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ORIGINAL PAPER

High diversity of diazotrophic bacteria associated with the carnivorous plant *Drosera villosa* var. *villosa* growing in oligotrophic habitats in Brazil

U. Albino · D. P. Saridakis · M. C. Ferreira · M. Hungria · P. Vinuesa · G. Andrade

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Abstract *Drosera villosa* var. *villosa* A. St.-Hil is a carnivorous plant that grows in Brazilian flooded soils very poor on nutrients, including low levels of N. Under these conditions, the plant shows vigorous growth, low root number, low number of captured prey (less than 50%) and a great assemblage of bacteria associated with the roots and leaves that grow in N-free medium. These preliminary results have led us to investigate the number of colony forming units (log CFU) in the roots (rhizosphere and endorhizosphere) and leaves (phyllosphere and endophyllosphere) of *D. villosa* var. *villosa* by the tenfold serial dilution technique in two N-free culture media. The results showed that the phyllosphere had 6.65 log CFU g dry leaf^{-1} and the rhizosphere 6.47 log CFU g dry soil^{-1}, with the lowest value detected in the endophyllosphere (4.39 log CFU g dry leaf^{-1}). Sixty-three different bacteria morphotypes were isolated from the surface and interior of the roots and leaves and the amplification of the DNA with specific primers detected the *nifH* gene in 34 of those strains. The DNAs of the 34 strains were compared by the BOX-PCR technique and a great diversity was observed, with the bacteria clustering at a final level of similarity of only 12%. The strains were also submitted to the partial sequencing of the 16S rRNA gene and several genera of N_2-fixing bacteria were detected, including *Bacillus*, *Burkholderia*, *Methylobacterium*, *Paenibacillus*, *Pseudomonas* and *Sphingomonas.*

Keywords Biological nitrogen fixation · *nifH* · 16S rRNA · *Drosera villosa* var. *villosa*

U. Albino · D. P. Saridakis · G. Andrade (✉)
CCB, Depto. Microbiologia, Lab. Ecologia Microbiana, Universidade Estadual de Londrina, CP 6001, Londrina, PR 86051-990, Brazil
e-mail: andradeg@uel.br

M. C. Ferreira · M. Hungria
Embrapa Soja, Londrina, PR, Brazil

P. Vinuesa
Nitrogen Fixation Research Center, Universidad Autónoma de Mexico, Mexico DF, Mexico

Introduction

The challenge at the start of the new millennium is to optimize sustainable agricultural practices by finding techniques that improve soil and food quality, and biological N_2 fixation (BNF) fits into this purpose. The high cost of synthetic N-fertilizers limits food production in poor countries, however, many plants, especially legumes may benefit from the BNF process. However, it is very important to detect new free-living N_2-fixing bacteria with potential for use as inoculants in non-legume plants. Several genera of N_2-fixing

bacteria have been shown to be associated with both mono (Barraquio et al. 1997; Chaintreuil et al. 2000; Gyaneshwar et al. 2001; Cruz et al. 2001) and dicotyledoneous plants (Gough et al. 1997; O'Callaghan et al. 2000; Estrada-De Los Santos et al. 2001).

The *Drosera villosa* var. *villosa* is a carnivorous dicotyledoneous plant inhabiting flooded acid areas showing low levels of nutrients as P, S and especially poor on N (Adamec 1997). Although the captured insects contribute to the plant nutrition (Perica and Berljak 1996), survival under N-limiting conditions suggests the association with diazotrophic bacteria. Molecular techniques such as the use of repetitive sequences of the DNA, the detection of *nif* genes and sequencing of the 16S rRNA have allowed major advances in the identification of diazotrophic bacteria (Caballero-Mellado et al. 1999). In this context, we have started to investigate the association of *D. villosa* with diazotrophic bacteria. Bacteria associated with the plant were isolated in N-free culture media, following the genetic characterization of the putative diazotrophs. As *D. villosa* var. *villosa* grows under low fertile conditions, bacteria associated with the host plant may have developed adapted strategies to compete with other microorganisms and might be potentially useful as inoculants under similar conditions, as for recovering riparian woods in degraded tropical soils.

