THE RESURRECTION PLANT *Tripogon spicatus* (POACEAE) HARBORS A DIVERSITY OF PLANT GROWTH PROMOTING BACTERIA IN NORTHEASTERN BRAZILIAN CAATINGA

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ABSTRACT

Plant species that naturally occur in the Brazilian *Caatinga* (xeric shrubland) adapt in several ways to these harsh conditions, and that can be exploited to increase crop production. Among the strategic adaptations to confront low water availability, desiccation tolerance stands out. Up to now, the association of those species with beneficial soil microorganisms is not well understood. The aim of this study was to characterize *Tripogon spicatus* diazotrophic bacterial isolates from the *Caatinga* biome and evaluate their ability to promote plant growth in rice. Sixteen bacterial isolates were studied in regard to their taxonomic position by partial sequencing of the 16S rRNA gene, putative diazotrophic capacity, *in vitro*

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indole-acetic acid (IAA) production and calcium phosphate solubilization, metabolism of nine different C sources in semi-solid media, tolerance to different concentrations of NaCl to pHs and intrinsic resistance to nine antibiotics. Finally, the ability of the bacterial isolates to promote plant growth was evaluated using rice (*Oryza sativa*) as a model plant. Among the 16 isolates evaluated, eight of them were classified as Enterobacteriaceae members, related to *Enterobacter* and *Pantoea* genera. Six other bacteria were related to *Bacillus*, and the remaining two were related to *Rhizobium* and *Stenotrophomonas*. The evaluation of total N incorporation into the semi-solid medium indicated that all the bacteria studied have putative diazotrophic capacity. Two bacteria were able to produce more IAA than that observed for the strain BR 11175^T of *Herbaspirillum seropedicae*. Bacterial isolates were also able to form a microaerophilic pellicle in a semi-solid medium supplemented with different NaCl concentrations up to 1.27 mol L^{-1}. Intrinsic resistance to antibiotics and the metabolism of different C sources indicated a great variation in physiological profile. Seven isolates were able to promote rice growth, and two bacteria were more efficient than the reference strain *Azospirillum brasilense*, Ab-V5. The results indicate the potential of *T. spicatus* as native plant source of plant growth promoting bacteria.

Keywords: biological nitrogen fixation, inoculant, diazotrophic bacteria, Semi-arid, desiccation tolerant plants.

RESUMO: *A PLANTA REVIVESCENTE Tripogon spicatus (POACEAE) ABRIGA UMA DIVERSIDADE DE BACTÉRIAS PROMOTORAS DE CRESCIMENTO VEGETAL NA CAATINGA, REGIÃO NORDESTE DO BRASIL*

As espécies vegetais que ocorrem naturalmente na Caatinga brasileira apresentam diversas adaptações às condições adversas que podem ser exploradas para aumentar a produção vegetal. Entre essas estratégias adaptativas para o enfrentamento da baixa disponibilidade hídrica, a tolerância à dessecação pode ser destacada. Até o momento, a associação entre essas espécies e os microrganismos benéficos do solo não é bem entendida. Os objetivos deste trabalho foram caracterizar bactérias diazotróficas de Tripogon spicatus e avaliar a capacidade de promoção do crescimento delas em plantas de arroz. Dezesseis isolados bacterianos foram estudados quanto a seu posicionamento taxonômico por meio do sequenciamento parcial do gene 16S rRNA, potencial diazotrófico, produção de ácido indol acético (AIA) e solubilização de fosfato de cálcio in vitro, metabolismo de nove diferentes fontes de C em meio semissólido, tolerância a diferentes concentrações de NaCl a pHs e resistência intrínseca a nove antibióticos. A habilidade em promover o crescimento vegetal foi avaliada utilizando o arroz (Oryza sativa) como planta-modelo. Dentre os 16 isolados avaliados, oito foram classificados como pertencentes à família Enterobacteriaceae, relacionados aos gêneros Enterobacter e Pantoea. Seis outros isolados foram relacionados a Bacillus e outros dois a Rhizobium e Stenotrophomonas. A avaliação da incorporação do N ao meio semissólido demonstrou o potencial diazotrófico de todos os isolados. Duas bactérias produziram mais AIA do que a estirpe BR 11175^T de Herbaspirillum seropedicae. Isolados bacterianos foram capazes de formar película em meio semissólido suplementado com concentrações de NaCl de até 1.27 mol L^{-1}. A resistência intrínseca a antibióticos e o metabolismo de diferentes fontes de C indicaram um perfil fisiológico muito variável. Sete isolados foram capazes de aumentar o crescimento das plantas de arroz, mais eficientemente do que a estirpe de referência Ab-V5 de Azospirillum brasilense. Os resultados indicaram o potencial de T. spicatus como uma espécie nativa fonte de bactérias promotoras de crescimento vegetal.

