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REGULAR ARTICLE

Genetic diversity and plant growth promoting traits of diazotrophic bacteria isolated from two *Pennisetum purpureum* Schum. genotypes grown in the field

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Abstract
Background and aims Some elephant grass (*Pennisetum purpureum*) genotypes are able to produce large amounts of biomass and accumulate N derived from BNF when growing in soil with low N levels. However, information about the diazotrophic bacteria colonizing this C4 plant is still very scarce. This study aimed to characterize the plant growth promoting traits of a fraction of culturable diazotrophs colonizing the genotypes CNPGL F06-3 and Cameroon.
Methods A total of 204 isolates were obtained from surface sterilized leaves, stems and roots after culturing on five different N-free semisolid media. These were then analyzed by BOX-PCR, and the 16S rRNA and *nif*H sequences of representative isolates were obtained. The functional ability of the isolates to reduce acetylene, produce indole and to solubilize phosphate was also determined.
Results The diazotrophic bacterial population varied from 10^2 up to 10^6 bacteria g^{-1} fresh tissues of both genotypes. The BOX-PCR analysis suggested a trend in the genetic diversity among the 204 diazotrophic strains colonizing the different genotypes and plant tissues. Sequencing of 16S rRNA fragments confirmed the presence of *Azospirillum brasilense* and *Gluconacetobacter diazotrophicus* and revealed for the first time the occurrence of *G. liquefaciens, G. sacchari*, *Burkholderia silvatlantica*, *Klebsiella sp., Enterobacter cloacae* and *E. oryzae* in elephant grass. Interestingly, several *nif*H sequences from isolates identified as *G. liquefaciens* and *G. sacchari* showed homologies with *nif*H sequences of *Enterobacter* species. The majority of the isolates (97%) produced indole compounds, 22% solubilized phosphate and 6.4% possessed both characteristics.

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Conclusions The results showed the occurrence of novel diazotrophic bacterial species colonizing different tissues of both genotypes of elephant grass. In addition, the study revealed the presence of several bacteria with growth promoting traits, and highlighted their potential to be exploited as biofertilizers.

Keywords Elephant grass · PGPB · BOX-PCR analysis · FBN · P solubilization · Indole production

Introduction

The fast-growing C4 grass *Pennisetum purpureum* Schum., commonly known as elephant grass, grows well in semi-humid or humid tropic regions (Morais et al. 2011). It is highly efficient at fixing atmospheric CO_2 and is capable of accumulating more than 60 Mg ha^{-1} of dry matter per year when grown under optimal conditions including high N fertilization and an abundant water supply (Andrade et al. 2005). Nevertheless, some genotypes are able to accumulate more than 30 Mg ha^{-1} dry matter after two cuts per year, when grown in soils with low available N (Morais et al. 2009). Various genotypes of elephant grass have been shown to accumulate from 50 to 130 kg of N ha^{-1} via Biological Nitrogen Fixation (BNF) (Morais et al. 2009, 2011), which reduces the need for fertilizer application to this crop, which is both economically and environmentally advantageous (Boddey 1995). Moreover, in addition to supplying N to this energy crop, BNF also helps contribute to its positive energy balance (21.1:1- energy output/input ratio) which is much higher than that observed for ethanol produced from switchgrass grown in temperate regions (Samson et al. 2005). However, given its apparent partial dependence on BNF, the variation in growth of various elephant grass genotypes under soil N-limiting conditions may be affected by populations of plant associated diazotrophs (Morais et al. 2009, 2011), and hence studying the cultivable diazotrophic bacterial population associated with this plant may lead to the development of an inoculant similar to that developed for sugarcane (Oliveira et al. 2002).

Only a few studies have examined the occurrence of culturable diazotrophic bacteria associated with elephant grass. The first one compared the populations of N-fixing bacteria during a growing season and found the highest numbers in roots (up 10^5 bacteria g^{-1} fresh weight) with lower numbers (undetectable to 10^4 bacteria g^{-1} fresh weight) in aerial tissues (Kirchhof et al. 1997a). These authors observed that the culturability of diazotrophic populations was dependent on the type of semi-solid media utilized to enumerate the bacteria. In a subsequent study, using molecular microbiological techniques, Kirchhof et al. (1997b) confirmed the presence of the same diazotrophic bacterial species colonizing the internal tissues of *P. purpureum* plants grown in the field in Brazil. Further studies carried out by Reis et al. (2001) detected from 10^3 to 10^7 (bacteria g^{-1} fresh weight) of diazotrophic bacteria (in roots and stems) and confirmed the capacity of different *Pennisetum* genotypes to obtain varying inputs from BNF. A similar survey carried out on *Miscanthus*, another energy crop, grown in Germany, showed the occurrence of two new species of diazotrophic bacteria: *Herbaspirillum frisingense* (Kirchhof et al. 2001) and *Azospirillum doebereinerae* (Eckert et al. 2001).