Materials and methods

Experimental design

The plants were collected in the county of Itararé, State of São Paulo (24°05′00″S and 49°11′57″W), and Jaguariaíva, State of Paraná (24°23′23″S and 49°51′21″W), Brazil. Five plants were randomly chosen from three different areas in each county. The bacteria assemblage that grew in two N-free media of soil samples adhering to the root surface were considered as rhizosphere (R), from the root cortex as the endorhizosphere (ER), from the leaf surface as phyllosphere (P) and from the phylloderm as endophyllosphere (EP). A total of 60 samples from roots and 60 from leaves were analyzed.

Isolation of free-living N_2-fixing strains

The collected plants were placed in plastic pots and kept at 5°C until sample processing. The roots used for endorhizosphere (ER) sampling were washed, surface disinfected (700 ml l^{-1} ethanol for 1 min, washed three times in sterile distilled water), dried with sterile absorbent paper and crushed throughout in saline solution (8.5 g l^{-1} NaCl). After growth, the colonies were compared with those from soil samples. To obtain the phyllosphere (P) samples, the leaves were immersed in sterile saline solution and agitated gently in vortex for 1 min. After washing the surface, the leaves were surface sterilized by immersion in 700 ml l^{-1} ethanol for 1 min, washed three times in sterile distilled water, dried, crushed in sterile saline solution and the suspension was considered as the EP.

One gram of each sample was suspended in 9 ml of sterile saline solution and the tenfold serial dilution was performed for each suspension obtained with the soil, roots and leaves. One hundred-microliter aliquots of the 10^{-4}–10^{-7} dilutions were inoculated in two N-free culture media: Burk's (Wilson and Knight 1952), that uses glucose as the C source, and Nfb medium, that uses malate as the C source (Döbereiner and Day 1976). The plates were inoculated in duplicate, and incubated at 28°C for 7 days under low O_2 conditions (wrapped in plastic for less O_2 diffusion). The number of colonies was assessed on the third and seventh days of incubation (Andrade et al. 1997a).

The colonies grown in the two N-free media were counted, isolated and grouped according to the morphological similarities of the cells and colonies (Andrade et al. 1997b). The log CFU g $sample^{-1}$ was submitted to Tukey's test at 5% of significancy.

The isolated strains were successively replicated in N-free medium (ten times) to ensure good growth conditions, and afterwards cryogenized in sterile glycerol (20%) and stored at –80°C in deep freezer and in liquid nitrogen.

Molecular characterization

Sixty-three strains were grown in liquid YM medium, and after 2 days DNA was extracted as described before (Kaschuk et al. 2006) and amplified by PCR reaction in a thermocycler (MJ Research Inc., Watertown, MA, USA, PTC-100) using the *nifH*-19f (forward) and *nifH*-407R (reverse) primers (Ueda et al. 1995). The PCR products were submitted to horizontal electrophoresis in 15 g l^{-1} agarose gel and visualized and photographed under UV light after staining with ethydium bromide (0.05 μg ml^{-1}).

To detect diversity at the strain level, the strains that amplified with the *nifH* primer were also analyzed by the BOXA1R-PCR technique, as described before (Kaschuk et al. 2006). The same strains were also submitted to the sequencing analysis of the 16S rRNA gene, as described before (Chen et al. 2000). The sequences obtained were deposited in the GenBank database (www.ncbi.nlm.nih.gov/BLAST) where they received the accession numbers AY117575 to AY117608.

The phylogenetic position of each strain was inferred by the comparison with the following type strains (accession number of the GenBank in parenthesis): *Pseudomonas fragi* (AF094733/ATCC 4973^T); *Sphingomonas* sp. (AJ001053/C28242^T); *Methylobacterium organophillum* (AJ276806/JCM2833^T); *Burkholderia pseudomallei* (U91839/1026b^T); *Burkholderia mallei* (AF110188/ATCC23344^T); *Burkholderia tropicalis* (AF164045/Ppe8^T); *Burkholderia cepacia* (AF097530/ATCC25416^T); *Burkholderia glathei* (AB021374/ATCC 29195^T); *Bacillus* sp. (AF411118/Bch 1^T); *Paenibacillus chinjuensis* (WN9^T); *Paenibacillus borealis* (AJ011323/KK19^T).

