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Culturable diazotrophic bacterial community associated with *Agave sisalana* P. plants from semi-arid regions in Brazil

Adailson Feitoza de Jesus Santos[1], Zayda Piedad Morales Moreira[2], Jorge Teodoro de Souza[3], Lenaldo Muniz de Oliveira[4], Heloiza Ramos Barbosa[5], Eliane de Souza Silva[6], Rafael Mota da Silva[7], Ana Cristina Fermino Soares[6]

[1] Universidade do Estado da Bahia, Departamento de Tecnologia e Ciências Sociais, Juazeiro, BA, Brazil. E-mail: adailsonmicrobiologia@gmail.com
[2] University of Saskatchewan, College of Agriculture and Bioresources, Department of Food and Bioproduct Sciences, Saskatoon, SK, Canada. E-mail: z.morales@usask.ca
[3] Universidade Federal de Lavras, Departamento de Fitopatologia, Lavras, MG, Brazil. E-mail: jgeteodoro@gmail.com
[4] Universidade de Feira de Santana, Horto Florestal, Laboratório de Cultura de Tecidos Vegetais, Feira de Santana, BA, Brazil. E-mail: lenaldo.uefs@gmail.com
[5] Universidade de São Paulo, Instituto de Ciências Biomédicas, Departamento de Microbiologia, São Paulo, SP, Brazil. E-mail: hrbarbos@icb.usp.br
[6] Universidade Federal do Recôncavo da Bahia, Centro de Ciências Agrárias, Ambientais e Biológicas, Laboratório de Microbiologia Agrícola, Cruz das Almas, BA, Brazil. E-mail: any.biologia@hotmail.com; ferminosoares@gmail.com
[7] Faculdade da Região Sisaleira, Conceição do Coité, BA, Brazil. E-mail: rafaelmotasilva@hotmail.com

ABSTRACT: The aim of this study was to isolate, quantify, identify and characterize the culturable diazotrophic bacterial community associated with sisal (*Agave sisalana* P.) and assess its potential for plant growth promotion and tolerance to abiotic stress. Nitrogen-free Burk's medium was used for bacteria isolation and quantification. Isolates carrying the *nif*H gene were typed by BOX-PCR and sequenced for the 16S rRNA region. Additionally, the bacteria was tested for growth promotion in maize plants and for salinity tolerance in cucumber seedlings. The representative isolates were identified as members of *Pantoea*, *Rhizobium*, *Burkholderia*, *Leifsonia* and *Bacillus* genera. All isolates were positive for at least two of the nine plant growth promoting (PGP) traits tested. All isolates showed positive traits for tolerance to abiotic stress. *Leifsonia* sp. (S1.5) significantly increased root length, stem length and total fresh matter, and it was involved in the reduction of deleterious salt effects (60 mM NaCl) in cucumber seedlings. Diazotrophic bacterial community associated with sisal plants is genetically diverse, exhibits several plant growth-promoting traits and shows potential to support plant growth in adverse environments (salinity).

Key words: abiotic stress; PGPB; rhizospheric bacteria

Comunidade bacteriana diazotrófica cultivável associada com *Agave sisalana* P. de regiões semi-áridas do Brasil

RESUMO: O objetivo deste estudo foi isolar, quantificar, identificar e caracterizar a comunidade bacteriana diazotrófica cultivável associada com sisal (*Agave sisalana* P.) e avaliar o seu potencial para promoção de crescimento vegetal e tolerância a estresse abiótico. Para isolamento e quantificação das bactérias foi utilizado o meio de cultura Burk's livre de nitrogênio. Isolados contendo o gene *nif*H foram caracterizados usando BOX-PCR e em seguida a região 16S rRNA foi sequenciada. As bactérias foram testadas para promoção de crescimento em plantas de milho e para indução de tolerância a salinidade em mudas de pepino. Os isolados representativos foram identificados como membros dos gêneros *Pantoea*, *Rhizobium*, *Burkholderia*, *Leifsonia* e *Bacillus*. Todos os isolados foram positivos para pelo menos duas das nove caractetísticas de promoção de crescimento vegetal (PCV) testadas. Todos os isolados apresentaram características positivas para tolerância a estresse abiótico. *Leifsonia* sp. (S1.5) aumentou significativamente o comprimento da raíz, caule e matéria fresca total e reduziu os efeitos deletérios do sal (60 mM) nas mudas de pepino. A comunidade bacteriana diazotrófica associada com plantas de sisal é geneticamente diversa, possui diversas características para promoção de crescimento e apresenta potencial para manter o cresicmento de plantas em condições adversas (salinidade).

Palavras-chave: estresse abitótico; BPCP; bactéria rizosférica

Introduction

The Northeast region of Brazil has an extensive area with a semi-arid climate with average rainfall ranging from 400 to 800 mm (Rodal et al., 2018). The vegetation is composed of small trees, plants belonging to Cactaceae family, and perennial shrubs with spines, most of them deciduous in the dry season (Queiroz, 2006).

Sisal (*Agave sisalana* Perrine) is an Asparagaceae introduced from Mexico that has been grown for hard fiber production in large areas of the Northeastern semi-arid region of Brazil (Suinaga et al., 2006), being the main commercial crop for local farmers. The Northeast is the only Brazilian region where sisal is commercially produced, and the state of Bahia is responsible for approximately 95% of the national production with 197.748 hectares of harvested area (IBGE, 2017).

