 U Taq DNA polymerase (5 U μL^{-1}) and template DNA (20 ng μL^{-1}). The PCR amplification was performed in an Applied Biosystems 2720 Thermal Cycler (Life Technologies, CA, USA), being initiated by incubating the reaction mixture at 94 °C for 3 min followed by 30 cycles of denaturation (94 °C for 50 s), annealing (57 °C for 50 s) and extension (72 °C for 60 s), and finished with a final extension step at 72 °C for 7 min.

The PCR products were confirmed by electrophoresis on a 1.2% agarose gel containing SybrGold® (Sigma-Aldrich, MO, USA) and viewed by exposure to UV light on transilluminator. The 1 kb Plus DNA ladder (Promega Corporation, WI, USA) was used as molecular weight marker. The PCR products were purified and sequenced by Macrogen (Seoul, Korea) and StabVida (Lisbon, Portugal). All sequences generated were compared with GenBank database for similarity search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment was carried out with SeqMan, BioEdit, and ClustalW programs. The neighbor-joining phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) 5.1 program.

Plant growth-promotion and biocontrolling tests

Bacterial isolates identified as described previously were considered to biochemical characterization (plant growth-promotion and biocontrolling tests). The phosphate solubilization was tested using the National Botanical Research Institute's Phosphate (NBRIP) medium containing insoluble tricalcium phosphate as the single phosphorus source [22]. After incubation at 28 °C for 72 h, all isolates forming clear halo zones were identified as phosphate-solubilizing bacteria. Based in halo zone and colony diameters, the index of phosphate solubilization was calculated. IAA production was quantified using the Salkowski-based colorimetric technique [23]. For this, bacteria isolates were incubated in Tryptic Soy Broth (TSB) culture media supplied with L-tryptophan under shaking condition (150 rpm; 28 °C) for 48 h. A separate TSB broth inoculated with sterile water was used as control. IAA production was quantitated spectrophotometrically at 530 nm by using Salkowski's reagent (0.5 mM $FeCl_3$ and 35% $HClO_4$) and expressed as mg mL^{-1}.

The Chromeazurol-S (CAS) assay was used to detect siderophores [24]. Bacterial isolates were cultured in TSB broth containing CAS for 120 h (28 °C) and isolates forming yellow halo zones around the colony were identified as siderophore-producing bacteria. *N*-acyl homoserine lactones (AHL) production was checked by growing bacterial isolates in TSB agar medium containing X-Gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactoside) and the biosensor strain (*Agrobacterium tumefaciens* NT1) [25]. The production of AHL was confirmed by visualization of the blue halo around the well after 48 h. The ACC deaminase activity of bacterial isolates was screened based on their ability to use ACC as nitrogen source [26]. All isolates were inoculated in TSB medium supplemented with 3.0 mM ACC for 72 h (28 °C). Petri plates without ACC and with 0.2% (w/v) ammonium sulfate were used as the negative and positive control, respectively. Bacterial isolates with ACC deaminase activity were identified comparing with the negative and positive controls.

Nitrogenase activity was measured using the acetylene reduction assay [27]. For this purpose, aliquots of bacterial cultures (2.0 mL) were transferred into glass tubes and under a hypoxic atmosphere with 10% acetylene (C_2H_4) for 18 h. The quantification of the ethylene produced in the samples from each bottle was carried out in a gas chromatograph with flame ionization detector. Tubes without injected acetylene were used as negative control. After calculated, nitrogenase activity were expressed as ηmol C_2H_4 $hour^{-1}$. Catalase activity was checked by placing drops of hydrogen peroxide onto the bacterial colony streaked on TSB broth and the appearance of bubbles was positive for the test [20]. Gelatinase activity was detected in glass tubes using a TSB broth supplied with gelatin [20]. The enzyme liquefies the growth medium by hydrolyzing gelatin and therefore gelatin liquefaction was considered a positive result.

Amylase activity was detected in TSB broth containing 1% soluble starch [28]. The Petri dishes were incubated in a growth chamber (23°C; 5 days) under dark conditions. Amylase was identified by a translucent halo around the colony in contrast with a purple background. To chitinase activity [29], the isolates were grown in TSB broth supplied with 0.5% colloidal chitin and positive cultures were identified by observing transparent halo surrounding the colony. Lipase activity was observed in a TSB culture media supplemented with 1.0% Tween 20 and incubated at 23 °C in the dark for five days [29]. Lipase activity was detected by the formation of a clear halo around the bacterial colony. The protease activity was measured using TSB culture medium supplied with casein and casein hydrolysis was visualized by the formation of a transparent halo [30]. The isolates were grown in TSB culture media supplied with urea incubated for 48 h (20 °C) to measure urease activity [31]. After incubation, the presence of pink halo zones was positive for the test.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) preceded by the F test at the 1% and 5% probability levels. The data were analyzed using the Shapiro-Wilk test to evaluate normality. Scott-Knott test ($p < 0.05$) was used to analyze the quantitative data (phosphate solubilization, IAA production, and nitrogenase activity), and statistical analyses were performed using the SAS software version 8.2. The network was built by using Pearson correlation coefficients with the Euclidian dissimilarity indices using the free statistics Past software version 4.0 (https://folk.uio.no/ohammer/past/).