A genetical analysis performed on a few strains originating from *Pennisetum* and *Miscanthus* grown in Brazil and Germany showed a low genetical diversity amongst the isolates (Kirchhof et al. 2001; Eckert et al. 2001). These authors suggested that the highly selective conditions inside the grass species were the major factor regulating endophytic diazotrophic populations. However, it is also possible that the number of semisolid media (NFb, JNFb and LGI) used to isolate the aforementioned diazotrophs may have limited the isolation of a more diverse population of diazotrophs. Indeed, it is well known that these N-free semisolid media would have only allowed for the evaluation of a fraction of the culturable diazotrophic population colonizing any Poaceous species, such as elephant grass. In contrast, molecular strategies based upon PCR, cloning and sequencing of the *nif*H gene (Juraeva et al. 2006; Fischer et al. 2011) have provided a broader view of the relative abundance of both the culturable and unculturable diazotrophic community present in tissues of plants. Nevertheless, despite the potential bias introduced by the use of semisolid media they are still the easiest and most practical procedure for the isolation and selection of diazotrophic strains with potential for further application as biofertilizers (Döbereiner 1988).

In this study, we used five different nitrogen-free semisolid media to evaluate part of the culturable diazotrophic bacterial population associated with two

elephant grass genotypes. The BOX-PCR analysis was applied to determine the genetic diversity amongst the 204 isolates that were obtained from using these semi-solid media. Representatives from the main BOX-PCR groups were selected for further identification by sequencing their 16S rRNA and *nifH* genes. In addition, the ability of the isolates to solubilize P, to produce indoles and to fix N was measured in order to select strains with biofertilizer potential for future field inoculation experiments.

Materials and methods

Plant genotypes and field sampling

Two elephant grass (*P. purpureum*) genotypes, Cameroon and CNPGL F06-3, were chosen because of their high capacity to produce dry matter (62 and 59 Mg ha^{-1}, respectively) and accumulate high amounts of N derived from BNF (38% and 34%, respectively), during an 18-month growth period of cultivation on low N fertility soil (Morais et al. 2009). Samples were collected from leaf, stem and root tissues of both genotypes grown in a Typic Hapludult—Acrisol soil type (Itaguai series) at the Embrapa Agrobiologia Field Station, located in Seropédica (22°49′ 22″S and 43°38′42″W), RJ, Brazil. The soil was fertilized at planting, through application to furrows, at a rate of 132 kg ha^{-1} K (potassium chloride), 44 kg ha^{-1} P (single superphosphate) and 60 kg ha^{-1} micronutrients as FTE (fritted trace elements). No lime application was required. Hence, in theory, N was the limiting factor for plant development. The field experiment was established in October 2005 and was sampled in March 2009, 3 months after the sixth cut was made in December 2008. There were three replicates for each genotype.

Quantification of the culturable diazotrophic bacterial population

Diazotrophic microorganisms were isolated using the serial dilution technique by utilising five semi-selective N-free semisolid media viz., NFb, JNFb, JMV, LGI and LGI-P (Döbereiner 1995). These media have been used to isolate *Azospirillum lipoferum* or *A. brasilense; Herbaspirillum* spp; *Burkholderia* spp; *A. amazonense* and *Gluconacetobacter diazotrophicus*, respectively. Samples of roots were washed in tap water to remove excess soil and then rinsed three times with distilled water for 10 min each. Leaf samples were washed in tap water and surface disinfested with 70% ethanol, whilst the stems were surface disinfested with 70% ethanol before peeling off their epidermis. Ten grams of each sample were suspended in 90 mL of saline solution (0.8% NaCl) and macerated with a blender. Aliquots (100 μL) of each dilution (up to 10^{-8}) were inoculated into vials containing 5 mL of each N-free semisolid medium described above. The population size was estimated by the Most Probable Number (MPN) method; this method relies upon the appearance of a typical diazotrophic bacterial pellicle in the subsurface of the semisolid medium after incubation for 7–10 d at 30ºC. The characteristic pellicles of 3 vials with the highest dilution were transferred to fresh N-free semisolid medium (NFb, JNFb, JMV, LGI or LGI-P, as appropriate), and after confirmation of bacterial growth, a loopfull of the new pellicle was streaked onto the corresponding solid medium containing a trace amount of yeast extract (20 mg) to isolate the target bacterium based on the phenotypic characteristics of the colonies (Döbereiner 1995). A single purified colony was again checked in the same N-free semisolid medium before being stored for further analyses.