Microorganisms associated with plants from arid and semi-arid region exhibit some traits that help them to survive and thrive under harsh climatic conditions. Native vegetation, as well as sisal plants, host a bacterial community which has been of special interest for researchers in recent years due to its role in plant growth promotion and tolerance to abiotic stress (Santos et al., 2014; Lima et al., 2015). Several studies have shown that microorganisms adapted to these regions can enhance the tolerance of plants to abiotic stress, such as high salt concentrations (Navarro-Torre et al., 2017). For example, AKM-P6, a thermotolerant *Pseudomonas* sp. strain leads to an increase in tolerance to high temperatures in sorghum seedlings. Inoculation of seedlings induced the biosynthesis of high molecular weight molecules in leaves under high temperatures, reducing cell membranes injuries and increasing cell metabolites levels, such as: chlorophyll, proline, sugars, amino acids and proteins (Ali et al., 2009). Thus, bacterial communities produce substances that not only play a role in their survival, but also induce plants to synthesize compounds involved in their adaptation to adverse environmental conditions.

Plant tolerance to arid and semi-arid environments could be improved by rhizospheric and endophytic microorganisms, known as plant growth promoting bacteria (PGPB) (Naveed et al., 2014; Timmusk et al., 2014). PGPB may indirectly or directly promote plant growth and development. Direct promotion of plant growth commonly involves mechanisms that allow the acquisition of nutrients from the environment, including nitrogen fixation, iron sequestration by bacterial siderophores, phosphate solubilization and the alteration of plant hormone levels such as cytokinin, auxin and ethylene. Indirect plant growth promotion occurs by a decrease or prevention of deleterious effects caused by plant pathogens (Gamalero & Glick, 2015) or by abiotic stress through different mechanisms (Naveed et al., 2014). For instance, nitrogen-fixing bacteria can play an important role since sisal has been grown in Brazil for many decades without any nitrogen fertilization (Santos et al., 2014).

The aim of this study was to isolate, quantify, identify and characterize diazotrophic bacteria associated with sisal in a semiarid region in Bahia, Brazil. Additionally, their potential to promote plant growth and role in abiotic stress tolerance were assessed for future applications in sisal or other crops grown in semi-arid environments.

Material and Methods

Sampling area

Sisal plants and rhizosphere soil were collected in a production area located in the municipality of Conceição do Coité (11º 31 '18.4" S; 39º 16' 09.4" W), Bahia State, Brazil. The local climate is characterized by high potential evapotranspiration (2,000 mm per year), an average rainfall of 700 mm per year (minimum 300 and maximum 1,000 mm) during three to five months a year and temperatures ranging from 23 to 27 ºC. The vegetation found in the region is characterized as open Caatinga without palm trees.

Bacterial quantification and isolation

Five samples of sisal rhizosphere, roots, stems, leaves, stalks, and bulbils were randomly collected and immediately transported to the laboratory in Styrofoam boxes. Ten grams of rhizosphere samples were added to 90 mL of saline solution (NaCl 0.85%) and serial dilutions were carried out. Samples containing 10g of each plant tissue were surface sterilized (alcohol at 70% for 1 min, NaOCl at 1% for 3 min, and three successive rinses with sterile distilled water), grounded in a mortar, and serially diluted in saline solution (0.85%). For the diazotrophic bacterial community isolation, aliquots of 100 µL from 10^{-2} to 10^{-4} dilutions of all samples, including soil and surface-sterilized plant tissues, were spread in triplicate into tubes containing Burk's semi-solid nitrogen free selective medium (g L^{-1}: 10 g of glucose, 0.41 g of KH_2PO_4, 0.52 g of K_2HPO_4, 0.05 g of Na_2SO_4, 0.2 g of $CaCl_2$; 0.1 g of $MgSO_4.7H_2O$, 0.005 g of $FeSO_4.7H_2O$, 0.0025 g of $Na_2MoO_4.2H_2O$, 1.8 g of agar, with pH adjusted to 7.0) (Wilson & Knight, 1952) and incubated at 28 ºC during 7 days. In order to confirm the disinfection protocol, aliquots of sterile water used in the final rinse were plated on 10% TSA (Tryptic Soy Agar) and incubated at 28 ºC during 48 h.

Bacterial quantification was performed using the most probable number (MPN) method. Aliquots of 50 µL of the growth subsurface pellicle were transferred to Petri dishes containing solid Burk's medium and incubated at 28 °C for 7 days. Morphologically distinct colonies were picked and purified in solid Burk's medium and incubated at 28 ° C for 7 days. Individual colonies were stored at -80 ºC in 40% glycerol and TSB (Tryptic Soy Broth) for further experiments.

Presence of the nifH gene, diversity, and molecular identification

DNA extraction and amplification were performed as previously described by Santos et al. (2014). Fragments of the *nif*H gene were amplified by nested PCR in two steps: 1) a

reaction with primers PolF – (5'-TGCGAYCCSAARGCBGACTC-3') and PolR – (5'-ATSGCCATCATYTCRCCGGA-3') that target a 360-bp fragment of this gene from a wide range of diazotrophic microorganisms; 2) a reaction with the product amplified in the first step using the pair of primers *nif*HFor (5'-ACCCGCCTGATCCTGCACGCCAAGG-3') and *nif*HRev (5'-ACGATGTAGATTTCCTGGGCCTTGTT-3') that amplify a 314 -317-bp fragment.