Palavras-chave: fixação biológica do nitrogênio, inoculante, bactérias diazotróficas, Semiárido, plantas tolerantes à dessecação.

INTRODUCTION

The natural biome which encompasses most of the Northeast region of Brazil is the Brazilian Steppic Savanna, known as the *Caatinga* biome. The main features of this natural environment are the short and concentrated rainy season associated with high incident solar radiation that causes high diurnal temperatures and water deficit (Ab'Saber, 2006). Due to these environmental characteristics, plant species inhabiting the *Caatinga* exhibit several adaptations to drought and other abiotic stresses for successful colonization of the biome.

Among drought tolerant plants, a few of them can survive long periods of severe desiccation. This group is called "desiccation tolerant plants" or

"resurrection plants" (Gaff, 1987). These species are able to maintain viable quiescent vegetative tissues throughout long desiccation periods due to the activation of several mechanisms, including strong light protection of leaf tissues (Aidar et al., 2010, 2014), synthesis of osmoprotectants, membrane and protein stabilizers (Morse et al., 2011). *Tripogon spicatus* Ekman Nees is a resurrection grass naturally occurring in the Americas from the south of Texas (USA) to the Patagonia Desert (Argentina). In Brazil, this species is found in the Semi-arid (Northeast) and seasonally flooded *Pantanal* (Central-West) regions (Gaff, 1987). The recovery of photosynthetic activity of *T. spicatus* soon after rehydration may involve association with microorganisms that facilitate some important functions.

In addition to physiological mechanisms, plant tolerance to harsh environmental conditions can be influenced by plant association with soil plant growth promoting bacteria (PGPB), which are able to fix nitrogen release insoluble nutrients, secrete analogs to plant hormones, and control soil-borne pathogens, among other mechanisms (Kuss et al., 2007; Puente et al., 2009; Grover et al., 2011; Gumiere et al., 2014; Santos et al., 2015). Several studies that isolated and evaluated the diversity and biotechnological potential of PGPB in Brazil have already been carried out on crop species, mainly with grasses such as maize, rice, wheat, and sugar cane (Baldani et al., 2009; Hungria et al., 2010).

Regarding wild non-legume species, the search for bacterial isolates able to promote plant growth has been carried out worldwide since the 1980's (Reinhold et al., 1987; Bilal et al., 1990; Doty et al., 2009; Lopez et al., 2011; Jha et al., 2012; Zahid et al., 2015). In Brazil, few plant groups have been studied only in the last few years (Costa and Melo, 2012; Fernandes Júnior et al., 2013; Kavamura et al., 2013). Recently, bacteria from native cactus species growing in the *Caatinga* were isolated, and evaluation of their physiological features and ability to promote plant growth indicated the presence of bacteria with biotechnological applications that promote the growth of cowpea (Costa and Melo, 2012) and maize (Kavamura et al., 2013). In spite of those physiologically peculiar characteristics, the resurrection plants inhabiting the *Caatinga* biome have not yet been evaluated in regard to the presence of PGPB.

Isolation and characterization of the PGPB from *T. spicatu*s can advance the understanding of the physiology and ecology of this resurrection plant. In addition, evaluation of the biotechnological potential of those bacteria can indicate isolates to be applied as inoculants to crops. In this study, the physiological characteristics and taxonomic position of putative diazotrophic bacteria from *T. spicatus* collected in the *Caatinga* biome were evaluated, as well as their ability to promote the growth of rice.

MATERIAL AND METHODS

Bacterial isolates and partial 16S rRNA gene sequencing

The bacterial isolates used in the present study were previously isolated by Fernandes Júnior et al. (2012a). They were obtained from healthy *Tripogon spicatus* plants collected in the municipality of Lagoa Grande (Pernambuco, Brazil), from 8º 48' 11.6" and 8º 48' 23.9" S and from 40º 14' 48.4" and 40º 14' 58.7" W in an area of *Caatinga*, in the rainy seazon of 2011. To obtain putative diazotrophic isolates, the authors adopted an isolation strategy using a semi-solid medium (Döbereiner et al., 1995; Baldani et al., 2014). Isolation was carried out by crushing the washed and surface disinfected roots of *T. spicatus* in 0.15 mol L^{-1} NaCl and inoculating them in BMGM semi-solid culture medium (Estrada-De Los Santos et al., 2001).

Amplifications were conducted using the universal 27F and 1492R for partial 16S rRNA gene sequencing (Weisburg et al., 1991). The PCR products were purified using the commercial HiYield™ PCR DNA Mini Kit (Real Biotech Corp, Taipei, Taiwan). Sequencing was carried out using the 27F primer in a 3730 *xl* sequencer (Applied Biosystems, Drive Foster City, CA, USA) according to manufacturer's instructions.