Diazotrophic isolates and reference strains

In addition to the isolates originating from the plant tissues, the following reference strains were used as positive controls for the physiological and molecular analysis: *Burkholderia tropica* (Ppe8), *B. silvatlantica* (SRMrh-20), *B. kururiensis* (KP23), *B. vietnamiensis* (TVV75), *Herbaspirillum seropedicae* (HRC54), *H. rubrisubalbicans* (HCC103), *Azospirillum brasilense* (Sp7), *A. amazonense* (CBAmC), *Gluconacetobacter diazotrophicus* (PAL5) and *Azotobacter chroococcum* (AC1) (only for phosphate solubilization). The genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, USA) following the manufacturer's instructions.

Genetical analyses

BOX-PCR analysis

The PCR reactions were performed with the BOXA1R primer (5′-CTA CGG CAA GGC GAC GCT GAC

G-3′) (Versalovic et al. 1994). The PCR reactions were performed as described by Kaschuk et al. (2006) with 25 ng genomic DNA. Gel images from the BOX-PCR were analyzed using the GELCOMPAR II software (Applied Maths, Kortrijk, Belgium). Pairwise similarity matrices were obtained using the Dice coefficient at a tolerance of 1.5%. Cluster analysis of similarity matrices was performed by the unweighted pair group with arithmetical average (UPGMA) algorithm.

16S rDNA and nifH gene amplification and sequencing

The full length 16S rRNA gene was amplified using the universal pair of primers 27f (5′-AGA GTT TGA TCC TGGCTC AG-3′) (Furushita et al. 2003) and Amp2 (5′-AAG GAG GTG ATC CAR CCG CA-3′) (Wang et al. 1996) as described by Videira et al. (2009). The *nif*H gene was amplified using the primers PolF (5′-TGC GAY CCS AAR GCB GAC TC-3′) and PolR (5′-ATS GCC ATC ATY TCR CCG GA-3′) as described by Poly et al. (2001). PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA) and sequenced using an ABI 3500xL Genetic Analyzers (Applied Biosystems).

Similarity analysis

The 16S rRNA and *nif*H gene sequences were automatically aligned using ClustalW, and then corrected manually. The sequences were submitted to BLAST analysis (Altschul et al. 1997) with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov), and the closest relatives were included in the phylogenetical analysis. Phylogenetical analysis of 16S rRNA and *nif*H was performed using the neighbor-joining method and Kimura two-parameter (K2P) with the MEGA5 software package (Tamura et al. 2011). A total of 1000 bootstrap replicates were performed. The sequences of *Bacillus pumilus* were used as an outgroup for both genes.

Plant growth promoting traits

Nitrogen-fixing ability

The nitrogen-fixing ability of the isolates was tested using the acetylene reduction assay (ARA) as described by Boddey (1987). Each bacterial isolate was evaluated in the same N-free semisolid medium used for isolation. The cultures were incubated for either 48 h (JNFb medium) or 72–96 h (LGI, LGI-P and JMV media) at 30°C before the ARA assay. Nitrogenase activity as determined by ethylene production was measured 30 (JNFb medium) and 60 min (LGI, LGI-P and JMV media) after acetylene injection using a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer) equipped with a Poropak Q column. There were three replicates for each bacterial strain.

Production of indole compounds

Production of indoles was assayed as described by Sarwar and Kremer (1995). All strains were cultured in triplicate in 5 mL DYGS liquid medium, supplemented with 100 μM of L-tryptophan, and incubated at 30°C in the dark with shaking for 72 h. Culture supernatants were recovered after centrifugation at 6000 x g for 10 min. Five microliters of supernatant was mixed with 10 μL of 1 M NaOH and 5 μL of distilled water. This mixture was incubated at 90°C for 5 min. The samples were kept at room temperature and subsequently mixed with 180 μL of Salkowski's reagent. This suspension was incubated at room temperature for 20 min in the dark and the color intensity was measured on a spectrophotometer at 535 nm. There were three replicates for each bacterial strain.