Isolates carrying the *nif*H gene were typing by BOX-PCR A1-R to assess the genomic diversity of the diazotrophic bacterial community, according to Santos et al. (2014). The banding patterns on the gel were converted into a binary matrix. The similarity matrix was calculated using the Jaccard's coefficient (Legendre & Legendre, 1983) and the dendrogram was obtained with the UPGMA algorithm using the software FreeTree version 0.9.1.50.

16S rRNA gene was amplified from DNA samples by PCR using 27F (5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-TACGGYTACCTTGTTACGACTT) primers (with expected amplicon size of 1465bp). Each PCR reaction was carried out according to Weisburg et al. (1991). PCR products were sequenced by Macrogen (Seoul, South Korea) using a 3730 *xl* sequencer (Applied Biosystems, Drive Foster City, CA, USA). Sequences were analyzed using the Sequence Scanner Software v. 2.0 (Applied Biosystems) and the contigs assembled using the BioEdit software v 7.0 (http://mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were deposited in GenBank, a database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank) under the accession numbers KR094730 to KR094773 (Fig 2). Bacterial identification at the genus level was performed with BLAST searches (Altschul et al., 1990) in public databases. Sequence alignment, model selection and tree reconstruction were performed using the software Mega version 5.2.1 (Tamura et al., 2011).

EPS production, drought, salinity and temperature tolerance

Exopolysaccharide (EPS) production was measured as described by Paulo et al. (2012). The tested isolates were inoculated into sterilized filter paper discs of 5 mm Ø placed on the surface of culture medium modified by Guimarães et al. (1999) containing (2% yeast extract, 1.5% K_2HPO_4, 0.02% $MgSO_4$, 0.0015% $MnSO_4$, 0.0015% $FeSO_4$, 0.003% $CaCl_2$, 0.0015% NaCl, 1.5% agar and 10% sucrose, with pH adjusted to 7.5) followed by incubation at 30 °C for 48 h. A mucoid layer formed around the paper discs suggested EPS production. In order to confirm the presence of EPS, the mucoid layer was transferred to a tube containing 2 mL absolute ethanol. The EPS presence was confirmed by the formation of a precipitate. The previously described experiments were settled in triplicate and performed twice. Drought tolerance was assessed by growth in a medium with reduced water activity (A_w). Each isolate was spread on 10% TSA medium supplemented with sorbitol at different concentrations: 285, 405, 520 and 780 g L^{-1}, producing A_w values corresponding to 0.963, 0.93, 0.912 and 0.837, respectively. The plates were incubated at 30 ºC for 48 h. Salinity tolerance was tested by plating the isolates on nutrient agar (NA) supplemented with 0, 2.5, 5.0 and 10% of NaCl. The plates were incubated at 28 ºC and after 48 h were assessed for bacterial growth. Tolerance to high temperatures was evaluated by incubating NA plates containing each isolate at 28 °C (control), 37 °C, 42 °C and 50 °C.

Physiological characterization

Acetylene Reduction Assay (ARA) - All bacterial isolates were transferred to 10 mL test tubes containing 4 mL of semi-solid nitrogen-free medium and were incubated during 72 h at 28 ºC. Ten percent of the gas phase in the tested tubes was replaced with acetylene and isolates were incubated for 48 h at 30 ºC. The gas phase (0.1 mL) was analyzed for ethylene production using a Porapak-N 80/100 – INOX column with a gas chromatograph (Shimadzu GC-14A) (Thuler et al., 2003). The assays were performed in triplicate.

ACC Deaminase - Each isolate was grown during 4 days at 28 °C on minimal medium containing 1-aminocyclopropane-1carboxylate (ACC) as the sole carbon source (Glick, 1995). Bacterial growth in this medium indicates ACC deaminase activity.

Production of siderophores - Each isolate was grown in TSB medium at 28 °C for 24 h under stirring at 180 rpm. Following this incubation period, chrome-azurol sulphonate agar plates were inoculated with five µL of suspension (10^9 cells mL^{-1}) of each isolate tested and incubated during 48 - 72 h at 28 °C as described by Louden et al. (2011). An orange-yellow zone around the bacterial colonies confirmed siderophore production. Strains of *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 25922 were used as negative and positive controls, respectively.

Production of indolic compounds - Each bacterial isolate was inoculated in TSB medium supplemented with 5 mM L-tryptophan and incubated for 72 h at 28 °C shaking at 180 rpm. The supernatant was separated by centrifugation and used to determine the content of indole compounds by the Salkowski's assay as described by Gordon & Weber (1951). For color development, the mixtures were kept in the dark at room temperature for 30 min. The amount of indole compounds was previously estimated with the aid of a standard curve using pure IAA (0, 5, 10, 15, 20, 25 µg mL^{-1}).

Inorganic phosphate solubilization - Cell suspensions of each isolate were transferred to the medium GL containing a calcium phosphate precipitate produced by adding 0.57 M K_2HPO_4 and 0.90 M $CaCl_2$ at pH 6.5 (Sylvester-Bradley et al., 1982) and incubated for 10 days at 28±2 °C. The development of a clear zone around the colonies was considered as a positive reaction for phosphate solubilization. *Bacillus megaterium* ATCC 19213 was used as a positive control.

Statistical Analysis - All samples were evaluated in triplicate and the data were analyzed by variance analysis (ANOVA) and the Scott-Knott´s test at 5 % probability was used to compare the means using the statistical software SAS (SAS Institute).