Cluster analysis of the BOX-PCR products was performed using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium) with the UPGMA algorithm (unweighted pair-group method with arithmetic mean) and the coefficient of Jaccard. The 16S rRNA sequences obtained with the strains from this study and those of the reference strains were also analyzed using the Bionumerics program and the UPGMA algorithm.

Results

Nutrient supply by hunting (insects) in the field was relatively low, since less than 50% of the plants had insects captured in their leaf-traps in the summer (data not shown); however, plant vigor was high.

Bacterial populations, evaluated by the log CFU g $sample^{-1}$ were similar in both C sources tested for growth (Fig. 1). The population in the endophyllosphere (EP) was statistically lower than from the other parts of the plant (Tukey's test, 5%), in both MCS (Nfb medium, with malate as C source) and GCS (Burk's medium, with glucose as C source). Log CFU g $sample^{-1}$ was lower inside the roots in both MCS as GCS medium, although differences were not statistically significant. Similar results were obtained for the leaves, and log CFU g $leaf^{-1}$ at the surface (P) was significantly higher than inside (EP) the leaves.

Isolates were classified in 63 morphotypes, and the DNA of 34 of them amplified with *nifH* primer; 56% had been isolated from ER, 20% from R, 18% from EP, and only 6% from P (Table 1). Regarding the C source used, 59% of the 34 strains were obtained from the MCS and 41% from the GCS medium (Table 1). The morphological analysis of the 34 strains demonstrated that 73.5% were Gram-negative and 37.5% Gram-positive (Table 1).

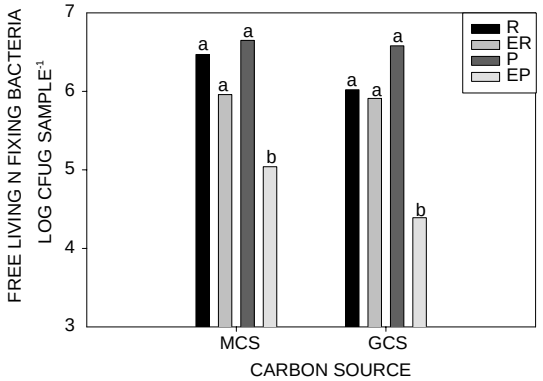

Fig. 1 Log CFU number obtained on four plant parts [rhizosphere (R), endorhizosphere (ER), phyllosphere (P) and endophyllosphere (*EP*)]. Two C sources were used for the isolation of bacteria, malate (MCS) and glucose (GCS). *Different letters* indicate statistical difference at $P \leq 0.05$ (Tukey's test) for each C source

Table 1 General properties of bacterial strains isolated from roots [rhizosphere (R) or endorhizosphere (ER)], and from leaves [phyllosphere (P) or endophyllosphere (EP)] of the carnivorous plant *Drosera villosa* var. villosa. Medium culture contained either malate (MCS) or glucose (GCS) as carbon source

Strain[a]	Genus[b]	General properties			
		Gram	Niche	MCS	GCS
LEM 18	*Methylobacterium*	–	R	–	+
LEM 19	*Paenibacillus*	+	R	–	+
LEM 21	*Bacillus*	+	ER	–	+
LEM 22	*Bacillus*	+	R	–	+
LEM 24	*Burkholderia*	–	ER	–	+
LEM 25	*Burkholderia*	–	ER	–	+
LEM 26	*Burkholderia*	–	ER	–	+
LEM 27	*Sphingomonas*	–	ER	–	+
LEM 40	*Paenibacillus*	+	ER	–	+
LEM 41	*Bacillus*	+	ER	–	+
LEM 42	*Paenibacillus*	+	ER	–	+
LEM 57	*Burkholderia*	–	EP	–	+
LEM 58	*Sphingomonas*	–	EP	–	+
LEM 59	*Sphingomonas*	–	EP	–	+
LEM 2	*Burkholderia*	–	R	+	–
LEM 3	*Burkholderia*	–	R	+	–
LEM 4	*Burkholderia*	–	ER	+	–
LEM 5	*Methylobacterium*	–	R	+	–
LEM 6	*Burkholderia*	–	ER	+	–
LEM 7	*Burkholderia*	–	ER	+	–
LEM 9	*Burkholderia*	–	ER	+	–
LEM 10	*Burkholderia*	–	ER	+	–
LEM 11	*Burkholderia*	–	ER	+	–
LEM 13	*Burkholderia*	–	ER	+	–
LEM 14	*Pseudomonas*	–	R	+	–
LEM 15	*Sphingomonas*	–	ER	+	–
LEM 28	*Bacillus*	+	ER	+	–
LEM 29	*Paenibacillus*	+	ER	+	–
LEM 37	*Bacillus*	+	ER	+	–
LEM 48	*Pseudomonas*	–	P	+	–
LEM 52	*Burkholderia*	–	P	+	–
LEM 64	*Burkholderia*	–	EP	+	–
LEM 66	*Pseudomonas*	–	EP	+	–
LEM 67	*Pseudomonas*	–	EP	+	–