The quality of the sequences was verified using the Sequence Scanner Software v. 2.0 (Applied Biosystems). Good quality sequences with no less than 400 bases were used for bacterial identification (Silva et al., 2013). The sequences were compared through the GenBank sequences database (National Center for Biotechnology Information) by means of the Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/Genbank) (Altschul et al., 1990). The sequences were deposited in the GenBank under the accession numbers from KF878276 to KF878293.

Plant growth promotion (PGP) traits: putative diazotrophic capacity, *in vitro* production of indole-acetic acid (IAA) analogs and calcium phosphate solubilization

The bacteria were grown in liquid DYGS medium (Rodrigues Neto et al., 1986) for three days at 120 rpm in an orbital shaker for assessment of bacterial plant growth promoting traits and other phenotypic evaluations. The bacterial broth was centrifuged (6,000 *g* for 3 min), the supernatant was discarded and the pellet was re-suspended in saline solution (0.15 mol L^{-1} NaCl). Cell suspensions were used for inoculation in solid, semi-solid, or liquid medium for all phenotypic evaluations described below.

The diazotrophic capacity of the bacterial isolates grown in semi-solid medium was assessed

through quantification of total N content after pellicle formation of the putative diazotrophic bacteria (Kuss et al., 2007; Silva et al., 2013). The bacteria were grown in vials containing 10 mL of BMGM semi-solid medium at 28 °C in a growing chamber for 7 days. After the incubation period, the medium were homogenized handly, frozen at -20 °C, and heated in a microwave oven for 2 min. The medium was homogenized again and a 5 mL aliquot was taken for quantification of total N content in the medium by the modified *semi-micro* Kjeldahl method (Liao, 1981). A positive control treatment with the diazotrophic *A. brasilense* (Ab-V5) and a negative control treatment with the non-pellicle forming strain CIAT 899^T of *Rhizobium tropici* were also evaluated. An aliquot of non-inoculated culture medium was also digested and distilled as blank to calculate the N concentration in the culture medium.

The production of indole-acetic acid (IAA) analogs *in vitro* was evaluated according to the spectrophotometric method described by Sarwar and Kremer (1995) at 520 nm reading wavelength. The cell suspensions (100 µL) were inoculated in NFb liquid medium (Döbereiner et al., 1995) supplemented with 168 mg L^{-1} of synthetic L-tryptophan (Vetec Química Fina, Rio de Janeiro, Brazil) without bromothymol blue. The bacterial isolates were grown under constant shaking for five days. To construct the calibration curve, solutions with known concentrations of synthetic IAA (Sigma Aldrich, St Louis, USA) were prepared with ultra-pure water, mixed with the Salkowski solution, incubated, and read as described for the samples.

Solubilization of calcium phosphate was assessed according to Sylvester-Bradley et al. (1982) using the GL medium supplied with $CaCl_2$ (150 mL at 0.1 mol L^{-1}) and KH_2PO_4 (150 mL at 0.75 mol L^{-1}). Aliquots of 10 µL of the bacterial suspensions were inoculated at equidistant points in each of the Petri dishes containing the GL medium. The inoculated plates were incubated at 28 °C for 5 days, and the solubilization index was calculated by the ratio between the diameter of the translucent area surrounding the colony and the colony diameter.

These experiments were performed in a completely randomized design with three replications.

In vitro growth in the semi-solid BMGM medium under different conditions and intrinsic antibiotic resistance

For assessment of bacterial growth in a semi-solid medium, as described above, the cell suspension was inoculated at the same time in BMGM semi-solid medium, modified by the conditions described below and incubated in a growth chamber at 28 °C for 7 days.

To assess the growth of the isolates at different salt concentrations, a 10 µL aliquot of the cell suspension was inoculated in BMGM semi-solid medium, supplemented with 0.09, 0.17, 0.52, 0.87, and 1.27 mol L^{-1} of NaCl. Tolerance to different pHs was also assessed through bacterial inoculation in a semi-solid BMGM medium with pHs adjusted to 4.0 and 9.0. Bacterial growth using different C sources was also analyzed using semi-solid BMGM medium supplemented with mannitol, glucose, malic acid, sucrose, fructose, maltose, xylose, arabinose, or citric acid (Vetec Química Fina, Rio de Janeiro, Brazil) as sole carbon sources.

For these experiments, the vials with pellicle formation, which characterizes microaerophilic diazotrophic ability of the associative bacteria were considered as "positive" and the non-forming tubes were considered "negative". The assays were conducted with three replications in a completely randomized design. Those treatments in which positive or negative results were not observed in all three replications were repeated to ensure consistency of the results.