RESULTS

Isolation of bacterial endophytes

Fifty-six bacterial isolates were obtained from leaves (21 isolates) and stems (35 isolates) of sugarcane (Supplementary tables 2 and 3). The isolates only grew in the NFb, JNFb, and LGI-P, while no isolates were found in the LGI or JMV broths. A total of 30 and 18 bacterial isolates were, respectively, grown in NFb and JNFb broths, while eight bacterial isolates were grown in LGI-P broth (IPA-CC49, IPA-CC51, IPA-CC52, IPA-CC55, IPA-CC56, IPA-CF61, IPA-CF62, and IPA-CF65).

Morphological and physiological traits

The morphological and physiological traits of bacterial isolates are summarized in Figure 1. Thirty-nine colonies of bacterial isolates were smaller than 1.0 mm, including 13 isolates from leaves and 26 isolates from stems, while 17 colonies were larger than 1.0 mm (Figure 1A). The majority of colonies isolated in leaves (78%) and stems (90%) were circular (Figure 1B), with a whole border (more than 70% of all isolates) and a smooth surface (85% and 92% of isolates from leaves and stems, respectively) (Figure 1B). Filamentous borders were found only in three bacterial colonies isolated from stems (isolates IPA-CC11, IPA-CC30B, and IPA-CC30C), and only one bacterial colony (IPA-CC11 from stems) displayed a filamentous surface. In general, bacterial colonies from leaves were translucent (60%), while bacterial colonies from stems were proportionally opaque (37%), translucent (29%), or transparent (34%). As showed in Figure 1C, yellow and cream were most prevalent colors observed in the bacterial colonies. The majority of bacterial isolates were able to produce acids (Figure 1D) and unable to produce mucus (Figure 1E). Furthermore, bacterial colonies displayed fast growth (88%) and were Gram-positive (63%) (Figure 1E and 1F).

Biochemical traits

Thirty-five bacterial isolates, 15 endophytes from leaves and 20 isolates from stems, were biochemically evaluated and presented characteristics of diazotrophic and plant growth-promoting bacteria (PGPB). In leaves, three isolates were recognized as diazotrophs and 12 bacterial isolates were recognized as PGPB. In contrast, 12 and 14 isolates from stems were identified as diazotrophs and PGPB, respectively. Isolates of *Brevibacillus agri* and *Methylobacterium organophilum* were present only in leaves, while isolates of *Herbaspirillum seropedicae* were found only in stems (Figure 2D). All bacterial isolates from the leaves and stems of the sugarcane plants synthesized IAA (Figure 3A). Except in IPA-CF45A, all bacterial isolates showed a positive nitrogenase activity (Figure 3B).

Bacterial isolates from leaves (80%) and stems (55%) solubilized phosphate (Figure 3C). Six isolates were identified as efficient siderophore-producing bacteria (Figure 4A and 4B). Fifty-four percent of diazotrophic endophytes (60% and 50% from leaves and stems, respectively) synthesized *N*-acyl homoserine lactones (AHL) (Figure 4C). Overall, 71% of diazotrophic bacterial endophytes were positive for ACC deaminase (67% and 75% of endophytes from leaves and stems, respectively). Over 50% of endophytes displayed activity of catalase, lipase and protease. Additionally, 17%, 23%, 14%, and 26% of bacterial isolates were positive for amylase, gelatinase, chitinase, and urease, respectively. The network analysis showed correlations between diazotrophic endophytes and PGPB (Figure 5).