[a] Based on morphotypes

[b] Based on the partial sequencing of the 16S rRNA gene

The electrophoretic profiles of the DNA of the 34 strains obtained with the amplification with the BOXA1R-PCR primer originated a dendrogram (Fig. 2), with six main groups joining at a very low level of similarity (12%), suggesting a great genetic diversity among the bacteria. The alignment of the partial 16S rRNA sequences (257–319 bp) of the strains from this study with those of reference strains deposited in the GenBank also resulted in a dendrogram (Fig. 3), and confirmed that the great number of both morphotypes and of BOX-PCR profiles were associated to several species of diazotrophic bacteria.

Information about the sequencing analysis (Table 1 and Fig. 3) was used for the description of BOX-PCR results (Fig. 2). In the BOX-PCR analysis, group I included four bacteria, strain 6, identified by sequencing of the 16S rRNA gene as belonging to the *Burkholderia* genus, strain 59, belonging to the genus *Sphingomonas* and strains 66 and 67, of the *Pseudomonas* genus. While the profile of strain 6 was 34% similar to that of the strain 59, strains 66 and 67 presented 80% similarity.

The second BOX-PCR group was the most diverse and included five genera, *Bacillus*, *Methylobacterium*, *Sphingomonas*, *Pseudomonas* and *Paenibacillus*. The diversity of genera was confirmed by the low level of similarity among the electrophoretic profiles of the strains in this