Plant growth promotion potential

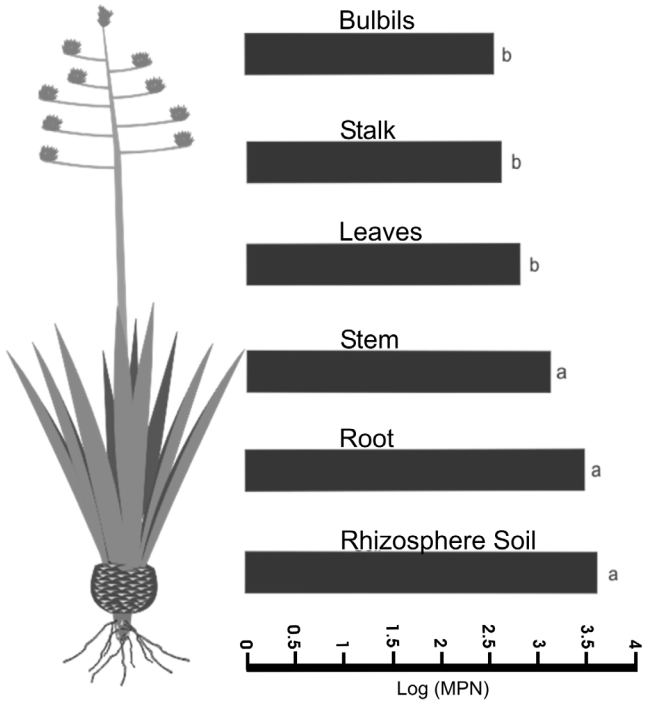
Two experiments were performed in order to check the potential for plant growth promotion by the isolates. The first

one was set up *in vitro* to evaluate the ability of the isolates to promote plant growth on a saline environment. For this purpose, cucumber (*Cucumis sativus*) seeds were surface sterilized by immersion in 70% ethanol for 2 minutes, and with 1% sodium hypochlorite for 5 min followed by three successive rinses with sterile distilled water. In order to test seed germination under salt stress, two surface-sterilized seeds were placed in tubes containing 0.8% water-agar with 0, 30, 60, 90, 120 and 150 mM NaCl and incubated at 30 °C for 5 days. The concentrations 60 and 90 mM NaCl were selected to evaluate seedling growth, since they had deleterious effects on the rate of germination and development of the plants. Based on preliminary results (data not shown), seven rhizospheric isolates (S1.2, S1.4, S1.5, S1.9, S3.5, S3.6 and S5.1) were selected for this experiment. The growth of cucumber seedlings in the presence and absence of bacteria was evaluated in tubes containing 0.8% water-agar with 60 and 90 mM NaCl and incubated at 30 °C. Surface-sterilized seeds were inoculated with bacterial suspensions containing 10^9 cells mL^{-1}. The control was inoculated with sterile distilled water. Growth was evaluated after seven days. The experiment was set up in a random design, and carried out using ten replicates with each tube containing at least two seeds. The second experiment was set up in a greenhouse aiming to test the potential of the isolates in promoting maize (*Zea mays* L.) plants growth when facing the soil bacterial community. The isolates S1.2, S1.4, S1.5, S1.9, S3.5, S3.6 and S5.1 were grown in TSB medium at 28 °C for 24h under shaking at 150 rpm. Cell suspensions containing 10^9 cells mL^{-1} were prepared with sterile distilled water and 0.1% xanthan gum for increasing bacterial adherence to seeds. Surface sterilized seeds were immersed in the bacterial suspensions during 2 h. A control was established by immersing disinfested seeds in sterile water containing xanthan gum (0.1%). After inoculation, seeds were placed in plastic pots containing 3 kg of soil. Plants were grown in a greenhouse for 45 days. The experimental design was entirely randomized with ten replicates and one plant per pot. The parameters used for evaluation of growth promotion were root length, stem height and shoots and roots dry matter. For both experiments, data were submitted to variance analysis (ANOVA) and the Scott-Knott test at 5% probability was used to compare the means using the SAS statistical software (SAS Institute).

Results and Discussion

The presence of nitrogen-fixing bacteria was detected in all internal tissues of the plant (root, stem, leaf, stalk and bulbils) and in the rhizosphere ranging from 2.58 to 3.65 log_{10} number of bacteria g^{-1} wet weight (Figure 1). The highest densities of nitrogen-fixing bacteria were found in rhizosphere, roots and stems; lower densities were found in leaves, stalks and bulbils (Figure 1).

In this study, diazotrophic bacteria associated with *Agave sisalana* were found to occur at higher densities in rhizospheric soil, inside roots and stems. The organisms were found at lower densities inside stalks, leaves and bulbils. A total of 191 isolates were obtained from the analyzed samples. These isolates were selected according to morphological patterns exhibited by the colonies. Leaves, rhizospheric soil and bulbils were the samples that presented the highest amount of morphologically distinct colonies, representing the highest amounts of selected isolates, 24.5%, 24.0% and 16.4%, respectively. The remaining 35.2% were isolated from other parts of the plants.



Means followed by the same letter are not significantly different by the Scott-Knott test ($p < 0.05$). Values represent the average of five evaluated plants.

Figure 1. Most probable number (MPN) of endophytic diazotrophs (log_{10}) associated with different sisal compartments.

The *nif*H trial of the 191 isolates resulted in 176 positive isolates for the presence of the *nif*H gene. Those isolates that exhibited negative results for the presence of *nif*H were obtained from leaves and bulbils. BOX-PCR analysis from the 176 *nif*H-positive isolates generated 45 genomic profiles. One isolate of each genomic profile was selected for further molecular and physiological characterization.

