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Characterization of plant growth-promoting bacteria inhabiting *Vriesea gigantea* Gaud. and *Tillandsia aeranthos* (Loiseleur) L.B. Smith (Bromeliaceae)

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Abstract: Microorganisms that live inside and around a plant can supply it with essential substances, such as phytohormones and essential nutrients. The present investigation aimed to isolate and characterize the phyllosphere, the endophytic, and the water tank bacteria associated with *Vriesea gigantea* and *Tillandsia aeranthos*. The bacteria were tested for siderophore and indole-3-acetic acid (IAA) production, phosphate solubilization, and presence of the *nifH* gene. Genetic diversity of the bacterial isolates was evaluated by rep-PCR. Sixty-eight bacterial strains were isolated from 3 different microhabitats of *V. gigantea* and from 2 microhabitats of *T. aeranthos* bromeliad plants. Gram-positive, spore-forming bacilli comprised most bacterial isolates. All isolates produced IAA *in vitro* in presence of very low amounts of tryptophan. More than 70% of the evaluated bacteria presented the ability of siderophore production and phosphate solubilization, and possessed the *nifH* gene. It was not possible to distinguish well-defined groups of isolates based on the bromeliad species and microhabitat they inhabit using genetic characterization by rep-PCR. Water tanks presented the most abundant diversity compared with phyllosphere and endophytes, probably due to the high nutrient concentration, which promotes an ideal environment for complex microbial communities.

Keywords: bromeliads, PGPB, diazotrophic bacteria, water tank, siderophore, IAA.

GIONGO, A., BENEDUZI, A., GANO, K.A., VARGAS, L.K., UTZ, L.R.P. & PASSAGLIA, L.M.P. Caracterização de bactérias promotoras de crescimento vegetal em *Vriesea gigantea* Gaud. e *Tillandsia aeranthos* (Loiseleur) L.B. Smith (Bromeliaceae). *Biota Neotrop.* 13(3):<http://www.biotaneotropica.org.br/v13n3/pt/abstract?article+bn01413032013>

Resumo: Microrganismos que habitam o interior e a superfície podem fornecer substâncias essenciais ao crescimento das plantas, como fitormônios e nutrientes essenciais. O presente trabalho teve como objetivo isolar e caracterizar as bactérias da filosfera, do ambiente endofítico e a água de tanque associadas à *Vriesea gigantea* e *Tillandsia aeranthos*. As bactérias foram submetidas a testes de verificação de produção de sideróforos e de ácido indol acético (AIA), solubilização de fosfatos, e a presença do gene *nifH*. A diversidade genética dos isolados bacterianos foi analisada por rep-PCR. Sessenta e oito microrganismos foram isolados de 3 microambientes distintos de *V. gigantea* e de 2 microambientes de *T. aeranthos*. A maioria das bactérias isoladas foram bacilos formadores de esporos, gram-positivos. Todos os isolados produziram AIA *in vitro* na presença de quantidades pequenas de triptofano. Mais de 70% das bactérias analisadas produziram sideróforos, solubilizaram fosfatos e possuíam o gene *nifH*. Não foi possível distinguir grupos definidos de microrganismos baseados no microhabitat e na espécie de bromélia de onde foram isolados usando rep-PCR. A água do tanque apresentou maior diversidade microbiana quando comparada com a filosfera e o ambiente endofítico, provavelmente devido à alta concentração de nutrientes, que promove um ambiente favorável para o desenvolvimento de comunidades microbianas complexas.

Palavras-chave: bromélias, PGPB, bactérias diazotróficas, água de tanque, sideróforos, AIA.

Introduction

Bromeliaceae is a diversified family of plants with terrestrial or epiphytic habitats, encompassing about 2,900 described species (Holst & Luther 2004). The species *Vriesea gigantea* Gaud., in the subfamily Tillandsioideae, as well as other bromeliads, has a high ornamental value, thus is threatened of extinction in some regions of Brazil (Rio Grande do Sul 2006). *Tillandsia aeranthos* (Loiseleur) L.B. Smith, another member of the Tillandsioideae subfamily, is broadly distributed, occurring in upper rocks, rocky high walls, and in riparian forests (Cronquist 1981).