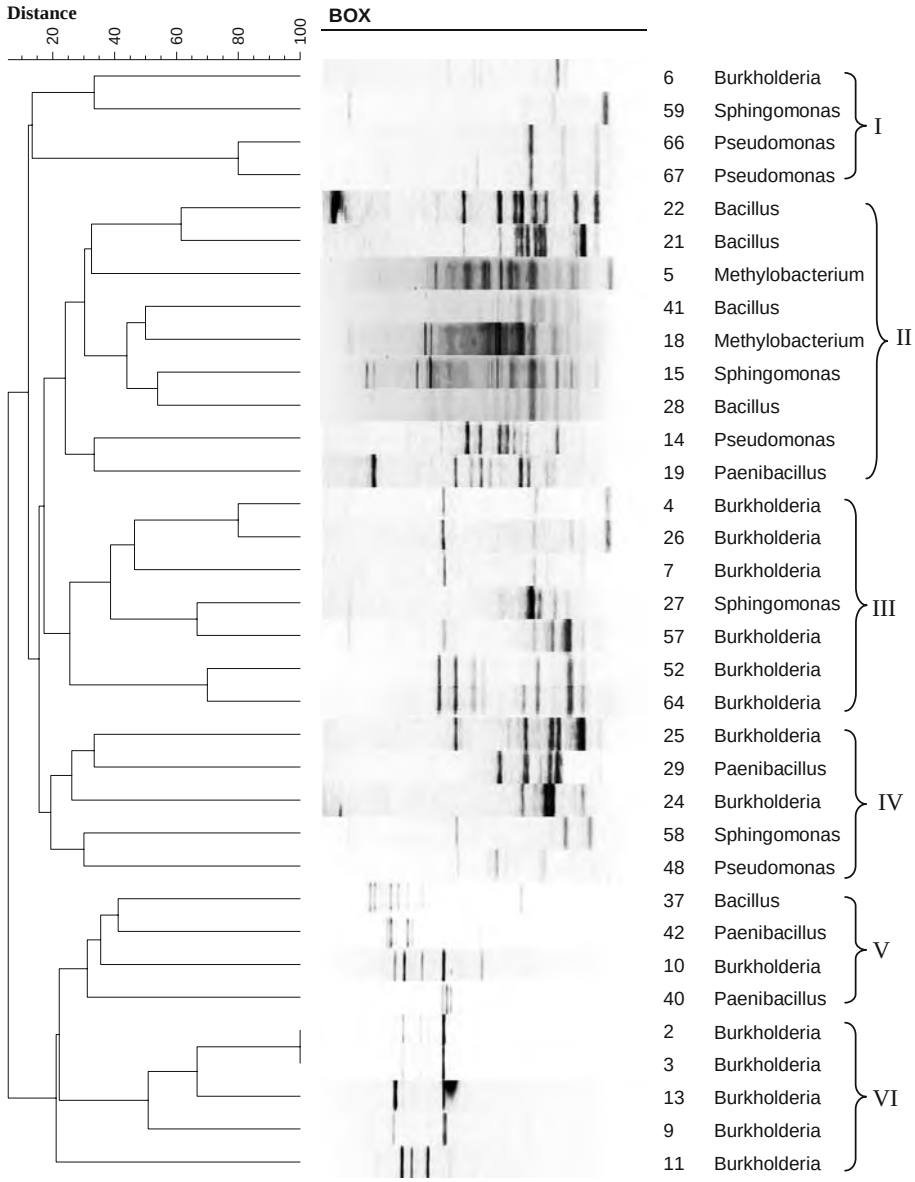

Fig. 2 Dendrogram obtained with the BOX-PCR profiles of 34 diazotrophic bacteria isolated from the carnivorous plant *Drosera villosa* var. *villosa*. Analysis performed using the UPGMA algorithm and the coefficient of Jaccard

group. The two strains with the greatest level of similarity (62%) were 21 and 22, both identified as *Bacillus* by the sequencing analysis (Figs. 2, 3).

The third group included seven different strains, six belonging to the *Burkholderia* and one to the *Sphingomonas* genera. The greatest similarity (80%) was found between two *Burkholderia* strains, 4 and 26.

Group IV was composed by five bacteria, belonging to the *Burkholderia*, *Paenibacillus*, *Sphingomonas* and *Pseudomonas* genera, joined at a level of similarity of only 20%. The fifth group with four bacteria belonging to the *Burkholderia*, *Bacillus* and *Paenibacillus* genera joined to the sixth group, formed only by *Burkholderia*, at a level of similarity of 24% and strain 11, identified as *Burkholderia*, joined the groups V and VI at a very low level of similarity, indicating a considerate variability in relation to the other strains of *Burkholderia*.