Intrinsic antibiotic resistance was assessed by the diffusion disc method. The cell suspensions were prepared as described above. The suspensions were optically adjusted with the aid of the MacFarland scale (point 0.5) and a 100 µL aliquot of the adjusted broth was inoculated in Petri dishes containing solid DYGS medium and spread using a Drigalski loop. At the inoculated plates were added three antibiotics filter paper discs (to avoid the overlap of the inhibition zones). Nine antibiotics were evaluated: Erythromycin (15 µg), Rifampicin (30 µg), Gentamicin (10 µg), Neomycin (30 µg), Streptomycin (10 µg), Chloramphenicol (30 µg), Vancomycin (30 µg), Ampicillin (10 µg) and Nalidixic acid (30 µg) (Cefar Diagnósticos Ltda., São Paulo, Brazil). The plate dishes were incubated for 5 days and antibiotic susceptibility or resistance was determined by the presence or absence of the inhibition zone around the discs, respectively.

Growth promotion of rice (*Oryza sativa* L.) in greenhouse conditions

Rice (*Oryza sati*va L.) cv. BRS Tropical was used as a model plant, and the experiment was set up using non-sterile soil as a substrate under greenhouse conditions. The soil sample was collected from the soil surface horizon (0.0-0.2 m) in a *Argissolo Amarelo* (Ultisol). The fertility characteristics of the soil were: pH(H_2O) 6.3; electrical conductivity 0.11 dS m^{-1}; organic matter 12.3 g kg^{-1}; P 44.62 mg kg^{-1} (Mehlich-1); K 140 mg dm^{-3} (Mehlich-1); Ca^{2+} 2.0 $cmol_c$ dm^{-3} (1 mol L^{-1} KCl); Mg^{2+} 0.4 $cmol_c$ dm^{-3} (1 mol L^{-1} KCl); Al^{3+} 0.05 $cmol_c$ dm^{-3} (1 mol L^{-1} KCl); H+Al 0.66 $cmol_c$ dm^{-3}; cation exchange capacity 3.44 $cmol_c$ dm^{-3}; base saturation 81 %. The soil was placed in 500 mL pots for planting. The surface of the seeds was disinfected with hydrogen peroxide and

washed 10 times in autoclaved distilled water before sowing the seeds. The surface disinfected seeds were then pre-germinated in Petri dishes with agar-water for 3 days. The germinated seeds were then sown in the pots (three seeds per pot) and inoculated with 1 mL of the bacterial culture broth previously grown in liquid NFb medium (around 10^9 cells per mL).

Inoculated plants received a NH_4NO_3 solution at the rate of 50 mg/plant of N (50 % of the amount required by rice) 7 days after the planting. The following experimental treatments were evaluated: single inoculation with the 16 bacteria isolated from *T. spicatus* (from ESA 0001 to ESA 0016); single inoculation of *Azospirillum brasilense* strain Ab-V5 (1); non-inoculated controls with full N application (100 mg/plant) (1); and non-inoculated control with 50 mg/plant N. On the 12th day after emergence, the plants were thinned, leaving only one plant per pot. The experiment was conducted until the 50th day after plant emergence. The parameters evaluated were shoot dry weight, root dry weight, and total dry weight (shoots + roots). A completely randomized experimental design with four replications was used.

Statistical analysis

The data from all quantitative evaluations of the plant growth promoting traits and the greenhouse condition experiment were analyzed by ANOVA and the Scott-Knott mean comparison test through the Sisvar software v. 5.0.

RESULTS

Identification of bacterial isolates by partial 16S rRNA gene sequence

Among the 16 bacterial isolates analyzed, nine were related to the Enterobacteriaceae family, showing the closest relation to the genus *Enterobacter* or *Pantoea*, with sequence similarities from 99 to 100 % (Table 1). Five isolates showed similarities from 99 to 100 % with the 16S rRNA sequences of the *Bacillus* genus in the GenBank database. The isolate ESA 0009 was most closely related to the *Stenotrophomonas* genus (99 % of similarity), and the isolate ESA 0015 showed similarity of 100 % with the *Rhizobium* genus.

In vitro plant growth promotion traits

The diazotrophic capacity of the isolates was confirmed by determination of the total N content incorporated in the culture medium (Table 2). Of the 16 isolates, 13 exhibited similar N incorporation when compared statistically to the diazotrophic *A. brasilense* reference strain, ranging from 23.3, for the isolates ESA 0002 and ESA 0004, to 42.0 µg of N per mL of medium (µg mL^{-1}), for ESA 0014.