Bromeliads are remarkably tolerant and adaptable plants. They can be found along seashores subjected to salt sprays, or in extreme heat and drought deserts. Some are terrestrial, others are saxicolous, but most of them are epiphytes (Benzing 2000). Epiphytes form a highly diverse group of plants especially common in humid tropical forests (Benzing 1990). In those habitats characterized by a high absolute precipitation, their growth in tree crowns without a contact with the soil is equivalent to highly intermittent water and nutrient supply. A suite of anatomical, morphological, and physiological adaptations allows epiphytes to cope with this irregular resource supply. One example is the 'tank' presented in many bromeliads which is an impounding structure formed by overlapping leaf bases (such as *V. gigantea*). Tank bromeliads generally show low contents of nutrients (Stuntz & Zotz 2001), and grow very slowly even under near-optimal conditions (Hietz et al. 2002, Schmidt & Zotz 2002). These are typical features of stress-tolerant plants associated with nutrient-poor habitats (Grime 2001). In this group of plants, nutrient uptake capacities are normally tuned towards the capture of short pulses, and such a combination of high uptake of nutrients and slow potential growth frequently leads to an accumulation of reserves (Chapin 1980). Some bromeliads, such as *Tillandsia* spp., do not have tanks for water absorption. Instead, those plants have trichomes, specialized hairs adapted to absorb water in the surface of narrow leaves (Benzing 1990).

It is widely accepted that some microorganisms can improve plant health and nutrition. The term plant growth promoting rhizobacteria (PGPR) was coined for the bacterial bio-control agents of rhizosphere (Kloepper et al. 1980) but some years later, the term plant growth promoting bacteria (PGPB) was proposed to encompass bacteria which enhance plant growth by other means (Bashan & Holguin 1998). PGPB are usually classified into two groups according to the way they benefit their host plant, directly or indirectly. The plant response to PGPB is a very complex phenomenon resulting from the combination of mechanisms that affect several aspects of mineral nutrition and root development (Cleyet-Marel et al. 2001, Mantelin & Touraine 2003). The exact mechanisms by which PGPB promotes plant growth are not totally understood (Glick 1995, Ahmad et al. 2006), although several studies already proved that these bacteria improve plant development by nitrogen fixation, phytohormone and siderophore production, mineral solubilization and disease control (Kloepper et al. 1992, Lippmann et al. 1995, Bashan & Holguin 1998, Barea et al. 2005, Inselsbacher et al. 2007). The ability of nitrogen fixation, for example, is an important PGPB characteristic mainly for epiphytic plants since these plants depend on foliar absorption of nutrients (Bashan et al. 2008). *Vriesea gigantea* has a water collector tank that contributes for its energy supply, as the water is favorable to organic matter decomposition (of leaves, flowers, seeds, small animal), and the microorganisms that live in the tank and on the leaf surfaces can supply the plant with many essential substances, such as phytohormones and proteins (Lindow & Brandl 2003).

Few studies have identified PGPB or other bacteria associated to bromeliad species (Tapia-Hernandez et al. 2000) and to the best of

our knowledge, none study has described or selected PGPB or other bacteria associated to these two species of bromeliads. For this reason, the aims of the present investigation were to isolate and characterize the phyllosphere, endophytic, and water tank bacteria associated with individuals of *V. gigantea* and *T. aeranthos* collected in southern Brazil. Those two species were chosen due to the morphological differences in the water uptake method: a water tank in *V. gigantea* and trichomes in *T. aeranthos*.

Material and Methods

1. Collection site

Bacterial community of *V. gigantea* and *T. aeranthos* was sampled in a native forest located in Viamão, Rio Grande do Sul, Brazil (30° 04' 52" S and 51° 01' 24" W). The region climate is classified by Köppen as Cfa, humid subtropical without dry season, with a raining fall about 1,300 mm per year (Mota 1951). A specimen of *V. gigantea* was cultivated in pots at the Campus do Vale gardens, Universidade Federal do Rio Grande do Sul, Brazil (UFRGS), Porto Alegre, Rio Grande do Sul, (30° 04' 40" S and 51° 09' 00" W). Bacterial isolates were named as follow: *Vriesea* – Campus do Vale (VC), *Vriesea* – Viamão (VV) and *Tillandsia* – Viamão (TV).