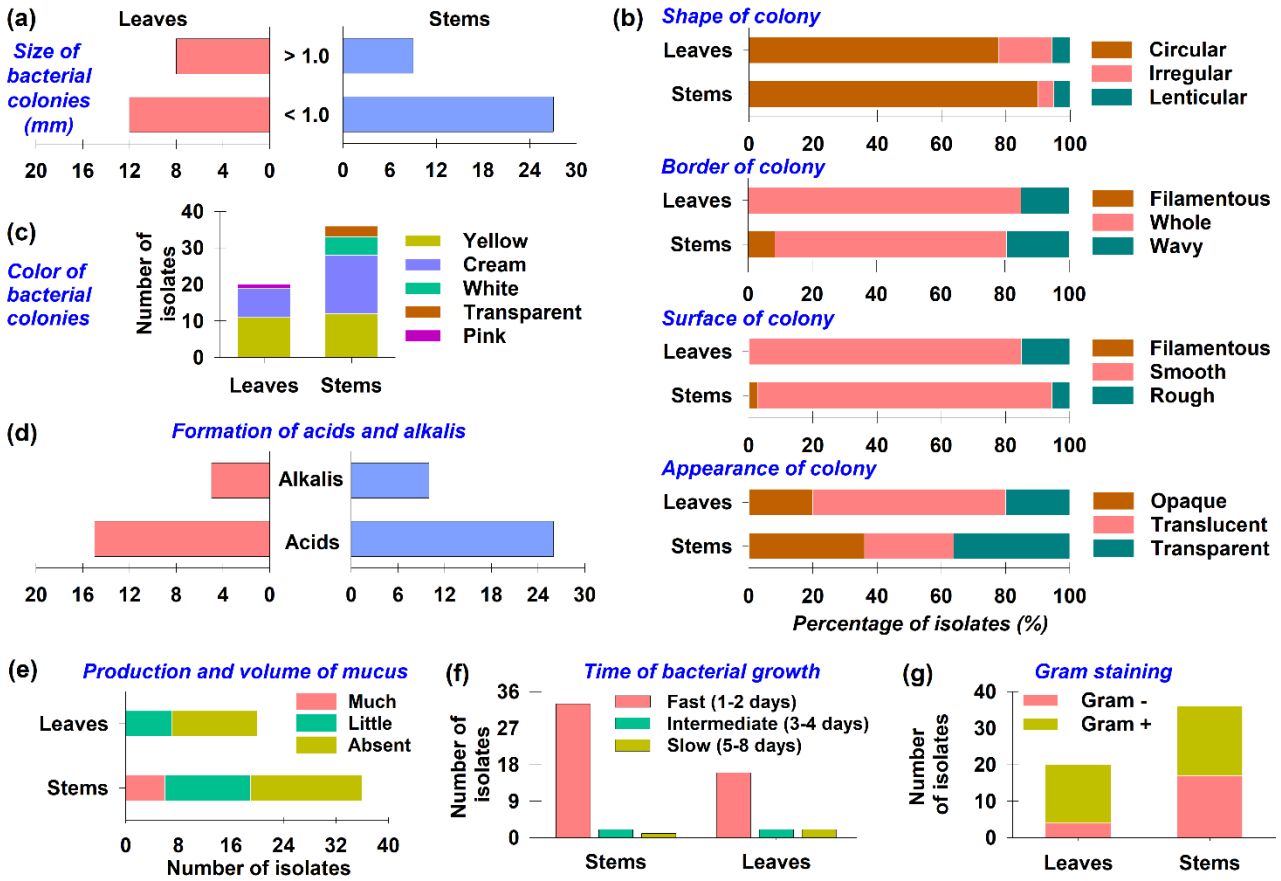


Figure 1. Morphophysiological aspects of diazotrophic bacterial isolates: (a) Size of bacterial colonies; (b) Shape, border, surface and appearance of bacterial colonies; (c) Color of colonies; (d) Formation of acids and alkalis from bacterial isolates; (e) Production and volume of mucus; (f) time of bacterial growth; and (g) Gram staining.

DISCUSSION

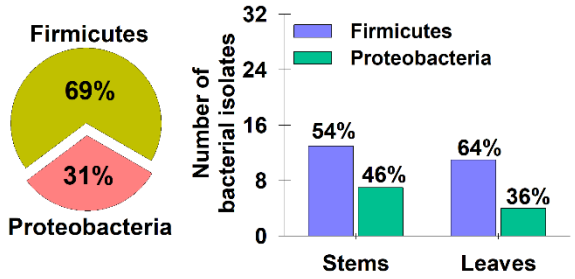
Isolation of bacterial endophytes

In this study, 56 bacterial isolates were obtained from sugarcane tissues, being 21 and 35 isolates found in leaves and stems, respectively. Interestingly, we found a higher number of endophytic bacterial isolates in sugarcane tissues than those found in previous studies with different crops, such as sorghum [32] and sugarcane [33]. For instance, Patel and Archana [34] isolated, through NFb broth, 31 endophytic diazotrophic bacteria in leaves, stems, and roots from Poaceae plants. By comparison, Leite and coauthors [11] found 24 endophytic diazotrophic bacteria in stems and roots of sugarcane by using LGI-P broth, while Alam and coauthors [33] found 21 bacterial isolates in roots and stems of sugarcane by using LGI broth. A total of 30, 18 and 8 bacterial isolates grown, respectively, in NFb, JNFb and LGI-P broths. These results showed that the numbers of bacterial isolates varied considerably according to the different types of culture media. Previously, Magnani and coauthors [35] reported variations in the number of bacterial isolates obtained with different types of culture media and stated that NFb and JNFb were more effective than LGI.

Morphological and physiological traits

According to the morphological and physiological analyses, the majority of bacterial colonies were smaller than 1.0 mm. In sugarcane, endophytic bacterial isolates (C2HL2, C34MR1, and UT3R1) with a size between 2.0 to 3.0 mm were previously described by Muangthong and coauthors [5]. As shown in Figure 1B, the majority of colonies isolated in leaves (78%) and stems (90%) were circular. This result agrees with Patel and coauthors [34], who found 58% of endophytic bacterial colonies from roots of *Brassica rapa* being circular. Additionally, more than 70% of all bacterial isolates were translucent and displayed whole border and smooth surface (Figure 1B). Interestingly, only endophytes isolated from stems exhibited bacterial colonies with filamentous border. This variation in colony appearance is common and a previous study showed significant variation in endophytic isolates [9].

(a)



(b)

Isolate	Bacterial species/genus*
IPA-CF20	*Bacillus megaterium*
IPA-CF45A	*Bacillus megaterium*
IPA-CF65	*Bacillus megaterium*
IPA-CF42	*Bacillus methylotrophicus*
IPA-CF13A	*Bacillus* sp.
IPA-CF16	*Bacillus* sp.
IPA-CF18	*Bacillus* sp.
IPA-CF48	*Brevibacillus agri*
IPA-CF44	*Burkholderia* sp.
IPA-CF19	*Methylobacterium organophilum*
IPA-CF39	*Methylobacterium* sp.
IPA-CF14	*Paenibacillus* sp.
IPA-CF47	*Paenibacillus* sp.
IPA-CF62	*Paenibacillus* sp.
IPA-CF66	*Pseudomonas* sp.