Three phyla and eleven genera were identified among the 176 *nif*H-positive isolates (Table 1). The phylum Firmicutes was the most abundant, followed by Proteobacteria and Actinobacteria and the genus *Bacillus* was the most common, followed by *Leifsonia*, *Enterobacter* and *Rhizobium*. Most of the isolates from leaves and bulbils were *Bacillus*, whereas *Enterobacter* was the most common genus in stems and stalks, *Rhizobium* was predominant in roots, and *Leifsonia* was the most common and was only observed in the soil (Figure 2 and Table 1).

The predominant phylum associated with sisal was the Proteobacteria, also found in other studies with plants of Asparagaceae family (Santos et al., 2014; Desgarannes et al.,

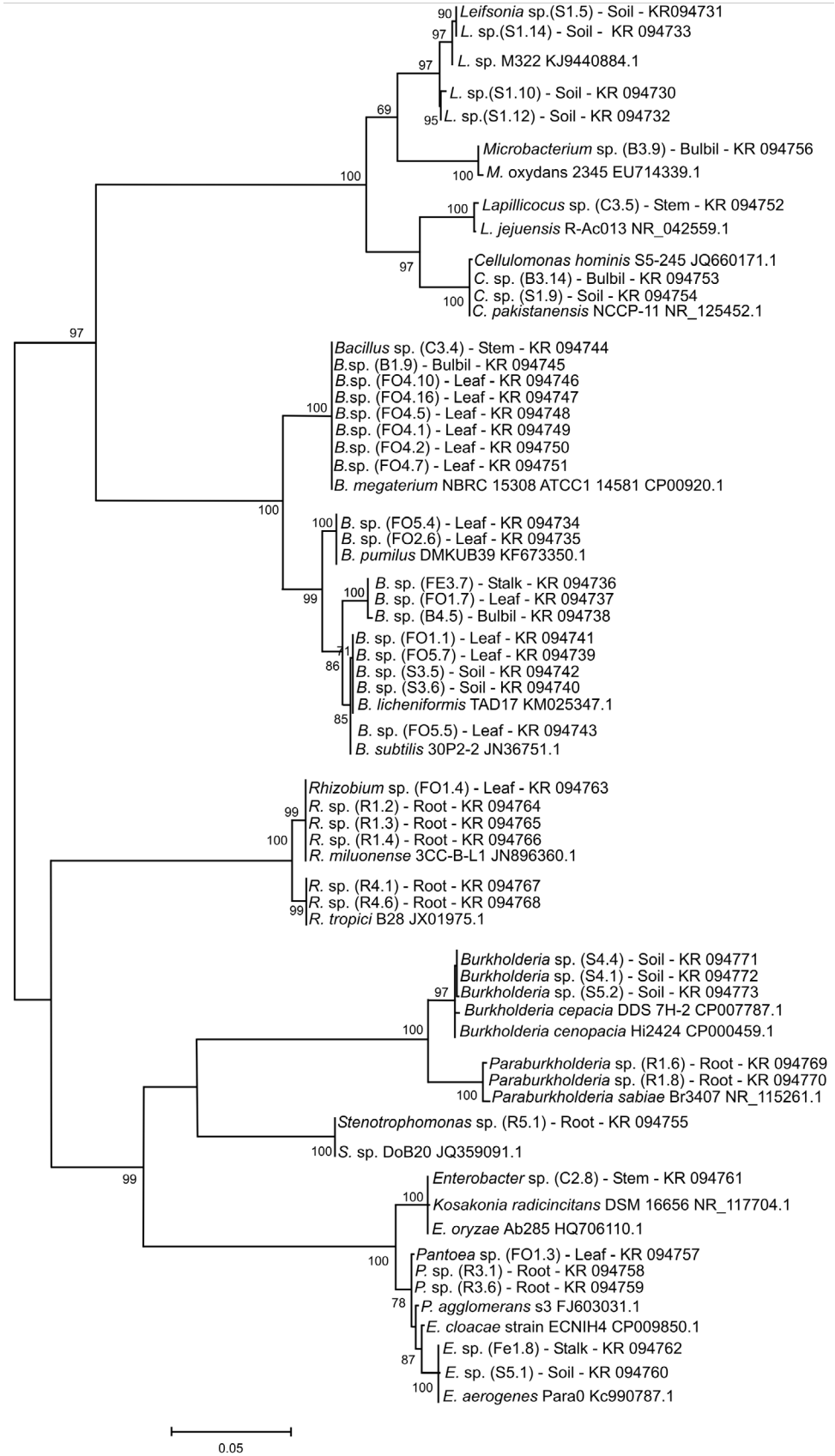


Figure 2. 16S rRNA phylogenetic tree of diazotrophic bacteria associated with sisal compartments (rhizosphere soil, roots, stem, leaves, stalk, and bulbils). The tree was generated with 1407-bp aligned nucleotides by using the maximum likelihood method and the substitution model T92+G. Bootstrap values higher than 70 % are shown on the appropriate branching nodes and were computed with 1,000 resampling. Sequences obtained from public databases for comparison purposes are shown in bold. The scale indicates the number of substitutions per site.

Table 1. Number of *nif*H-containing bacterial isolates classified in genera and phyla from soil and sisal tissues.