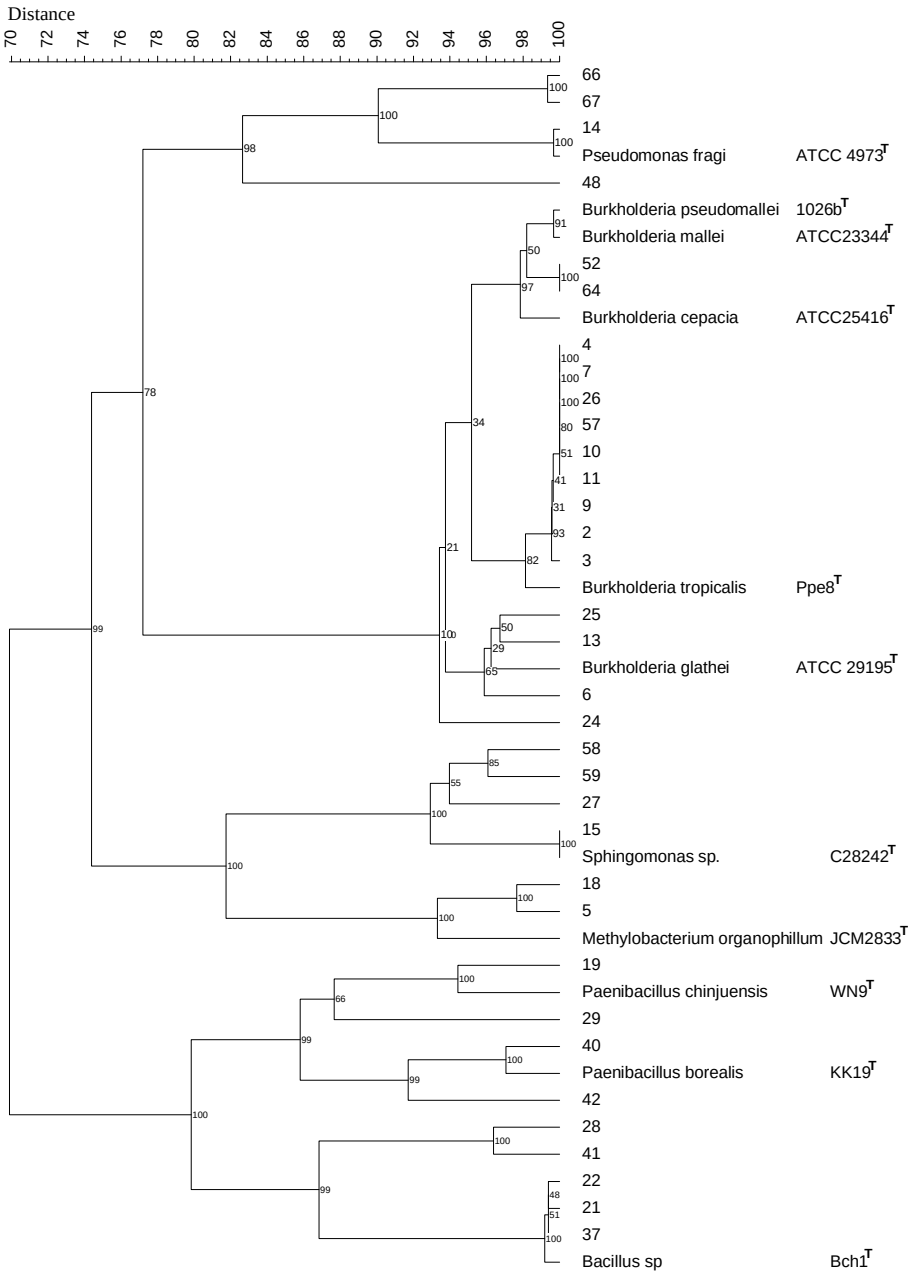

Fig. 3 Phylogenetic analysis of partial 16S rRNA sequences of 34 diazotrophic bacteria isolated from the carnivorous plant *Drosera villosa* var. *villosa* and of type strains of several species used as reference. GeneBank accession numbers are listed in the Sect. ''Materials and methods.'' Cluster analysis using the UPGMA algorithm and the *numbers* in the main branches indicate bootstrap values obtained with 100 replicates

Bacteria were also grouped in six different clusters in the dendrogram built with the 16S rRNA sequences. In the first group, the pair of strains 66 and 67 and of strain 14 with *P. fragi* ATCC 4973^T have shown high similarity of bases (99%), but strain 48 was quite dissimilar from the others; they were all joined at a final level of similarity of 82.5%.

The second and largest group clustered strains of *Burkholderia* and included 44% of the bacteria studied, while the third group included four strains with *Sphingomonas* C28242^T. This group

joined at 82% of similarity the fourth group, in which the strains 5 and 18 showed similarity with *M. organophillum*.

The fifth group was composed by the strains 19 and 29, which were joined to *Paenibacillus chijuensis* WN9^T, and by strains 40 and 42, clustered with *P. borealis* KK19^T. Finally, this sixth group clustered with *Bacillus* sp. strain Bch1^T and with five strains from this study, with 80% of similarity (Figs. 2, 3).

Discussion

Bacterial populations were higher outside (phyllosphere and roots) than inside (endorhizosphere and endophyllosphere) the plants. However, diversity was higher in bacteria isolated from inside than from outside the plants. It is possible that the hostile environment outside the plants limits bacterial growth. Few microorganisms would be adapted to the acidic and nutrient-limited conditions, establishing a low level of competitiveness, therefore few adapted individuals would prevail and show enhanced growth. On the other hand, the more favorable conditions inside the plant would result in greater diversity, but the higher competition among these microorganisms would result in a lower number of individuals of each established strain.