Table 1. Identification of 16 bacterial isolates from *Tripogon spicatus* by comparison of its partial 16S rRNA gene sequence with the sequences available in the GenBank database

Isolate	Fragment length	Genus	Similarity	*e* value
	bp		%	
ESA 0001	725	*Pantoea* sp.	99	0.0
ESA 0002	673	*Bacillus* sp.	100	0.0
ESA 0003	711	*Enterobacter* sp.	100	0.0
ESA 0004	690	*Enterobacter* sp.	99	0.0
ESA 0005	693	*Enterobacter* sp.	99	0.0
ESA 0006	714	*Enterobacter* sp.	99	0.0
ESA 0007	593	*Bacillus* sp.	99	0.0
ESA 0008	703	*Stenotrophomonas* sp.	99	0.0
ESA 0009	693	*Pantoea* sp.	100	0.0
ESA 0010	673	*Enterobacter* sp.	99	0.0
ESA 0011	567	*Enterobacter* sp.	99	0.0
ESA 0012	534	*Bacillus* sp.	100	0.0
ESA 0013	704	*Bacillus* sp.	99	0.0
ESA 0014	678	*Bacillus* sp.	100	0.0
ESA 0015	768	*Rhizobium* sp.	100	0.0
ESA 0016	770	*Bacillus* sp.	99	0.0

Three other isolates (ESA 0001, ESA 0003, and ESA 0009) showed lower N incorporated in the medium, ranging from 14.0 to 17.5 µg mL^{-1}, although these values were significantly higher than those observed for the non-pellicle forming *R. tropici* (3.5 µg mL^{-1}), indicating the diazotrophic capacity of these three isolates. The slight increase in N in the culture medium inoculated with *R. tropici* can be attributed to the N content in the inoculated cells and N remaining from cell suspension.

Regarding *in vitro* IAA production, the isolates ESA 0015 (82 µg mL^{-1} of IAA) and ESA 0012 (51.7 µg mL^{-1} of IAA) were ranked as the two highest clusters by the Scott-Knott mean range test ($p<0.01$), being statistically superior to the reference strain *H. seropedicae* BR 11175^T. Four other isolates also produced high amounts of IAA analogs, ranging from 36.8 to 42.7 µg mL^{-1} of IAA, and were ranked within the same group as the reference IAA-producing strain. IAA production was not detected for the isolate ESA 0009 by the method applied, but all other isolates exhibited production of at least 5.8 µg mL^{-1} of IAA.

Solubilization of calcium phosphate was observed for only four isolates. The nitrogen-fixing and IAA-producing isolate ESA 0003 showed the highest solubilization index, followed by the isolates ESA 0016, ESA 0001, and ESA 0009. The other isolates and the strains that used CIAT899^T and Ab-V5 grew

Table 2. Nitrogen incorporation in the culture medium, *in vitro* indole-acetic acid production, and calcium phosphate solubilization by 16 bacterial isolates from *Tripogon spicatus* and reference strains. Data are means of three replications.

Bacteria	N content	IAA content	Solubilization index
	μg mL^{-1}		
CIAT 899^T	3.5 c	nd	-
BR 11175^T	nd	37.3 c	nd
Ab-V5	35.0 a	nd	-
ESA 0001	14.0 b	5.9 g	1.2 b
ESA 0002	23.3 a	42.7 c	-
ESA 0003	17.5 b	36.8 c	2.1 a
ESA 0004	23.3 a	6.9 g	-
ESA 0005	31.5 a	30.8 d	-
ESA 0006	35.0 a	8.6 g	-
ESA 0007	25.7 a	5.9 g	-
ESA 0008	25.7 a	6.8 g	-
ESA 0009	14.0 b	-	1.1 b
ESA 0010	30.3 a	25.7 e	-
ESA 0011	30.3 a	40.8 c	-
ESA 0012	30.3 a	51.7 b	-
ESA 0013	30.3 a	5.8 g	-
ESA 0014	42.0 a	41.1 c	-
ESA 0015	30.3 a	87.0 a	-
ESA 0016	35.0 a	12.1 f	1.3 b
CV (%)	19.2	6.5	5.6

Means with the same letter, in the same parameter, do not differ by the Scott-Knott mean test ($p<0.01$). nd: not determined. CV: coefficient of variation.

in the GL medium but did not exhibit visible calcium phosphate solubilization.