(c)

Isolate	Bacterial species/genus*
IPA-CC30B	*Bacillus megaterium*
IPA-CC36	*Bacillus pumilus*
IPA-CC11	*Bacillus* sp.
IPA-CC1B	*Bacillus* sp.
IPA-CC35	*Bacillus* sp.
IPA-CC25	*Bacillus subtilis*
IPA-CC28	*Bacillus subtilis*
IPA-CC29	*Bacillus subtilis*
IPA-CC30C	*Bacillus subtilis*
IPA-CC3A	*Burkholderia gladioli*
IPA-CC10	*Burkholderia* sp.
IPA-CC27	*Burkholderia* sp.
IPA-CC49	*Burkholderia* sp.
IPA-CC8	*Herbaspirillum seropedicae*
IPA-CC9	*Herbaspirillum seropedicae*
IPA-CC22A	*Paenibacillus* sp.
IPA-CC23	*Paenibacillus* sp.
IPA-CC37	*Paenibacillus* sp.
IPA-CC6	*Paenibacillus* sp.
IPA-CC33	*Pseudomonas* sp.

(d)

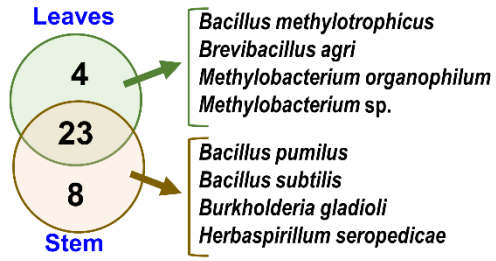


Figure 2. (a) Distribution of selected diazotrophic bacterial isolates in phyla. Diazotrophic bacterial endophytes from leaves (b) and stems (c). (d) Venn diagram showing bacterial isolates specific of leaves and stems.

The production of pigments by various organisms is a common phenomenon and can represent an evolutionary advantage for many species. In this study, bacterial colonies exhibited different colors, being yellow and cream the most prevalent (Figure 1C). Kumar and coauthors [36] found only three colors (white, cream, and yellow) in bacterial colonies isolated from different rice plant tissues. Three bacterial isolates from stems had colorless colonies (transparent) (isolates IPA-CC30B, IPA-CC30C, and IPA-CC56). Interestingly, only IPA-CF19 produced pink colonies (Figure 1C). According to Tuli and coauthors [37], canthaxanthin is a pigment found in pink bacterial colonies that displays important biological attributes, such as photo-protectant, antioxidant, and antimicrobial activities. Thus, this isolate could have these characteristics and could be a potential plant growth-promoter.

Bromothymol blue was used to identify the bacterial ability to produce acids and alkalis in the culture broth, and the majority of bacterial isolates were able to produce acids (Figure 1D). The ability to produce acids contributes to pH stability and is particularly important to bacterial endophytes living in plant apoplast. The sugarcane apoplast maintains substantial levels of carbohydrates, organic acids, and amino acids, and these compounds can provide energy and nutrients to endophytic bacteria [8, 38]. According to Geilfus [39], the apoplast is slightly acidic (pH 5.5) and can be alkalinized in response to biotic and abiotic stresses. Therefore, the bacterial endophytes, capable of producing acids, can symbiotically live in sugarcane apoplast without causing a pathogenic effect. Indeed, Silva and coauthors [38] reported that *Paraburkholderia tropica*, an endophytic diazotrophic bacteria with optimum growth in pH 5.0 to 5.8, altered its gene expression pattern in sugarcane apoplast, which allowed it to use the apoplast energy sources while avoiding host plant defense mechanisms.