Phylum	Genus	Soil	Roots	Stems	Leaves	Stalks	Bulbils	Total
Firmicutes	*Bacillus*	8		7	34	8	20	77
Actinobacteria	*Cellulomonas*	1					9	10
	Leifsonia	24						24
	Lapillicoccus			1				1
	Microbacterium						1	1
Proteobacteria (Alpha)	*Rhizobium*		11		2			13
Proteobacteria (Beta)	*Burkholderia*	8	1					9
	Paraburkholderia		2					2
Proteobacteria (Gamma)	*Enterobacter*	1		8		15		24
	Stenotrophomonas		7					7
	Pantoea		7		1			8
Total		42	28	16	37	23	30	176

2014). Although there is a great diversity of bacteria associated with plants, the predominant phyla usually are restricted to Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, and these are found in the rhizosphere, endosphere, and phylosphere of different plant species (Beckers et al., 2017; Correa-Galeote et al., 2018).

Only the phylum Bacteroidetes was not found in our study (Table 1). Since our objective was not performing an exhaustive survey but rather screening bacterial isolates with specific traits, we could be omitting Bacteroidetes strains in our study.

The distribution of bacterial genera in different plant tissues may be a reflex of the adaptation of these microorganisms to different microhabitats. A recent study has shown that factors as plant tissue and geographical distribution are determinants in the microbiome composition in *Agave* species (Coleman-Derr et al., 2016).

Bacillus was the predominant genus and it was mainly present in the aerial parts of sisal plants. Some isolates were also found in the rhizosphere. Therefore, the biochemical and physiological characteristics of *Bacillus* (e.g. endospore forming bacteria) can promote its survival in environments under stress conditions (Wang et al., 2014). It is important to verify the systemic distribution of *Bacillus* in sisal since endophytic *Bacillus* species can influence plant growth promotion, tolerance to water deficiency, and crop protection against diseases (Ribeiro et al., 2018; Vigani et al., 2018). Isolated with the same genetic pattern, later identified as *Enterobacter* sp. (Fe1.8), were found endophytically in the roots, stem, stalk and bulb of sisal, indicating their systemic distribution. The distribution pattern of these bacterial groups, as well as their physiological characteristics, can contribute to the maintenance of crops that are not adequately managed, as well as sisal.

Among all the bacterial genera found in this study, only *Actinobacteria*, *Cellulomonas* and *Lapillicoccus* have not been previously reported as atmospheric nitrogen-fixing bacteria. Furthermore, their capacity to fix nitrogen was confirmed by *nif*H gene presence and their positive reaction in the acetylene reduction assay.

Rhizobium species in association with leguminous plants constitute the classical model for bacterial nitrogen fixation. In this system, atmospheric nitrogen fixation only occurs when the bacteria is in symbiosis. However, it is well established in the literature that *Rhizobium* and other bacterial genera are able to fix nitrogen out of the classical symbiosis model in non-leguminous plant species (Rouws et al., 2014). Other mechanisms of nitrogen supply were recently demonstrated, in which endophytic bacteria act as a nutrient reservoir, including nitrogen. This mechanism has been observed in *Agave tequilana* with *Bacillus tequilensis,* where tests for hydrogen peroxide showed its presence during bacteria degradation in plant tissues, supporting the involvement of reactive oxygen in the degradation process and nitrogen becomes available (Beltran-Garcia et al., 2014). The presence of the *nif*H gene and ARA activity indicate that bacteria containing these traits are able to fix nitrogen in sisal tissues. Likely the nitrogen is supplied to sisal plants by both mechanisms: bacterial fixation and bacterial degradation by the plant.

Among the 44 isolates used in these *in vitro* screening assays, the most tolerant to abiotic stress were *Pantoea* and *Bacillus*. In contrast, representatives of the phylum Actinobacteria, with the exception of *Microbacterium*, were not tolerant to reduced water activity (Table 2). All isolates grew on 10% TSA medium without sorbitol (A_w 0.998), but none were able to grow on medium with 780 g L^{-1} of sorbitol (0.837 A_w) (Table 2). Most isolates (86%) were able to grow on medium with high salt concentration, with exception of some isolates of *Paraburkholderia*, *Leifsonia* and *Rhizobium* (Table 2). All isolates were able to grow at 37 ºC and 40 ºC (data not shown). Isolates of *Bacillus*, *Leifsonia*, *Burkholderia* and *Pantoea* grew at 50° C (Table 2), but *Bacillus* was the genus with highest tolerance to high temperatures. Production of EPS was observed in 81.8% of the isolates in medium supplemented with 10% glucose.

Eight traits supposedly associated with plant growth-promotion were included in this *in vitro* screening and only indole compounds (AUX) were produced by all 44 isolates tested (Table 3). The concentration of indole compounds ranged from 0.09 to 5.56 µg mL^{-1}. Overall, 78% of the isolates produced <1µg mL^{-1}. Nitrogenase activity measured via acetylene reduction was positive for 34 (77%) out of 44 tested isolates (Table 3), even though all isolates included

Table 2. Isolates with potential stress tolerance traits among 44 *nif*H -containing representative isolates defined using BOX-PCR fingerprinting. The table does not show the 37 °C test, since all 44 isolates were able to grow at this temperature.