Physiological characterization

The evaluation of diazotrophic ability, based on pellicle formation in the culture medium, showed that there is a number of high halotolerant bacteria among the isolates evaluated. The isolates ESA 0001, ESA 0003, ESA 0005, ESA 0007, ESA 0010, ESA 0012, ESA 0013, and ESA 0016 showed diazotrophic capacity in the BMGM semi-solid culture medium supplemented with up to 1.27 mol L^{-1} of NaCl. The bacteria ESA 0006, ESA 0011, and ESA 0015 exhibited a diazotrophic character in the medium with a maximum concentration of 0.86 mol L^{-1} of NaCl (Table 3). Low halotolerance was observed only for ESA 0008, which showed diazotrophic capacity in microaerophilic conditions in the medium supplemented with 0.09 mol L^{-1} of NaCl, in addition to the control treatment. The pH of the medium influenced pellicle formation for six isolates and ten bacteria were able to fix nitrogen microaerophilically at both pHs evaluated. By combining these evaluations, the isolates ESA 0005, ESA 007, ESA 0011, ESA 0013, and ESA 0016 stand out for their diazotrophic ability, both under salt and pH stress conditions.

Pellicle formation in culture media supplemented with nine C sources revealed a variable profile. Half of the bacteria evaluated were able to show diazotrophic ability in the culture medium supplemented with seven or more C sources evaluated (Table 3). In contrast, six bacteria showed the ability to form the pellicle in the modified BMGM semi-solid medium supplemented with four or fewer C sources. In regard to C sources, all supplemented media allowed the growth of more than nine isolates, except for fructose. All bacterial isolates showed microaerophilic growth in the culture medium supplemented with mannitol, malic acid, or glucose, the three C sources of the original BMGM medium.

Regarding the antibiotic resistance of the bacteria, the bacterial isolates ESA 0006 and ESA 0014 showed intrinsic resistance for a large number of antibiotics, being susceptible only to gentamicin and neomycin, respectively (Table 3). In addition, the isolate ESA 0007 was resistant only to ampicillin and susceptible to all other antibiotics tested. All bacterial isolates were resistant to ampicillin, while the antibiotics least tolerated by the isolates evaluated were gentamicin and neomycin, to which only three and four bacteria were resistant, respectively.

Ability to promote rice growth

Among the bacterial isolates, seven were able to increase rice root and/or shoot growth compared to the control treatments. Among these bacteria, five promoted the same plant growth as that observed in the plants inoculated with the Ab-V5 reference strain (Table 4). Surprisingly, two bacterial isolates were statistically higher than the treatment inoculated with the reference strain and equal to those treatments that received full N fertilization. Considering those two isolates, ESA 0001 stood out; it was the only one able to promote the growth of the shoots, roots, and complete plant at the same rate that was observed in the plants that received full mineral N fertilization. The dry biomass increase as a result of inoculation with the isolate ESA 0001 was 82 and 51 % when compared to non-inoculated plants and to the plants inoculated with Ab-V5 strain, respectively.

Together with ESA 0001, the isolates ESA 0016 and ESA 0015 are notable for their ability to promote plant growth. These bacteria were able to increase the shoot and total plant weight more than the increase promoted by inoculation with the Ab-V5 strain.

Table 3. Tolerance to different concentrations of NaCl and pHs *in vitro*, ability to metabolize nine carbon sources, and intrinsic resistance to different antibiotics for 16 bacteria isolated from *Tripogon spicatus*

	ESA 0001	ESA 0002	ESA 0003	ESA 0004	ESA 0005	ESA 0006	ESA 0007	ESA 0008	ESA 0009	ESA 0010	ESA 0011	ESA 0012	ESA 0013	ESA 0014	ESA 0015	ESA 0016
	Maximum NaCl concentration with microaerophilic growth (mol L^{-1})															
	1.27	0.17	1.27	0.52	1.27	0.86	1.27	0.09	0.17	1.27	0.86	1.27	1.27	0.52	0.86	1.27
pH of the medium																
4	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+
9	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Metabolism of carbon source[1]																
Malic acid	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Glucose	+	-	-	+	-	-	+	+	-	-	+	+	+	+	-	+
Mannitol	+	-	-	+	-	-	+	+	+	+	+	+	-	+	-	+
Arabinose	-	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+
Citric acid	-	+	-	+	-	+	+	-	+	-	+	+	+	+	+	+
Maltose	+	-	-	+	-	+	+	-	+	-	+	+	+	+	-	+
Xylose	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+	-
Sucrose	+	-	+	+	-	+	+	-	+	-	+	-	+	+	-	+
Fructose	-	-	-	+	-	-	-	-	+	+	-	-	+	+	-	-
Intrinsic antibiotic resistance[2]																
Nalidixic Acid	-	+	+	-	+	+	-	+	+	-	-	-	+	+	-	+
Streptomycin	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+
Ampicillin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gentamicin	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-
Vancomycin	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+
Chloramphenicol	+	+	-	+	+	+	-	+	+	+	+	-	-	+	+	+
Erythromycin	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+
Neomycin	-	-	-	+	-	+	-	-	-	+	-	-	+	-	-	-
Riphampicin	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	+

[1] Pellicle forming in semi-solid BMGM medium modified by the characteristics described, + is positive isolates (able to form pellicle); - is negative isolates (unable to form pellicle). [2] Intrinsic antibiotic resistance: + is resistant (absence of an inhibition zone around the disc); - is susceptible (presence of an inhibition zone around the disc).