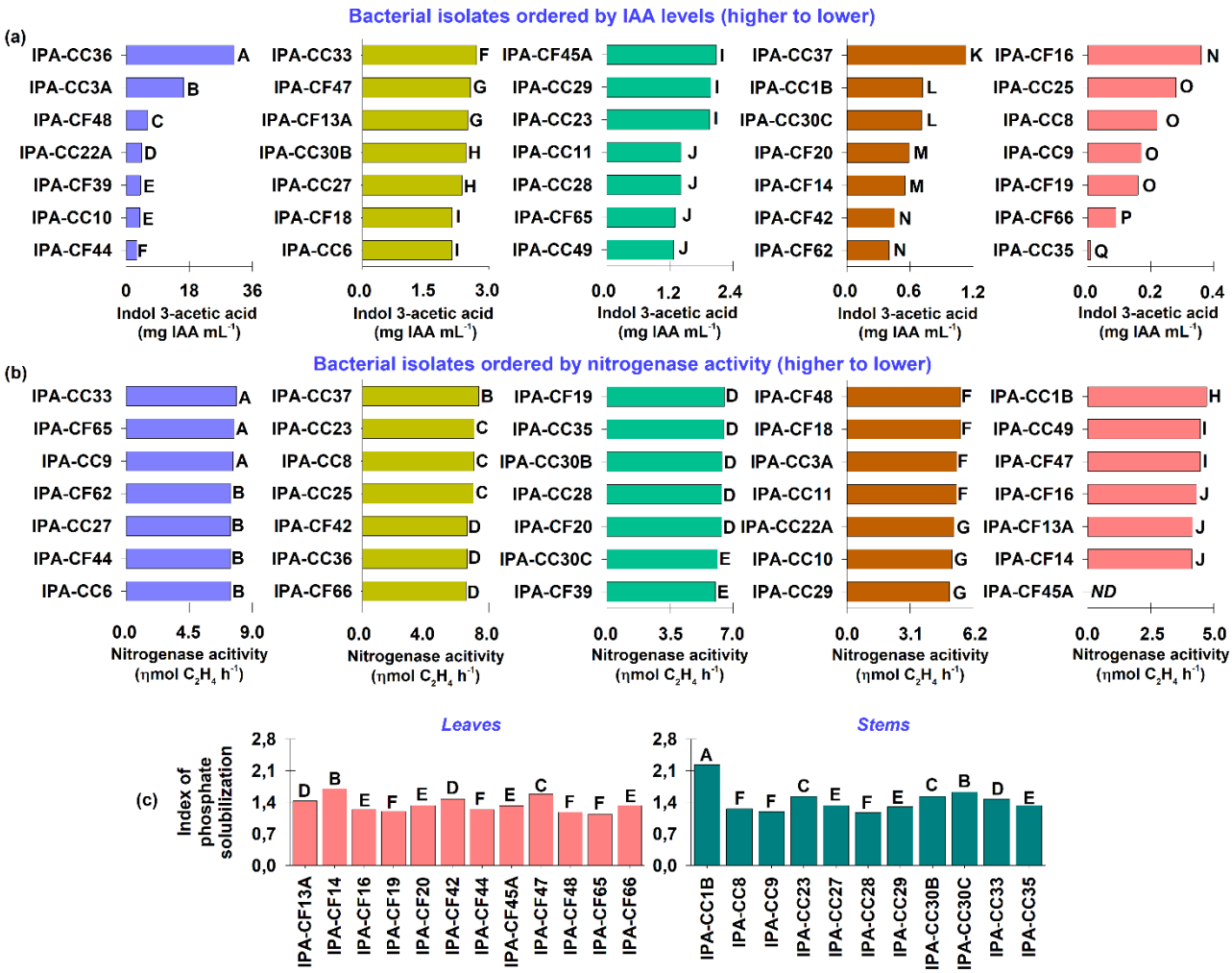


Figure 3. Production of indol 3-acetic acid (IAA) (a), nitrogenase activity (b), and index of phosphate solubilization (c) of bacterial diazotrophic bacterial endophytes from sugarcane tissues.

Except to six endophytes from stems (IPA-CC4, IPA-CC22A, IPA-CC22B, IPA-CC31, IPA-CC32, and IPA-CC34), bacterial isolates were unable to produce mucus (Figure 1E). Mucus or extracellular polymeric substances (EPS), such as polyamides, polyesters, inorganic polyanhydrides, and polysaccharides, are polymers with different and important biological functions that are produced and excreted by many bacterial strains [39]. Although EPS can protect the bacterial strains against abiotic stress, the exact physiological function of these biomolecules for endophytic bacteria is poorly understood. Tian and coauthors [40] report that EPS can create a favorable microenvironment and, therefore, contributes to the survival of endophytes inside the host plant. The EPS from endophytic bacteria helps to mitigate water loss by the host plant, which is critical in protecting the integrity of the xylem vessels in stems [7].

Endophytic bacteria from sugarcane leaves and stems showed fast growth and were mainly Gram-positive (Figure 1F and 1G). In contrast, Magnani and coauthors [35] and Alam and coauthors [33] reported that most of the endophytic bacteria from sugarcane are Gram-negative. On the other hand, the majority of bacterial endophytes from different plant tissues of rice were Gram-positive [37]. These results showed that Gram-positive bacteria are better able to adapt to stressful environments and can form spores when surrounding conditions are unfavorable, for example, in soil with low fertility. Thus, Gram-positive bacteria can survive and predominate in acidic environments, such as the apoplast.

Biochemical traits

After the morphophysiological characterization, 15 bacterial endophytes from leaves and 20 isolates from stems were biochemically evaluated. Previously, these isolates were molecularly characterized as belonging to phyla Proteobacteria and Firmicutes (Figure 2). According to Compant and coauthors [7], Proteobacteria and Firmicutes are common endophytes of various plant tissues. This result agrees with Kumar and coauthors [36] and Tian and coauthors [40], who found Proteobacteria and Firmicutes as predominant phyla in rice and tomato, respectively. In other studies, endophytic bacterial communities associated with maize,

rice, sorghum, and wheat were more diverse, and members of Proteobacteria, Firmicutes, Actinobacteria, and others were identified [8, 34].