Phosphate solubilization ability

Initial qualitative estimation of the P-solubilizing activity of the isolates was carried out on National Botanical Research Institute Phosphorus (NBRIP) agar, a minimal medium with insoluble β-tricalcium phosphate ($Ca_3(PO_4)_2$) as sole phosphorus source (Mehta and Nautiyal 2001). A total of 20 μL of bacterial cultures (10^8 ufc mL^{-1}) were inoculated into NBRIP agar plates. The appearance of a clear halo around the colonies, 12 d after incubation, indicated phosphate solubilization. Each isolate was tested three times on NBRIP agar medium to confirm the phosphate solubilization phenotype.

Results

Culturable diazotrophic bacterial population

The presence of diazotrophic bacteria colonizing leaves, stems and roots of both elephant grass

genotypes was demonstrated by the veil-like pellicle formed in all five N-free semisolid media (NFb, JNFb, JMV, LGI and LGIP). The culturable diazotrophic bacterial population colonizing these plants varied from 10^2 to up 10^6 bacteria g^{-1} fresh tissues. Statistically significant differences were observed in numbers of diazotrophic bacteria retrieved from both genotypes and tissues using the five different media (Table 1).

A total of 204 diazotrophic isolates were obtained from the different tissues (leaves, stems and roots) of both elephant grass genotypes. Of this number, 40 (19.6%) originated from roots, 40 (19.6%) from leaves and the majority, 124 (60.8%), were isolated from stems. It should be mentioned that the number of isolates originating from stems did not differ between the various stem parts assayed (Table 1), indicating that the sampling procedure did not favor any part of the plant. A total of 103 isolates were obtained from genotype CNPGL F06-3 (CN) and 101 from genotype Cameroon (CA). The analysis also showed that 68 isolates were obtained using JNFb, 25 with LGI, 43 with LGI-P and 63 with JMV media (Table 1). During the storage process, the majority of the diazotrophic strains originated from the NFb medium were lost, with only 6 isolates remaining viable. Considering that they were isolated on a medium containing malic acid, a carbon source also present in the JNFb medium, these isolates, identified by the code NF, were analyzed together with isolates from the JNFb medium.

Genetical analysis

All 204 of the isolates were clustered based on the BOX-PCR profiles generated within each semisolid medium used to isolate the diazotrophs (Fig. S1–S4). There was a large and diverse number (2 to 20) of

Table 1 Quantification of diazotrophic bacterial population colonizing two elephant grass genotypes using five different nitrogen free semisolid media (log number cells per g fresh weight)

Isolating media/ Genotype	Tissue					Average	Total of isolates
	Leaf	Stem			Root		
		apical	middle	basal			
NFb medium**							
CNPGL F06-3	3.78* bB	5.36 aA	5.60 aA	5.67 aA	5.49 aA	5.18 a	1
Cameroon	4.60 aB	4.67 bB	4.76 bB	4.90 bAB	5.59 aA	4.90 b	5
JNFb medium							
CNPGL F06-3	3.53 bC	5.09 aAB	5.04 aAB	4.57 aB	5.32 aA	4.71 a	39
Cameroon	4.40 aB	4.41 bB	4.59 aB	4.48 aB	5.40 aA	4.66 a	29
LGI medium							
CNPGL F06-3	3.18 aC	4.15 aAB	5.47 aA	4.27 aAB	5.06 aA	4.42 a	19
Cameroon	3.34 aBC	2.51 bC	4.57 aAB	4.78 aA	4.31 aAB	3.90 b	6
LGI-P medium							
CNPGL F06-3	4.34 aC	5.32 aAB	5.81 aA	4.48 aBC	5.73 aA	5.14 a	10
Cameroon	4.48 aA	4.57 bA	4.82 bA	4.52 aA	4.57 bA	4.59 b	33
JMV medium							
CNPGL F06-3	3.71 aB	5.03 aA	4.81 aAB	4.59 aAB	4.78 aAB	4.58 a	34
Cameroon	4.16 aA	4.01 bA	3.74 bA	4.45 aA	4.87 aA	4.25 a	28
Average	3.96C	4.51B	4.92AB	4.67B	5.11A	–	
Total of isolates	40	42	37	45	40	–	204

*Values represent mean values of three replicates. Similar letters did not differ at the Tukey test at 5% of probability. Lowercase letters separate genotypes within each selective media and part of the plant. Uppercase letters separate average within each line. The coefficient of variation for the semisolid NFb, JNFb, LGI and LGI-P, JMV was 6.60%, 5.88%, 13.09%, 7.99% and 10.60%, respectively