2. Bacteria isolation and reference strains

Bacterial isolates associated with the sampled bromeliad were isolated from three different habitats: phyllosphere (p), water tank (w) and endophytes (e). Since *Tillandsia* lacks a water tank, bacteria were isolated only from the phyllosphere (p) and endophytically (e).

To isolate phyllosphere microorganisms, leaves were extracted from the plants and washed with 3 mL 0.85% NaCl saline solution. After that, 100 µL of each serial dilutions (10^{-1} to 10^{-4}) were carried out and were inoculated into 15 mL tubes containing 3mL NFB (Baldani & Dobereiner 1980), a selective nitrogen-free medium for Gram-negative nitrogen fixing bacteria, or Thiamine-Biotin (TB) medium (Seldin et al. 1983) used to isolate Gram-positive nitrogen fixing bacilli. Endophytic microorganisms were isolated from leaves surface sterilized for 5 min with 70% ethanol and cut in 10×10mm squares. These pieces were kept in constant agitation in 100mL flasks containing 50mL of sterile water for 30 min at 28 °C. Three milliliters of tank water was collected using a sterile pipette and diluted.

Serial dilutions were plated on NFB or TB agar without nitrogen. After incubation at 28 °C for 48 h in NFB, bacteria were purified through repetitive streaking (Vincent 1970). To isolate Gram-positive bacteria, suspensions were kept at 80 °C for 10 minutes and after that inoculated in plates containing the TB medium at room temperature for 7 days in anaerobiosis. All isolates were stored at -20 °C in 25% glycerol-TB broth for further analysis.

3. Indol-3-acetic acid production

The production of indole-3-acetic acid (IAA) by the isolates was evaluated according to Asghar et al. (2002). Briefly, bacterial strains were grown in King B broth (King et al. 1954) supplemented with 0.05 mg mL⁻¹ of tryptophan. After 48 h, bacterial cultures were centrifuged at 10,000 rpm for 5 min and 60 µL of their supernatants were placed into micro plates to react with 40 µL of Salkowski reagent (2 mL 0.5 M FeCl₃ + 98 mL 35% HClO₄) for 30 min. The mixture was left in the dark for 30 min at room temperature. The visualization of a red color in the mixture was considered a positive result (Asghar et al. 2002).

4. Siderophore production

The capacity of production of siderophore was tested in the isolates using liquid King B medium (King et al. 1954) supplemented

with a complex chromazul S [CAS/iron(III)/hexadecyl-trimethyl ammonium bromide], as described by Schwyn & Neilands (1987). One single drop of bacterium culture grown in King B broth for 48 h at 28 °C was then deposited in tryptone soy agar (TSA) plates and incubated for seven days at 28 °C. Bacteria that were able to produce siderophore grew and formed a yellow halo in the blue-green medium. It was recorded as siderophore production (+) or no siderophore production (–) in relation to the controls.

5. Phosphate solubilization

The method described by Sylvester-Bradley et al. (1982) was used to identify isolates able to solubilize phosphate. Bacteria were grown in glucose-yeast (GY) broth, containing 10 g of glucose, 2 g of yeast extract, and 15 g of agar per liter. Two other solutions were prepared separately, one containing 5 g of K_2HPO_4 in 50 mL of distilled water, and the other containing 10 g of $CaCl_2$ in 100 mL of distilled water. These solutions were added to one liter of GY medium just before pouring into Petri dishes, forming insoluble calcium phosphate that made the medium opaque. Bacterial isolates previously grown in NFb broth were dropped (10 µL per culture) into the GY plates and incubated for seven days at 28 °C. Those isolates that formed visible clearing halos around their colonies were considered phosphate solubilizers.