	Nº isolates	0.995 A_w	0.963 A_w	0.912 A_w	0.859 A_w	0% NaCl	2.5% NaCl	5% NaCl	10 % NaCl	42 °C	50 °C
Burkholderia	3	3	1	0	0	3	3	3	3	2	1
Paraburkholderia	2	0	0	0	0	2	2	2	0	2	0
Enterobacter	3	3	3	0	0	3	3	3	3	3	0
Cellulomonas	2	0	0	0	0	2	2	2	2	1	0
Leifsonia	4	0	0	0	0	3	4	3	3	1	1
Rhizobium	6	4	2	0	0	6	6	5	5	2	0
Stenotrophomonas	1	0	0	0	0	1	1	1	1	1	0
Pantoea	3	3	3	1	0	3	3	3	3	3	1
Bacillus	18	18	18	15	0	18	18	18	18	18	16
Lapillicoccus	1	0	0	0	0	1	1	1	1	0	0
Microbacterium	1	1	1	0	0	1	1	1	1	0	0

Table 3. Isolates with putative plant growth promotion traits among 44 *nif*H-containing representative isolates defined using BOX-PCR fingerprinting. ARA = Acetylene Reduction Assay ; AUX = Auxin production; EPS = Exopolysaccharide production; Si = Siderophores; PS = Phosphate solubilization; ACC = ACC deaminase activity.

	Num of isolates	ARA	AUX	EPS	Si	PS	ACC
Burkholderia	3	2	3	2	1	3	3
Paraburkholderia	2	2	2	1	1	0	2
Enterobacter	3	2	3	2	1	0	3
Cellulomonas	2	1	2	2	0	0	2
Leifsonia	4	4	4	3	2	1	4
Rhizobium	6	4	6	5	0	0	2
Stenotrophomonas	1	1	1	1	0	0	0
Pantoea	3	2	3	3	1	0	3
Bacillus	18	15	18	15	4	3	18
Lapillicoccus	1	0	1	1	0	0	1
Microbacterium	1	1	1	1	0	0	1

the *nif*H gene. Inorganic phosphate solubilization activity was observed in 15.9% of the 44 tested isolates (Table 3). Phosphate solubilization indices ranged from 1.4 to 1.8 (Table S2- supplementary material). Twenty-four (82%) of the 44 tested isolates were siderophores and ACC deaminase producers (Table 3). The highest number of siderophore producers was observed in isolates from the rhizosphere soil. ACC deaminase activity was frequently observed, but some *Stenotrophomonas, Bacillus* and *Rhizobium* isolates were not able to produce ACC deaminase in detectable levels (Table 3).

Preliminary experiments carried out with salt concentrations of 30, 60, and 150 mM of NaCl showed that cucumber seed germination was approximately 88% for the first two concentrations and 25% for the highest tested concentration. In further experiments, cucumber seedlings were exposed to concentrations of 60 and 90 mM of NaCl and treated with seven selected bacterial isolates (Table 4). The salt stress had more dramatic effect on root length than on stem length. Only *Leifsonia* sp. S1.5 significantly increased cucumber root length (64.5%) when compared to the positive control at 60 mM NaCl, with no effect of any bacterial isolate at

Table 4. Negative control (C-) - treatment without NaCl, Positive Control (C+) – treatment with NaCl, Root length (RL), shoot length (SL) and total fresh weight (TFW) of cucumber seedlings treated with seven selected diazotrophic bacteria and exposed to two salt concentrations. Means with the same letter in a column are not significantly different by Scott-Knott´s test ($p \geq 0.05$).

Treatments	60 mM (NaCl)			90 mM (NaCl)		
	RL (cm)	SL (cm)	TFW (g)	RL (cm)	SL (cm)	TFW (g)
C-	8.05 a	4.85 a	0.27 a	8.05 a	4.85 a	0.27 a
C+	4.60 b	4.25 a	0.15 b	3.05 b	3.30 b	0.08 c
Leifsonia sp. (S1.2)	6.30 b	6.20 b	0.26 a	2.60 b	3.25 b	0.19 b
Leifsonia sp. (S1.4)	5.87 b	6.60 b	0.27 a	2.85 b	3.30 b	0.19 b
Leifsonia sp. (S1.5)	7.57 a	7.35 b	0.27 a	3.30 b	2.20 b	0.11 c
Cellulomonas sp. (S1.9)	5.02 b	5.60 b	0.25 a	0.98 b	3.45 b	0.12 c
Bacillus sp. (S3.5)	5.55 b	5.62 b	0.19 b	3.45 b	4.80 a	0.10 c
Bacillus sp. (S3.6)	5.30 b	5.90 b	0.19 b	3.34 b	3.30 b	0.12 c
Enterobacter sp. (S5.1)	6.65 b	6.25 b	0.28 a	2.90 b	3.30 b	0.17 b
CV%	22	27	19	28	28	32

90 mM NaCl. For stem length, all isolates were able to reduce the negative effects at 60 mM NaCl and only the isolate S3.5 of *Bacillus* reduced it at 90 mM NaCl. The total fresh weight was similar when compared to the untreated control in all tested isolates, except for two *Bacillus* at 60 mM NaCl. At 90 mM NaCl, three isolates, two of *Leifsonia* sp. (S1.2 and S1.4) and one of *Enterobacter* sp (S5.1), were able to reduce the stress on cucumber seedlings (Table 4).

Although salinity negatively affects all stages of the plant growth by interfering in biochemical and physiological aspects, seed germination and seedling growth are the most sensitive stages (Parihar et al., 2015). Until this date, there is any report in the published literature pointing a member of the genus *Leifsonia* with ability to reduce negative effects of salinity in plants.

Most bacterial genera tested in our study showed the capacity to promote plant growth and reduce *in vivo* salt stress, corroborating with the presence of several traits putatively involved in stress tolerance and *in vitro* growth promotion. Bacteria with these traits can promote plant growth and reduce the deleterious effects of high salt concentrations (Naili et al., 2018). In the present study, it was not provided information regarding all mechanisms present in each isolate. Further studies should focus on the development of biotechnological products with these isolates for the low-input family-based farmers of the semi-arid region.