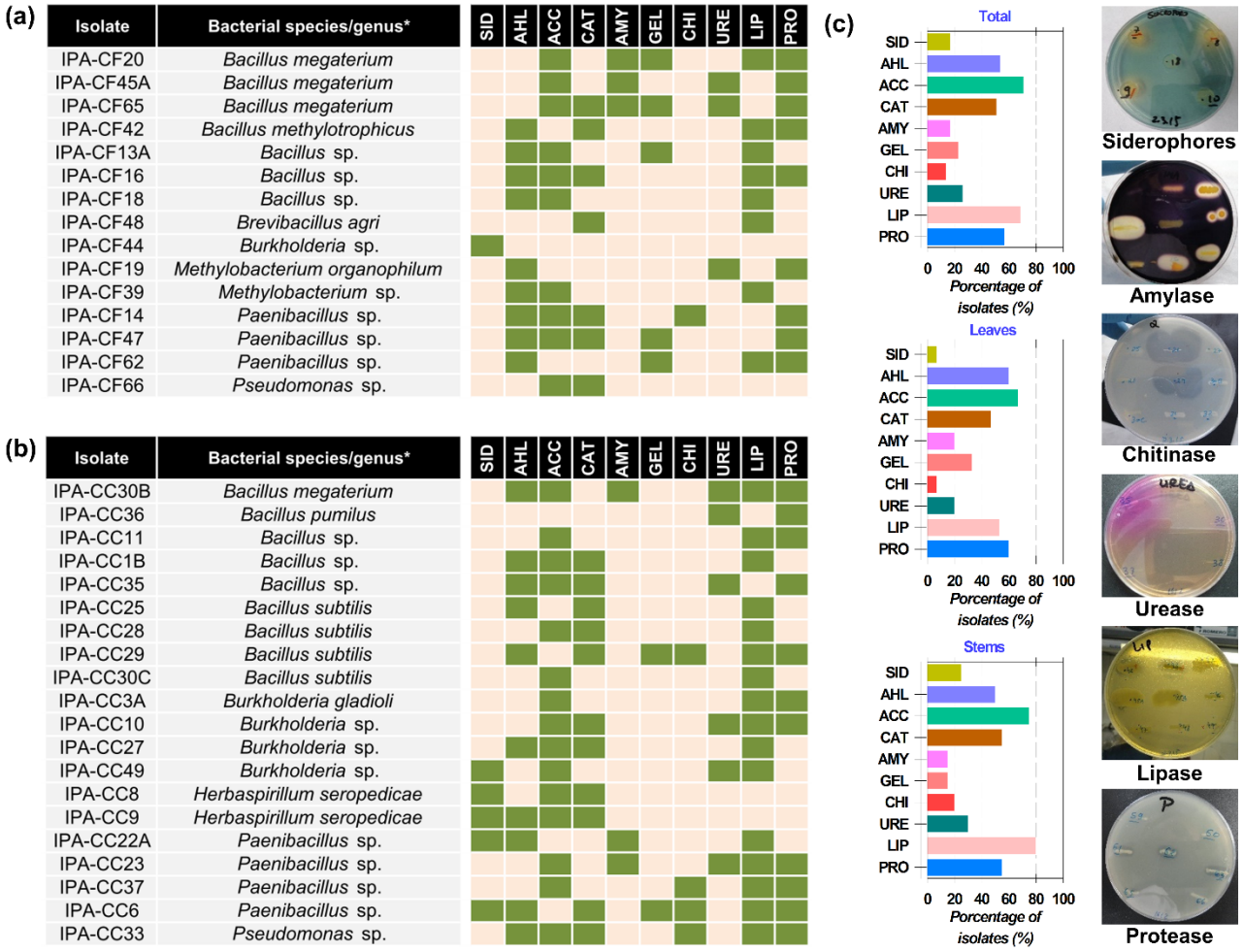


Figure 4. Biochemical characterization of bacterial diazotrophic bacterial endophytes from leaves (a) and stems (b) of sugarcane. In c, percentage of bacterial isolates positive for siderophores (SID), N-acyl homoserine lactones (AHL), ACC deaminase (ACC), catalase (CAT), amylase (AMY), gelatinase (GEL), chitinase (CHI), urease (URE), lipase (LIP), and protease (PRO).

Overall, it is possible observe characteristics of diazotrophic and PGPB in bacterial endophytes isolated from sugarcane tissues. In leaves, three isolates were recognized as diazotrophs and 12 bacterial isolates as PGPB. In contrast, 12 and 14 isolates from stems were identified as diazotrophs and PGPB, respectively. *Bacillus* was the predominant genus of endophyte in sugarcane tissues (Figure 2). The existence of tissue-specific endophytes was observed when the endophytic bacterial communities from leaves and stems of sugarcane were compared. It is noteworthy that isolates of *Brevibacillus agri* and *Methylobacterium organophilum* were present only in leaves, while isolates of *Herbaspirillum seropedicae* were found only in stems (Figure 2D). *Methylobacterium* and *Herbaspirillum* are genera of diazotrophs belonging to the phylum Proteobacteria. This phylum includes bacterial endophytes that perform nitrogen-fixation in different tissues of monocots, including rice, maize, sorghum, and sugarcane [8, 33, 34].