**During the storage process, the majority of the diazotrophic strains originated from the NFb medium was lost

fragments among the BOX-PCR profiles and it was possible to discriminate, at about 70% similarity coefficient, most of the bacteria. Isolates showing very similar profiles were considered to have a high degree of genetical similarity. This strategy permitted the selection of representative isolates from each cluster for the similarity analysis using partial 16S rRNA gene sequences (600–900 bp) (isolates indicated with "*" in the Fig. S1–S4). This allowed for the taxonomical identification of 45 isolates with confidence at least to the genus level, although in many cases the species could be inferred based upon the size of the fragment sequenced (Table 2). Analysis of these data revealed the predominance of diazotrophs belonging to the genus *Gluconacetobacter* (followed by the genera *Azospirillum* and *Enterobacter*) colonizing the plant tissues of both genotypes. No clear effect of plant genotypes (CNPGL F06-3 and Cameroon) or of plant tissues (leaves, stems or roots) were observed on the diazotrophic populations, as the isolates originating from each genotype and plant part were distributed randomly within each bacterial genus. Phylogenetical analyses based upon 16S rRNA sequences (~1,450 bp) of representative strains from each genus were compared with the most closely related bacterial strains retrieved from the database, and these confirmed the results presented in Table 2. It was thus confirmed that *G. diazotrophicus* (LP343) and diazotrophs closely related to the species *G. liquefaciens* (LP520, JM444, LG214) and *G. sacchari* (JM60, LP433) were present in elephant grass (Fig. 1). In addition, diazotrophs belonging to the species *Azospirillum brasilense* (JN73, JN246) and *Burkholderia silvatlantica* (JM241) were also shown colonizing elephant grass. In addition, diazotrophic bacteria phylogenetically similar to the species *Enterobacter clocae* (LG222, JM180 and LP161) and *E. oryzae* (JM489) were also detected among the bacterial isolates (Fig. 1).

To confirm that they were diazotrophs, all 204 isolates were screened for the presence of the *nifH* gene using the degenerate primers describe by Poly et al. (2001). The PCR reaction showed that all strains, except the isolate JN128, contained an amplifiable product of the expected size (~360 bp) on agarose gel electrophoresis (data not shown). Further *nifH* sequencing analysis of 13 strains characterized by their 16S rRNA gene (see above) showed that strains JN73 and JN246 had 99% homology with the *nifH* gene of *A. brasilense*, whereas JM242 showed >99% homology with the *nifH* gene of *B. silvatlantica* and that of LP343 was closely related to the *nifH* of *G. diazotrophicus* (Fig. 2). In contrast, the other strains, including the other *Gluconacetobacter* species, showed high homology with *nifH* genes of the species *E. clocae*, *K. pneumoniae* and Bacterium QZ25S, a strain isolated from sugarcane tissues (Fig. 2).

Plant growth promoting traits

Some features (nitrogen fixation, indole production and P solubilization), known to contribute to plant growth promotion, were tested among the 204 diazotrophic isolates with the aim to identify strains with potential to be exploited as biofertilizers. The results are summarized in Table 3.

The ability of the isolates to fix nitrogen, as measured by the ARA, showed that there was significant variations in nitrogenase activity among the isolates, and that this was influenced by the type of N-free semisolid medium used for the assay (Table 3). It is important to mention that the nitrogen-fixing ability was measured in the same medium used to isolate the bacteria. The isolates obtained from JNFb medium showed higher amounts of nitrogenase activity as compared to the other media. On average, the activity, expressed as nmol ethylene h^{-1} mg $protein^{-1}$, varied from 10.2 to 1,254 when tested in the JNFb medium, from 0.8 to 318 in the LGI-P medium, from 6 to 98.8 in the LGI medium, and from 1.4 to 267 in the JMV medium. All type strains showed ARA values above 100 nmol ethylene h^{-1} mg $protein^{-1}$ (Table 3).

Evaluation of these isolates for their ability to produce phytohormones indicated that 97% were able to produce indole compounds (Table 3), but their intrinsic ability to produce these substances in the presence of tryptophan varied greatly amongst the isolates. Values, expressed as μg indole mg $protein^{-1}$, varied from 0.31 to 19.02, with an average of 1.40, from the JNFb medium, 2.63 from the LGI, 6.66 from the LGI-P and 2.56 μg for isolates from the JMV medium.