Growth of maize plants was evaluated after the inoculation with same seven selected isolates (S1.2, S1.4, S1.5, S1.9, S3.5, S3.6 and S5.1). Stem length increased significantly in four isolates (*Leifsonia* sp. (S1.5), *Leifsonia* sp. (S1.2), *Bacillus* sp. (S3.5), *Leifsonia* sp. (S1.5)), and almost all isolates promoted root length (Figure 3). The increases in stem length varied from 7-10% and in root length from 32% to 58%. There were no significant differences in the shoot and root dry matter of inoculated plants when compared to the non-inoculated control plants.

Bacteria with different characteristics for plant growth promotion were isolated and selected. When cucumber seeds were inoculated, these isolates promoted significant increases in the stem and root length (Figure 3). The length and architecture enable roots to have better access to water and nutrients, factors that are limiting to plant growth due to the advantage over plants with smaller root system. The shoot and root growth could be associated with bacterial production of hormones or action on humic acids which cause the elongation of cells in the meristem by vacuolar turgor and would not cause significant effects on dry matter (Conceição et al., 2008). In plants under water deficit, a larger root system can promote a higher survival rate.

The fact that sisal is associated with a microbial community, with such physiological versatility that may affect its physiology; may explain, in part, how this crop has been cultivated for several decades without any input of soil fertilizers in the semi-arid region of Bahia state in Brazil.

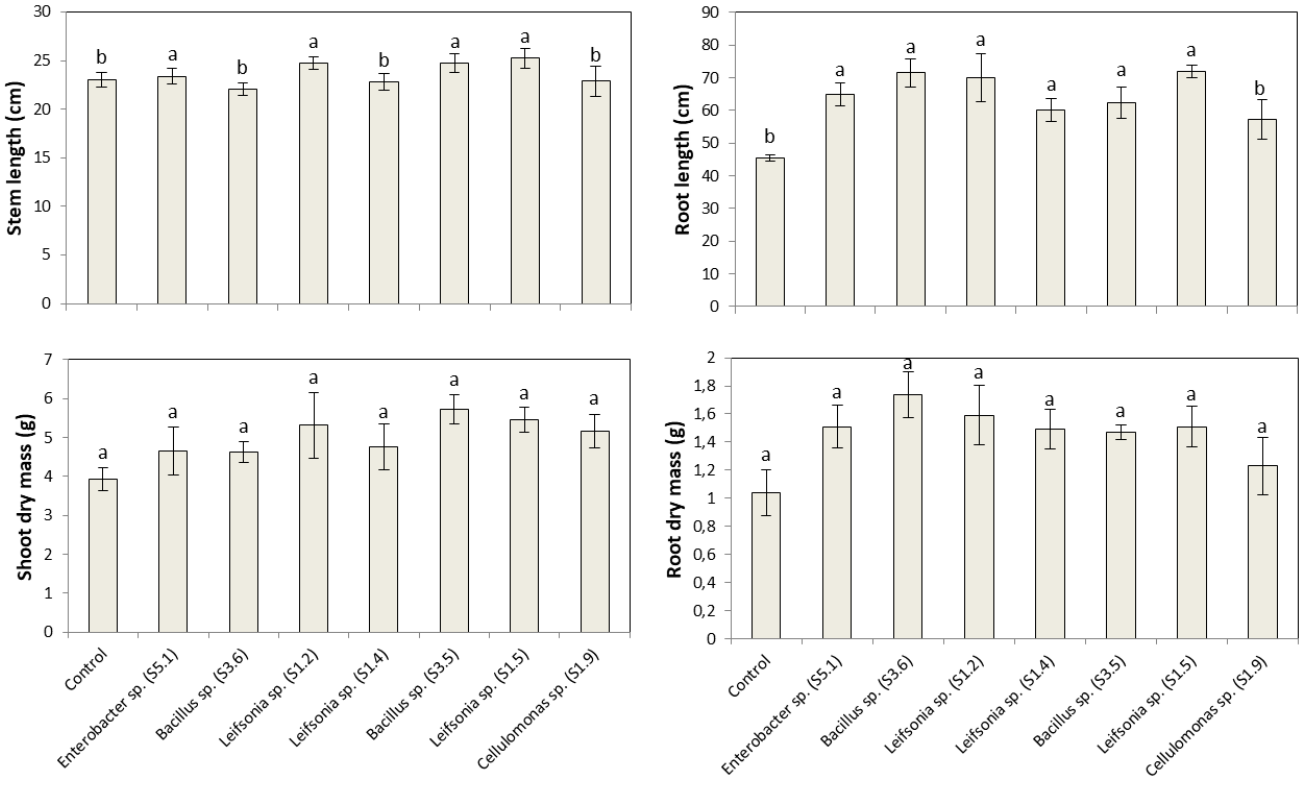


Figure 3. Stem length (SL), root length (RL), shoot dry matter (SDM), root dry matter (RDM) of maize seedlings after treatment with seven diazotrophic isolates. Evaluations were performed at 45 days after seed inoculation and sowing. Error bars indicate standard deviation based on ten replicates of plants grown. Different letters indicate difference between treatments according to Scott-Knott´s test ($p < 0.05$).

Conclusions

This study has shown that sisal plants host several diazotrophic bacteria that hold mechanisms other than nitrogen fixation that may be involved in plant growth promotion and tolerance to abiotic stress. These bacteria have the potential to be developed into biostimulants for sisal and other crops grown in semi-arid regions.

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