6. *nifH* gene amplification

Bacterial cells were grown in LB medium (Sambrook & Russel 2001) or TB broth at 28 °C for 48h at 128 rpm. Total DNA was extracted from pure cultures as described by Giongo et al. (2007). Selected primers PolF and PolR (Poly et al. 2001) were used to amplify a 360-bp region of *nifH* as described previously (Giongo et al. 2007).

7. rep-PCR

Rep-PCR reactions were carried out using enterobacterial repetitive intergenic consensus primers ERIC1-R and ERIC-2 (De Bruijn 1992) and enterobacterial repetitive sequences (BOX A1) primer (Versalovic et al. 1994). The reactions were performed in a 25- µL volume, containing 50 ng of DNA template, as described by Giongo et al. (2008). Fragments were visualized after electrophoresis on 1% agarose gel. All rep-PCR fingerprint patterns were converted into a two-dimensional binary matrix and analyzed by the Jaccard (*J*) coefficient. The matrix was analyzed by PAST—Paleontological Statistics Software Package for Education and Data Analysis (Hammer et al. 2001) and a dendrogram was built using the UPGMA algorithm (Nei 1987). To obtain a more detailed cluster analysis, the data of ERIC and BOX were combined.

Shannon diversity index (*H*) (Shannon & Weaver 1949) was estimated based on the number of isolates belonging to each group of profiles in rep-PCR dendrogram, considering a 70% of similarity in the cluster analysis (Alberton et al. 2006).

Besides the 68 isolates, four bacterial strains (*Bacillus* sp., *Burkholderia* sp., *Klebsiella* sp. and *Pseudomonas* sp.) available in the laboratory were submitted to the genotypic characterization.

Results and Discussion

The composition and PGBP traits of 68 bacterial strains isolated from three different microhabitats of *V. gigantea* and *T. aeranthos* bromeliad plants were evaluated. Twenty-three bacterial strains were isolated from *T. aeranthos*. Of these, 10 were isolated from the plant phyllosphere and 13 were endophyte bacteria. From *V. gigantea*, 45 bacterial strains were isolated, considering all samples (Table 1).

Gram-positive bacteria predominated in all the microhabitats studied. Most of them were spore-forming bacilli. The spore forming ability of these bacteria can increase the ability to survive in a limited environment added to the fact that bacilli are able to degrade complex biopolymers (Lindow & Brandl 2003). The predominance of Gram-positive isolates in the rhizosphere of several plants has been reported (Lucas-García et al. 2001).

In addition to Gram-positive bacilli, various microorganisms live around the plant tissue due to the rich nutrient availability, especially N and P (Glick 2003, Han et al. 2005). Although bacteria like *Rhizobium*, *Azotobacter* and *Azospirillum* enhance plant growth as a result of their ability to fix nitrogen, bacteria with mechanisms such as phytohormones production and other PGP activities can contribute to improve the ability of the host plant to live in extreme environments (Glick 1995, Bashan & Holguin 1998, Patten & Glick 1996, Asghar et al. 2002). All the isolates produced IAA *in vitro* in presence of very low amounts of tryptophan (Table 1). It will be important to explore the exact contribution of IAA production by those isolates in the bromeliad, although it is known that IAA alters root patterns and enhances nutrient absorption (Vargas et al. 2010).

Seventy-seven per cent of the isolates were able to produce siderophore in a medium provided with CAS (Table 1). Siderophore production may influence the plant growth by binding to the available form of Fe^{3+} . Through this process, iron is made unavailable to the phytopathogens in a process called induced systemic resistance (ISR), a plant-mediated, broad-spectrum resistance response activated by selected PGPB strains (Kloepper et al. 2004). At the same time, the siderophore protects the plant health (Siddiqui 2006). Press et al. (2001) reported that the catechol siderophore biosynthesis gene produced by *Serratia marcescens* is associated with resistance of cucumber against anthracnose.

Table 1. Morphological, physiological, and biochemical characteristics of the bacterial strains isolated from *Tillandsia* and *Vriesea* species in different microhabitats.