Biochemical traits are important to plant growth, and recent studies have reported these characteristics in bacterial endophytes [9, 37]. For instance, the ability to synthesize IAA, solubilize minerals (such as P and K), exudate siderophores, and produce AHL are important biochemical traits [37]. In this study, all bacterial isolates from sugarcane were able to synthesize IAA (Figure 3A). As a primary auxin in higher plants, IAA has marked effects on plant development. Previous studies on several plant species, such as chickpea, maize, rice, sorghum, sugarcane, tomato, and wheat, have reported bacteria releasing IAA and promoting plant growth [9, 33, 34, 40].

The isolates IPA-CC36 (30.8 µg mL^{-1}) and IPA-CC35 (0.01 µg mL^{-1}) exhibited highest and lowest IAA production levels, respectively (Figure 3A). These results are similar to previous studies that found values of IAA varying from 0.3 to 20.2 µg mL^{-1} with diazotrophic endophytic bacteria from sugarcane roots [12]. Silva

and coauthors [32] reported bacterial endophytes from sorghum, producing about 71.2 to 192.8 µg mL^{-1} IAA. In chickpea, Mukherjee and coauthors [9] found endophytes in seeds releasing a significant amount of IAA, with the highest value being 58.9 µg mL^{-1}. This is an important finding since the synthesis and liberation of IAA improve cell elongation and stimulate the growth of roots and shoots [8, 10]. In legumes, inoculation with rhizobia and endophytes, such as *Azospirillum*, *Bacillus*, and *Paenibacillus*, contributed to the production and secretion of IAA and improved nodulation [9].

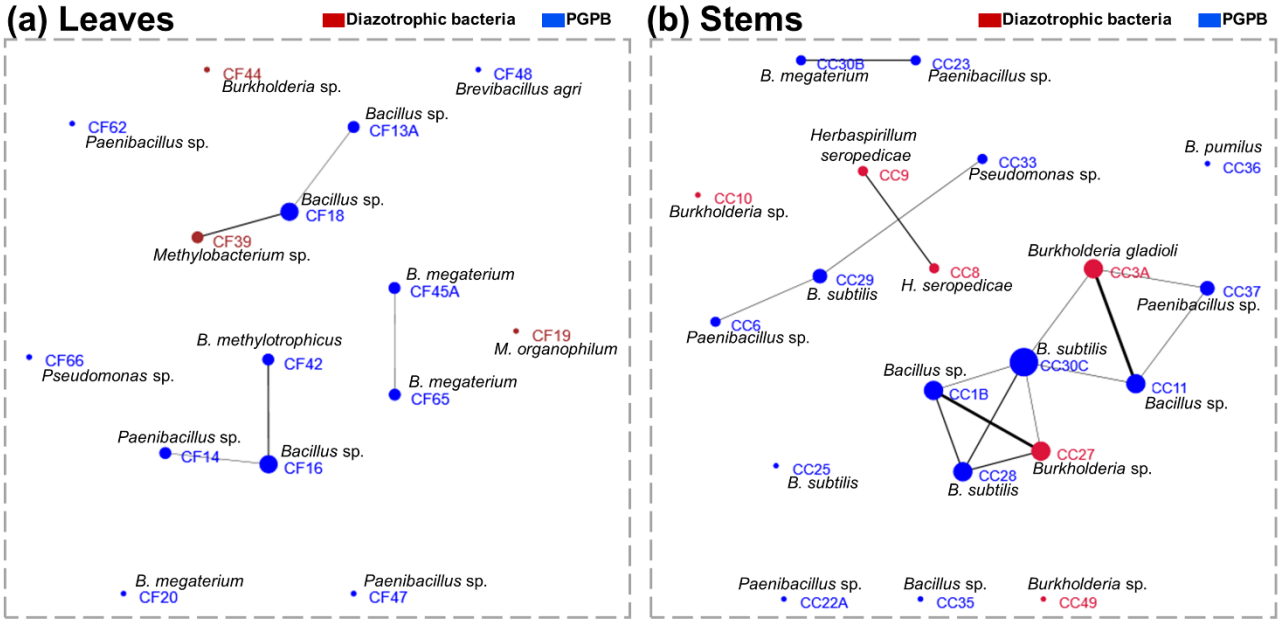


Figure 5. Network analyses of bacterial isolates from leaves (a) and stems (b) of sugarcane plants with basis in their biochemical characteristics. Blue circles represent diazotrophic endophytic plant growth-promoting bacteria (PGPB) and red circles shows diazotrophic endophytic bacteria. Big circles represents strong nodes, i.e., bacteria with strong influence in network. Line width is proportional to the strength of the correlation between bacterial isolates.

IAA produced by endophytes benefits the host plant and can also stimulate nitrogenase activity, i.e., high levels of IAA positively affect the N-fixing capability of the bacterial endophytes [8]. In this study, nitrogenase activity, by acetylene reduction assay, was measured in all bacterial isolates. Except in IPA-CF45A, all bacterial isolates showed a positive nitrogenase activity, and IPA-CC33 (*Pseudomonas* sp.), IPA-CF65 (*Bacillus megaterium*), and IPA-CC9 (*Herbaspirillum seropedicae*) were the most promising (Figure 3B). Additionally, this study found a positive correlation between IAA levels and nitrogenase activity (r = 0.99). This result confirms that IAA can stimulate nitrogenase activity in these endophytes, as reported by Defez and coauthors [8]. Muangthong and coauthors [5] detected nitrogenase activity in all endophytic bacteria from sugarcane and observed that the two isolates with the highest activity (C2HL2 and C7HL1) were also able to achieve high nitrogen contents in sugarcane leaves growing in N-free substrate.