DISCUSSION

Identification of the bacterial isolates indicated the predominance of bacteria belonging to Enterobacteriaceae and *Bacillus*. Other species native to the *Caatinga*, for example cactus species, also harbor plant growth promoting bacteria related to those taxa, belonging to the *Pantoea* and *Bacillus* genera (Kavamura et al., 2013). Furthermore, related bacteria were also isolated from Cactaceae and Poaceae at different sites around the world, including dry land environments (Peng et al., 2009; Puente et al., 2009; Lopez et al., 2011). In the present study, among the 16 bacterial isolates evaluated, only two were not related to Enterobacteriaceae or Firmicutes, and they were classified as *Rhizobium* and *Stenotrophomonas*. Both genera are known to include PGPB associated with non-legume crops and wild species (Ferrara et al., 2012; Jha et al., 2012; Silva et al., 2013).

Characterization of the bacterial isolates regarding their plant growth promoting (PGP) traits revealed that some isolates showed more than one feature. For example, the highest IAA analog producing isolate, ESA 0015, which belongs to the *Rhizobium* genus, was also clustered in the highest nitrogen-fixing group, according the statistical analysis. The same characteristics were observed in the *Bacillus* sp. isolate ESA 0014. These results corroborate results from other studies, in which efficient diazotrophic and phytohormone producing bacteria were isolated from cultivated (Kuss et al., 2007; Ferrara et al., 2012; Silva et al., 2013; Santos et al., 2015) and wild species (Doty et al., 2009; Jha et al., 2012).

Bacterial isolates with the capacity to fix atmospheric N and produce high amounts of IAA analogs can be applied in the field and help in seed germination and initial root development (Gumiere et al., 2014). At this point, the isolates ESA

Table 4. Growth of 'BRS Tropical' rice (*Oryza sativa*) plants as a result of inoculation of plant growth promoting bacteria isolated from *Tripogon spicatus*. Data are means of four replications.

Inoculation treatment	Dry weight		
	Shoot	Root	Whole plant
	g/plant		
ESA 0001	1.10 a	0.61 a	1.71 a
ESA 0016	0.77 b	0.46 a	1.23 a
ESA 0015	0.74 b	0.46 a	1.20 b
ESA 0007	0.70 c	0.50 a	1.20 b
ESA 0014	0.66 c	0.49 a	1.15 b
ESA 0011	0.57 c	0.57 a	1.14 b
ESA 0013	0.63 c	0.41 b	1.04 b
ESA 0004	0.62 c	0.41 b	1.03 c
ESA 0003	0.61 c	0.40 b	1.01 c
ESA 0012	0.58 c	0.39 b	0.97 c
ESA 0005	0.52 c	0.40 b	0.92 c
ESA 0008	0.53 c	0.39 b	0.92 c
ESA 0010	0.48 c	0.31 b	0.79 c
ESA 0006	0.48 c	0.30 b	0.78 c
ESA 0009	0.47 c	0.28 b	0.75 c
ESA 0002	0.42 c	0.31 b	0.73 c
Ab-V5	0.58 c	0.54 a	1.13 b
50 mg/plant of N	0.58 c	0.36 b	0.94 c
100 mg/plant of N	1.00 a	0.65 a	1.65 a
CV (%)	13.25	13.46	12.21

Means with the same letter, in the same parameter, do not differ by the Scott-Knott mean test ($p<0.05$). CV: coefficient of variation.

0015 and ESA 0014 can be tested for this application. The ability of the PGPB to use several mechanisms to promote plant growth is desirable regarding commercial inoculant production. In this case, the same isolate can promote growth in its host by means of different mechanisms and at different phases of plant development and establishment in the field.

However, considering the three *in vitro* PGP characteristics evaluated in the present study, none of the bacterial isolates performed best in all of them. This observation indicates that for the *T. spicatus* roots there is likely more than one microorganism acting concomitantly to promote plant growth with different and complementary mechanisms (Marasco et al., 2012; Philippot et al., 2013).