	<i>Tillandsia aeranthos</i>			<i>Vriesea gigantea</i>			
	phyllosphere (TV-p)	endophyte (TV-e)	water tank (VV-w)	phyllosphere (VV-p)	endophyte (VV-e)	water tank (VC-w)	phyllosphere (VC-p)
Gram-negative	3	4	3	2	3	6	3
Gram-positive	7(5)*	9(7)	3(3)	5(4)	5(2)	10(9)	5(4)
Siderophore production	10	8	4	7	5	13	6
IAA production	10	13	6	7	8	16	8
Phosphate solubilization	10	13	6	6	8	14	7
<i>nifH</i> gene	7	10	4	6	7	10	5
Total	10	13	6	7	8	16	8

*Parentheses represent number of Gram-positive spore-forming bacilli compared with the total Gram-positives.

More than 94% of the isolates showed the ability of phosphate solubilization (Table 1). This characteristic is particularly important in microhabitats where a low and irregular supply of nutrients demands an effective uptake mechanism (Winkler & Zotz 2009). The only four bacteria that lack this trait were isolated from the phyllosphere and the water tank of *Vriesea*.

The gene *nifH* is one of the most important genes in the biological nitrogen fixation system and encodes the nitrogenase reductase protein (Mortenson & Thornley 1979). Forty-nine bacteria (72%) presented *nifH* gene. Although the existence of the *nifH* gene does not necessarily represent effective nitrogenase activity, as this enzyme is regulated at both pre- and posttranslational levels (Dean & Jacobson 1992), its presence in the genome is evidence that the bacterium is a nitrogen-fixer. Analysis of the phyllosphere bacterial populations of *Tillandsia* species in mainland Mexico detected a single diazotrophic species, *Bacillus megaterium*, but only after a liquid enrichment of the entire leaf (Brighigna et al. 1992). Another study from Mexico identified *Pseudomonas stutzeri*, a nitrogen-fixing bacterium, isolated from the interior of the epiphyte *Tillandsia recurvata* (Puente & Bashan 1994). Pineapple plants were found to host *Acetobacter diazotrophicus*, isolated from inner tissues of surface sterilized roots, stems, and leaves of the bromeliad (Tapia-Hernandez et al. 2000). Most of the information about the nutrient uptake in epiphytic bromeliads is regarded as nitrogen uptake (Nyman et al. 1987, Endres & Mercier 2001, 2003). Inselsbacher et al. (2007) detailed uptake kinetics of various nitrogen compounds in the bromeliads tank of *V. gigantea*. In this case, NH_4^+ was found to be the most important N form for the N nutrition in this bromeliad.

The diversity among all the strains studied is shown in Figure 1. According to the dendrogram, two large groups (I and II) were observed with a similarity of 30%. Most of the isolates from water tank were clustered in Group I, where the strain *Bacillus* was also allocated. Group II was formed by 36 isolates mostly isolated from phylloplane and endophytic microorganisms clustered with the strains *Burkholderia*, *Klebsiella* and *Pseudomonas*. The vast number of rep-PCR profiles obtained suggested a high level of genetic diversity within populations. Although the isolates formed groups predominantly from the microhabitat they were isolated from, it was not possible to distinguish them based on the bromeliad they were obtained. Rep-PCR method has become a simple method to distinguish strains and to study their diversity in a variety of ecosystems, and it might be necessary to evaluate a larger number of bromeliad species to observe differences in the microbial genetic community living on them.

The Shannon diversity index was used to assess the diversity in the different microhabitats (Figure 2). Water tanks presented the most abundant diversity, followed by the phyllosphere and then by the endophytes. The water trapped in the tank has a high nutrient concentration, which promotes an ideal environment for complex microbial communities (Benzing et al. 1972). Although leaves have been considered a hostile environment to bacteria due the limitation of water and nutrient availability, and exposition to UV radiation (Lindow & Brandl 2003), 25 different bacteria were isolated from this microhabitat. Oppositely, a study about the carnivorous plant *Drosera villosa* suggested that the diversity was higher in bacteria isolated from inside (endophytes) than from outside the plants (phylloplane) (Albino et al. 2006). They suggest that the hostile environment in the phylloplane limits bacterial growth.