Additional screening of the isolates for their capacity to solubilize phosphate in vitro showed that only 45 (22%) produced a clear zone on NBRIP agar plates when measured 12 d after inoculation (Table 3). Solubilization indices varied from 0.6 to 2.80 (data not shown). The phosphate solubilization trait was encountered within isolates cultured in the LGI-P

Table 2 Similarity analysis of isolates from elephant grass tissues based on partial sequence of 16S rRNA gene

Isolate	Plant genotype	Source	16S rRNA (highest match %)	Access. n°.	% sim
JN125	Cameroon	Middle stem	*Azospirillum brasilense*	GU256444.1	97
JN135	Cameroon	Middle stem	*Azospirillum brasilense*	GU256444.1	96
JN15	Cameroon	Leave	*Azospirillum brasilense*	FR667913.1	93
JN246	Cameroon	Root	*Azospirillum brasilense*	GU256444.1	97
JN184	Cameroon	Basal stem	*Azospirillum brasilense*	GU256444.1	96
JN318	CNPGL	Leave	*Azospirillum brasilense*	GU256444.1	97
JN360	CNPGL	Apical stem	*Azospirillum brasilense*	GU256444.1	97
JN450	CNPGL	Basal stem	*Azospirillum brasilense*	GU256444.1	95
JN454	CNPGL	Basal stem	*Azospirillum brasilense*	FR745918.1	93
JN73	Cameroon	Apical stem	*Azospirillum brasilense*	GU256444.1	95
JN127	Cameroon	Middle stem	*Azospirillum* brasilense	NR042845.1	96
JN189	Cameroon	Basal stem	*Azospirillum* brasilense	NR042845.1	96
JN245	Cameroon	Root	*Azospirillum* brasilense	NR042845.1	96
JN500	CNPGL	Root	*Azospirillum* brasilense	GU256444.1	97
JN128	Cameroon	Middle stem	*Pseudomonas oryzihabitans*	GQ250598.1	98
LG166	Cameroon	Middle stem	*Azospirillum brasilense*	GU256444.1	97
LG208	Cameroon	Basal stem	*Enterobacter cloacae*	FJ532062.1	95
LG222	Cameroon	Basal stem	*Enterobacter cloacae*	FJ532062.1	97
LG210	Cameroon	Basal stem	*Enterobacter oryzae*	EF488760.1	87
LG214	Cameroon	Basal stem	*Gluconacetobacter liquefaciens*	AB626659.1	95
LG218	Cameroon	Basal stem	*Gluconacetobacter liquefaciens*	GU372344.1	95
LG469	CNPGL	Basal stem	*Gluconacetobacter liquefaciens*	GU372344.1	97
LG49	Cameroon	Leave	*Gluconacetobacter sacchari*	AF127412.1	98
LG387	CNPGL	Apical stem	*Klebsiella oxytoca*	GU459204.1	90
LP161	Cameroon	Middle stem	*Enterobacter cloacae*	FJ532062.1	99
LP518	CNPGL	Root	*Enterobacter cloacae*	FJ532062.1	99
LP432	CNPGL	Middle stem	*Enterobacter oryzae*	EF488760.1	99
LP343	CNPGL	Leave	*Gluconacetobacter diazotrophicus*	AM889285.1	96
LP431	CNPGL	Middle stem	*Gluconacetobacter liquefaciens*	AB626659.1	96
LP386	CNPGL	Apical stem	*Gluconacetobacter sacchari*	AF127412.1	92
LP433	CNPGL	Middle stem	*Gluconacetobacter sacchari*	AF127412.1	94
LP479	CNPGL	Basal stem	*Gluconacetobacter* sp	AF127400.1	93
LP45	Cameroon	Leave	*Gluconacetobacter* sp	AF127400.1	95
JM241	Cameroon	Root	*Burkholderia silvatlantica*	AY965242.1	94
JM242	Cameroon	Root	*Burkholderia* sp	JF772523.1	86
JM235	Cameroon	Root	*Enterobacter cloacae*	FJ532062.1	96
JM180	Cameroon	Basal stem	*Enterobacter oryzae*	EF488760.1	99
JM2	Cameroon	Leave	*Enterobacter oryzae*	EF488760.1	99
JM489	CNPGL	Root	*Enterobacter oryzae*	HQ706110.1	99
JM119	Cameroon	Middle stem	*Gluconacetobacter liquefaciens*	AB626659.1	92
JM393	CNPGL	Middle stem	*Gluconacetobacter liquefaciens*	AB626659.1	98
JM444	CNPGL	Basal stem	*Gluconacetobacter liquefaciens*	AB117967.1	93
JM486	CNPGL	Root	*Gluconacetobacter liquefaciens*	AB626659.1	96
JM491	CNPGL	Root	*Gluconacetobacter liquefaciens*	GU372344.1	94