Regarding their physiological features, the ability of eight isolates to fix N under microaerophilic conditions in culture media supplemented up to 1.27 mol L^{-1} of NaCl (75 g L^{-1}) indicates that some bacteria evaluated are highly halotolerant. The halotolerance of diazotrophic associative bacteria was already shown for isolates, especially for halophyte hosts such as *Leptochloa fusca* (kallar grass) (Reinhold et al., 1987), *Atriplex* sp. (Bilal et al., 1990), and *Salicornia brachiata* (Jha et al., 2012). Of the eight highest diazotrophic halotolerant bacteria found in the present study, seven belong to the Enterobacteriaceae group, and only one (ESA 0005) was classified as *Bacillus* sp. The other two Enterobacteriaceae characterized here were able to grow microerophilically in the semi-solid medium supplemented with up to 0.85 mol L^{-1} NaCl (50 g L^{-1}), and can also be considered as highly halotolerant. Among the *Bacillus* related isolates, halotolerance was variable since one isolate grew in the medium with 1.27 mol L^{-1} NaCl, two bacteria grew microaerophilically in the semi-solid medium supplemented with 0.52 mol L^{-1} NaCl, and one grew in the medium supplemented with up to 0.09 and 0.17 mol L^{-1} NaCl.

Halotolerance under diazotrophic conditions of Enterobacteriaceae and Firmicutes is quite variable and can range from total inhibition, through the addition of low concentrations of salt in the culture medium, to no effect (Bilal et al., 1990; Sorokin et al., 2008; Jha et al., 2012). High tolerance to salts *in vitro* can indicate promising bacteria for plant inoculation in saline field environments. The other physiological characteristics evaluated showed great metabolic variability of the isolates and indicated 10 bacteria that are able to fix N in a semi-solid medium under a wide range of pH, eight isolates able to fix N in a culture medium supplemented with at least seven different C sources, and great variability in regard to intrinsic antibiotic resistance.

Combining all physiological results, the data suggest that the isolates are able to persist in the soil or in the desiccated tissues of *T. spicatus* due to their tolerance to harsh environments and metabolic versatility in regard to the ability to metabolize different C sources and tolerance to antibiotics that may be produced by other soil micro-organisms (Fernandes Júnior et al., 2012b; Jha et al., 2012).

The ability of these isolates to promote rice growth showed that some bacteria can increase plant biomass at higher rates than those observed for the Ab-V5 strain that is currently used for production of commercial inoculants for rice in Brazil. Among the isolates evaluated, the N-fixing, halotolerant, and phosphate solubilizing *Pantoea* sp. ESA 0001 and *Enterobacter* sp. ESA 0016 stood out through their performance. The ability of these isolates to increase rice growth at the same rate observed for the plants that received 100 mg of N indicates that, in this experiment, these bacteria may be acting with more mechanisms to help plant development than the plants supplied with only N.

Furthermore, two other *Enterobacter* sp., two *Bacillus* sp., and a *Rhizobium* sp. were also able to promote rice growth. It is likely that, in the soils of the *Caatinga* where the plant samples were taken, those bacteria can colonize the naturally

occurring plants (e.g., *T. spicatus*) and act together with different mechanisms to promote plant growth (Philippot et al., 2013). For biotechnological applications, the use of inoculants that contain more than one PGPB may also be a strategy for increasing crop growth (Schultz et al., 2014).

There are results in the scientific literature showing the characteristics and application (in crop inoculation) of diazotrophic isolates obtained from wild species, for example halophytes (Jha et al., 2012), cacti (Puente et al., 2009; Costa and Melo, 2012; Kavamura et al., 2013), and relatives to crop species (Doty et al., 2009; Fernandes Júnior et al., 2013), including the bacteria isolated by Kavamura et al. (2013) and Costa and Melo (2012) from the *Caatinga* biome. Most of those studies were conducted in the last few years, indicating that prospection of diazotrophic bacteria associated with wild species is a new area of research, especially in Brazil. To our knowledge, the present study is the first report for characterization of diazotrophic bacteria associated with the resurrection plant *T. spicatus*, a promising source of PGP bacteria with biotechnological applications. To assure the potential of these bacteria as candidates for application in commercial inoculants, as well as to get a better understanding of their role in plant establishment in *Caatinga* soils, further inoculation experiments with crops and native species needs to be carried out.

CONCLUSIONS

Bacteria with plant growth promoting characteristics and a very diverse metabolic profile belonging to the genera *Enterobacter*, *Bacillus*, *Rhizobium*, *Stenotrophomonas*, and *Pantoea* are colonizing *Tripogon spicatus* roots.

The isolates ESA 0001 (*Pantoea* sp.) and ESA 0016 (*Rhizobium* sp.) stood out for their PGP ability for rice plants, indicating the resurrection plant *Tripogon spicatus* as a putative source of efficient plant growth promoting bacteria.

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