Sixty-three different morphotypes were observed among the strains isolated from *D. villosa* var. *villosa*. The DNA of 34 of those strains amplified with the *nifH* primer, an indicative of their capacity of fixing N_2. The putative diazotrophic bacteria were also characterized by a high level of genetic diversity, based on both the BOX-PCR profiles and the 16S rRNA sequencing analyses.

In general the clusters observed in the BOX-PCR analysis did not reflect the groups determined by the sequencing of the 16S rRNA genes. Some agreement in BOX-PCR and 16S rRNA clusterings was observed only with strains classified in the genera *Sphingomonas* and *Burkholderia*. However, strains from both genera were positioned in more than one cluster, often showing very low levels of similarity among them. Clustering in BOX-PCR seemed more related with the plant part, as the bacteria isolated from endophyllosphere and phyllosphere were restricted to the lower part of clusters I, III and IV.

The great majority of the strains (44%) showed higher similarity of the 16S rRNA genes with reference strains belonging to the genus *Burkholderia*. This genus is quite diverse and includes many species, with a variety of physiological roles. There are *Burkholderia* that may act as human pathogens (Chua et al. 2003), others that are classified as plant growth-promoting bacteria (Bending et al. 2002), and also N_2-fixing bacteria (Minerdi et al. 2001). Bacteria belonging to this genus have also a wide geographic distribution and are associated with several plant species (Estrada-De Los Santos et al. 2001). In Brazilian soils, there are descriptions of *Burkholderia* associated with cassava (*Manihot esculenta*) (Balota et al. 1999), banana (*Musa* spp.) and pineapple (*Ananas comosus*) (Cruz et al. 2001), coconut palms (*Cocos nucifera*) (Fernandes et al. 2001) and sugarcane (*Sacarum oficinalis*) (Boddey et al. 2003); furthermore, very effective symbioses of *Burkholderia* with Brazilian leguminous species have also been described (Menna et al. 2006). *B. cepacia*, used for comparison in this study, was reported as a growth promoter of common bean (*Phaseolus vulgaris*) when associated with rizobia, due to an improvement in the nodulation (Peix et al. 2001).

Two strains from this study have shown similarity of bases of the 16S rRNA with *M. organophillum*, a genus known for fixing N_2 with monocotyledons (Elbeltagy et al. 2000) and also in symbiosis with legumes (Sy et al. 2001; Menna et al. 2006). Therefore diazotrophic bacteria belonging to this species seem to benefit a wide-range of host plants and should be further investigated.

The presence of *Pseudomonas* and *Sphingomonas* in the rhizoplanes is well known, with description that the plants may benefit from the association with both species by accelerating root growth and inhibiting some soil pathogens (Adhikari et al. 2001). In addition, this study has shown that bacteria belonging to both genera may also bring benefits to plant growth by fixing N_2.

Two putative diazotrophic strains have shown higher similarity with *Paenibacillus chijuensis*

WN9^T, while two others clustered with *P. borealis* KK19^T (Yoon et al. 2002). The N_2-fixing characteristic of this genus in rhizoplanes has also been described (Rosado et al. 1998).

Finally, five strains were classified as *Bacillus*, confirming that this genus includes N_2-fixing bacteria (Achouak et al. 1999). Diazotrophic bacteria belonging to the *Bacillus* and *Pseudomonas* genera were isolated in the rizosphere of the arboreal species Scots pine (*Pinus sylvestris* L.) and common oak (*Quercus robur* L.) (Różycki et al. 1999).

The diversity of bacteria in association with *D. villosa* var. *villosa* described in this study may be related to some of the strategies that this plant uses to survive in localities very poor on nutrients. However, the dependence that the plant has on these diazotrophic bacteria needs to be assessed, and quantification experiments are in progress (Albino 2004).

The bacteria associated with *D. villosa* var. *villosa* roots and leaves are submitted to a strong selection pressure, both because the plant is not a legume and also because it grows in a nutrient-poor habitat where very few plants survive. Free-living N_2-fixing bacteria adapted to these stress conditions may show a great potential for the use as inoculants, in crops as irrigated rice (*Oryza sativa* L.) or in programs of recovery of degraded areas that suffer from periodic flooding such as river banks.

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