In this study, the majority of diazotrophic bacterial endophytes were able to solubilize phosphate, being IPA-CC1B (*Bacillus* sp.) the most efficient (Figure 3C). Previous studies have found bacterial endophytic communities able to solubilize phosphate in leaves, stems, and roots of rice [36], in roots and stems of sugarcane [33], in seeds of chickpea [9], and in roots of sorghum [32]. The *Bacillus* genera encompass various phosphate-solubilizing microorganisms, and their ability to increase the soluble phosphate in soil and then promote adequate and vigorous plant growth have been reported [8]. The application of efficient phosphate-solubilizers as microbial inoculants can increase the phosphorus-uptake in numerous crops [7].

Phosphate solubilization and siderophore production by endophytes can increase the accessibility and absorption of different types of nutrients [8]. Siderophores are metal chelating agents produced and secreted by bacteria that sequester and solubilize iron and other metals [7]. In this study, IPA-CF44 and IPA-CC49 (*Burkholderia* sp.), IPA-CC8 and IPA-CC9 (*Herbaspirillum seropedicae*), and IPA-CC22A (*Paenibacillus* sp.) were identified as efficient siderophore-producing bacteria (Figure 4A and 4B). The results support previous studies in bacterial diazotrophic endophytes from maize, wheat, sorghum, and rice [32, 34]. The ability to produce siderophores by Proteobacteria has been reported [34], and this is an important trait in beneficial microbes, whether they are endophytes or not [8]. Microbial siderophores can supply the iron demand of plants by increasing its bioavailability, and also inhibit attacks from phytopathogens [7]. Thus, the

siderophores produced by endophytes can be the basis of a sustainable strategy to help plants tolerate abiotic stresses.

Beneficial bacteria able to synthesize AHL and release ACC deaminase enzymes are potentially useful for promoting better plant development in adverse environmental conditions [8]. In this study, over 50% of diazotrophic bacterial endophytes were able to synthesize AHL (Figure 4C). Additionally, the majority of diazotrophic bacterial endophytes were positive for ACC deaminase, with this trait being registered in over 65% of bacterial endophytes from leaves and stems (Figure 4C). While AHL induces systemic resistance responses and contributes to disease suppression, ACC deaminase improves abiotic stress tolerance by diminishing ethylene levels while simultaneously increasing ammonia contents [10]. Thus, the endophytes from the sugarcane microbiome can benefit plant health and growth and are a promising tool for mitigating the negative effects of environmental stress.

The diazotrophic bacterial isolates were also tested for catalase, an enzyme involved in the antioxidant system, and considered an important indicator of stress tolerance in plants. The majority of the isolates were catalase positive and, therefore, showed the ability to convert hydrogen peroxide into water and molecular oxygen. In general, 47% of endophytes from leaves and 55% of isolates from stems were found to be catalase positive, and *Bacillus* isolates were predominant in this group (Figure 4). Previous studies have found that most bacterial endophytes in sugarcane [12], rice [36] and chickpea [9] are catalase positive. Plant exposure to a variety of biotic and abiotic stresses results in the production of reactive oxygen species (ROS), including hydrogen peroxide. Catalase prevents cell toxicity and can be useful protection against oxidative damage by ROS.

Commonly, inhibition of the growth of plant pathogens is related to the ability of endophytic bacteria to release various extracellular enzymes [8]. In this study, it was observed that bacterial isolates were positive for amylase, gelatinase, chitinase, urease, lipase and protease (Figure 4). Additionally, the isolates of *Bacillus*, *Paenibacillus* and *Pseudomonas* were able to secrete three or more of these enzymes (Figure 4A and 4B). The penetration, infection, and colonization of plant tissues by endophytes are reportedly assisted by the extracellular enzymes they produce [39]. These extracellular enzymes can also improve the uptake and turnover of nutrients [8]. More bacterial isolates positive for chitinase, lipase, and protease were observed in the stem than in the leaves (Figure 4). Stems are responsible for transporting water and nutrients between roots and leaves and, therefore, the extracellular enzymes from endophytes can be useful in translocating nutrients between different parts of the plant and provide better nutrition and carbon gain to the host. Interestingly, the network analysis showed the isolates of *Burkholderia*, *Herbaspirillum*, and *Methylobacterium* to have biochemical correlations with *Bacillus*, mainly in stems (Figure 5).

Overall, we demonstrated that bacterial endophytes isolated in sugarcane leaves and stems presented distinct morphological, physiological and biochemical traits. Interestingly, except to isolate IPA-CF45A, all bacterial endophytes were positive for nitrogenase activity, which is an important trait related to biological nitrogen fixation. All bacterial endophytes exhibited important plant growth-promoting traits, such as phosphate solubilization, production of IAA, AHL, and siderophores, as well as ACC deaminase, catalase and extracellular enzyme activity. Further studies should explore the potential of these endophytes for sustainable agriculture, providing nutrients, mainly phosphorus and nitrogen, protecting against phytopathogens, and promoting plant growth, particularly in sugarcane fields.

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