There are few studies on the microbial diversity in Southern Brazilian bromeliaceous plants (Ambrosini et al. 2007), many of them describing yeast species (Landell et al. 2006, 2009, 2010, Mautone et al. 2010). Nevertheless, the present study emphasizes the high level of genetic diversity in bacterial populations in the

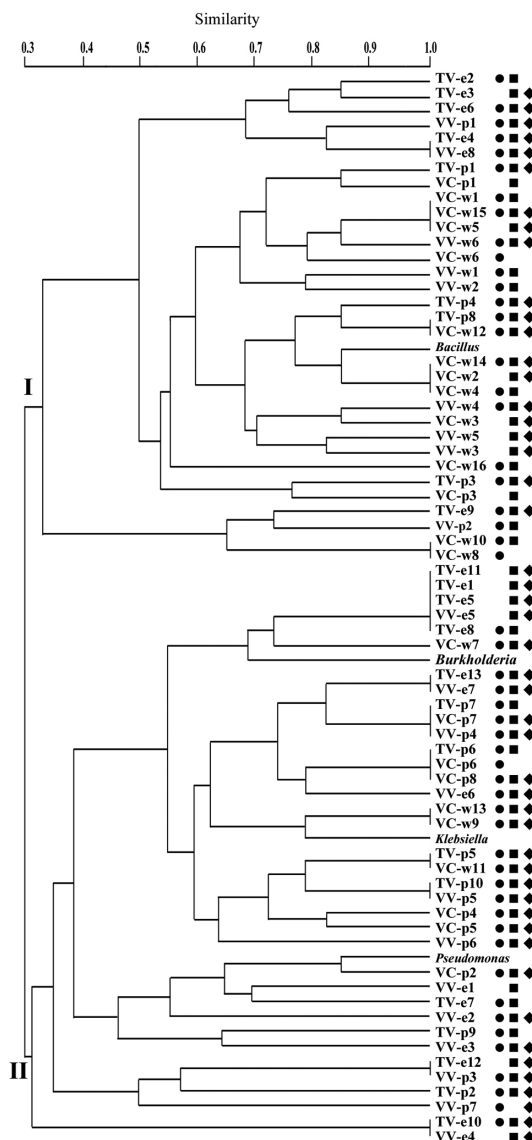


Figure 1. Dendrogram based on UPGMA cluster analysis using the rep-PCR data obtained from 68 isolates plus four known bacterial strains. Circle represents siderophore production, square represents phosphate solubilization and triangle represents the presence of *nifH* gene. Bacterial isolates were named as *Vriesea* – Campus do Vale (VC), *Vriesea* – Viamão (VV) and *Tillandsia* – Viamão (TV). Different habitats: phyllosphere (p), water tank (w) and endophytes (e).

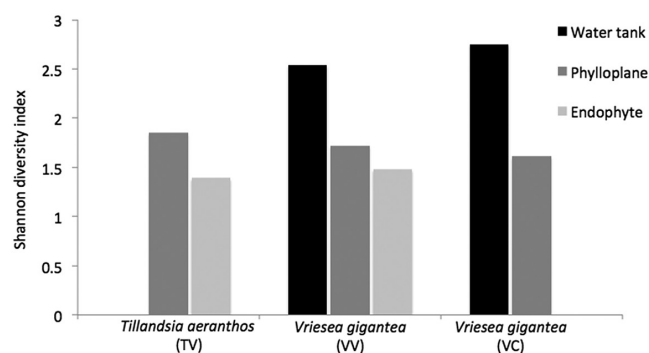


Figure 2. Shannon bacterial diversity index for the different microhabitats in *Tillandsia* and *Vriesea*. Bacterial isolates were named as *Vriesea* – Campus do Vale (VC), *Vriesea* – Viamão (VV) and *Tillandsia* – Viamão (TV).

studied bromeliads, corroborating the high level of diversity in morphology, physiology, and genetic properties. The understanding of the diversity of microorganisms inhabiting the phylloplane and the tanks of bromeliad species has both ecological and economic importance since this information could be useful in the management and conservation of the bromeliads themselves.

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