Table 2 (continued)

Isolate	Plant genotype	Source	16S rRNA (highest match %)	Access. n°.	% sim
JM349	CNPGL	Apical stem	*Gluconacetobacter sacchari*	AF127412.1	94
JM60	Cameroon	Apical stem	*Gluconacetobacter* sp	EF493039.1	91
JM355	CNPGL	Apical stem	*Klebsiella oxytoca*	EU931550.1	97

medium (38%), followed by JMV (33%) and LGI (29%), but no P solubilization was observed amongst the isolates cultured in the JNFb and NFb media; this was similar to the reference strains of the genus *Herbaspirillum* (HCC103 and HRC54) and *Azospirillum brasilense* (Sp7). In contrast, P solubilization was positive for the reference strains *Azotobacter chroococcum* (AC1), *Gluconacetobacter diazotrophicus* (PAL5) and *Burkholderia* (Ppe8 and SRMrh-20) which were used as positive controls.

Discussion

Isolation of culturable diazotrophic bacteria and their genetical diversity

In this study, a culture-dependent approach was used to enrich, count and isolate a fraction of the bacterial community present in the roots, stems and leaves of *P. purpureum* grown in field conditions. The MPN bacterial counting method using N-free semisolid media

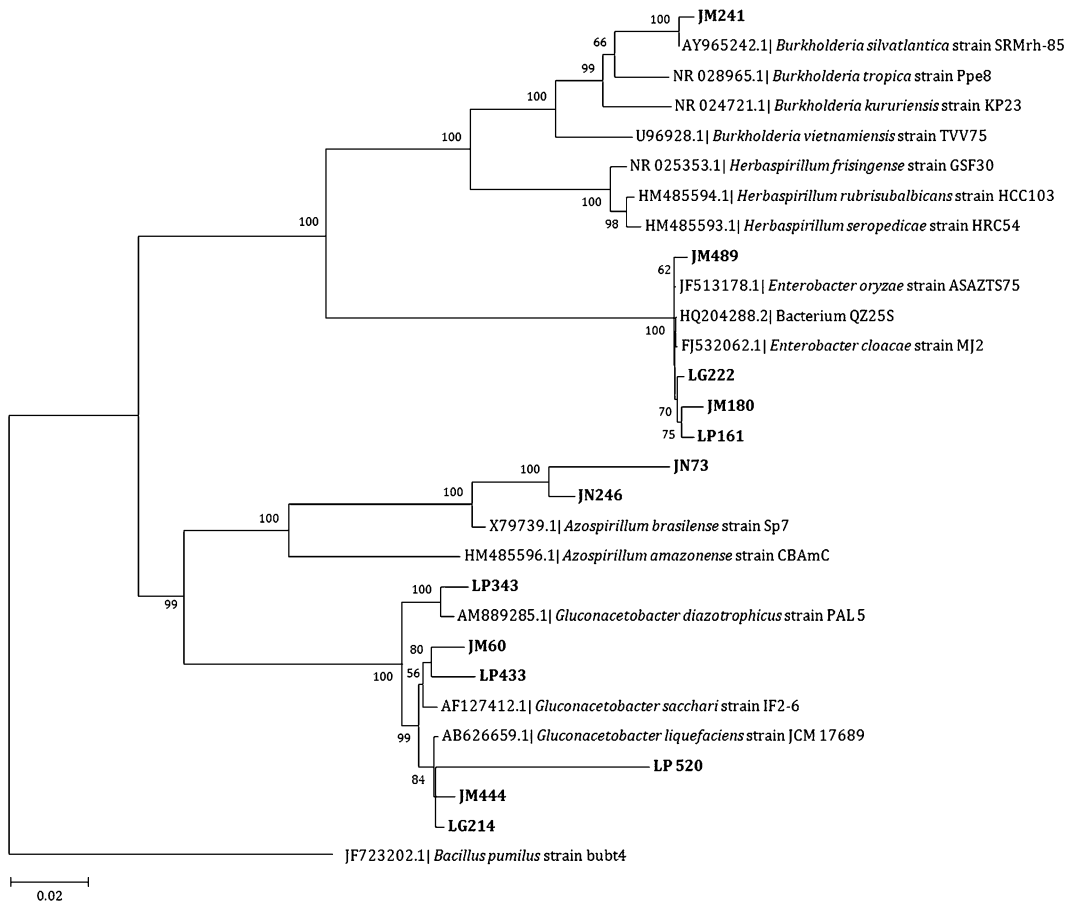

Fig. 1 Neighbor-joining phylogenetical tree based on bacterial 16S rRNA sequences (~ 1,450 bp), including sequences obtained in this study and from the most closely related 16S rRNA genes of previously cultured diazotrophic strains. 16S rRNA sequences of representative isolates obtained in this study are shown in bold: Numbers at branches represent bootstrap values >50% from 1,000 replicates. The scale bar shows the number of nucleotide substitutions per site

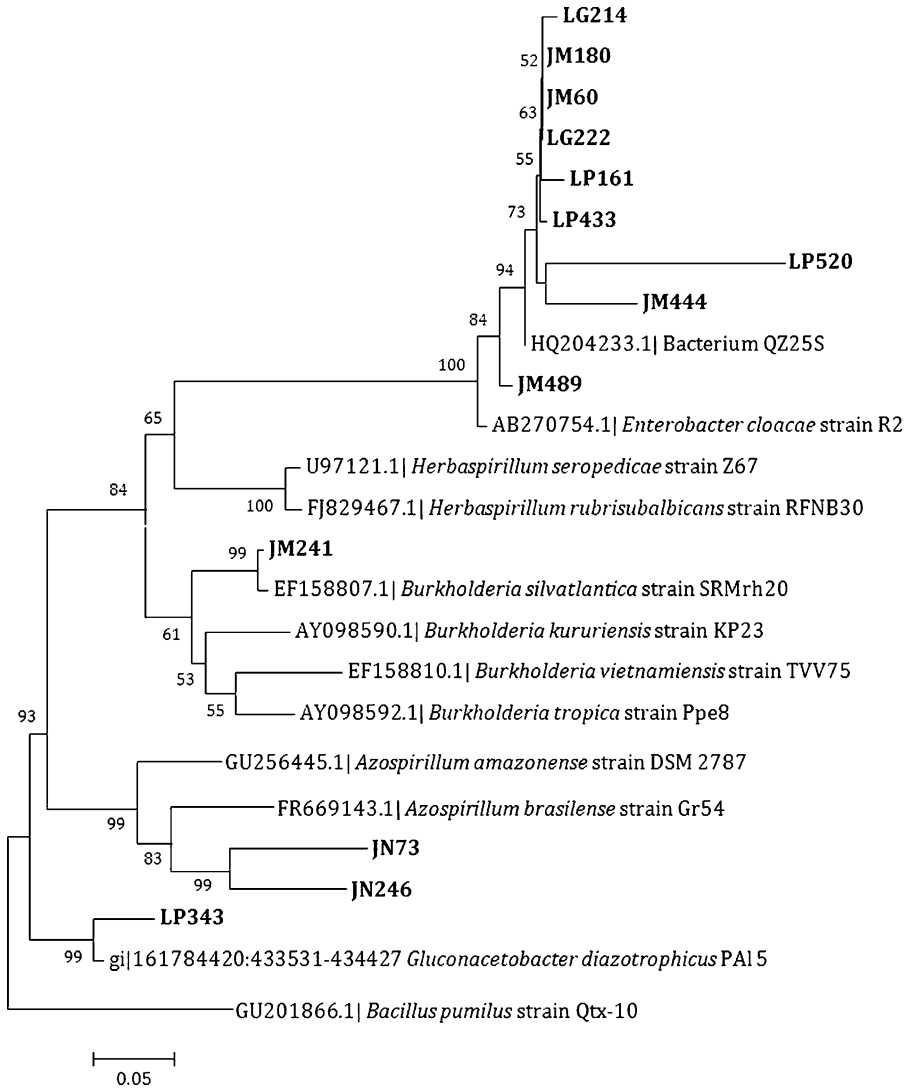

Fig. 2 Neighbor-joining phylogenetical tree based on bacterial *nif*H sequences (~ 300 bp), including sequences obtained in this study and from the most closely related *nif*H genes of previously cultured diazotrophic strains. *nif*H sequences of representative isolates obtained in this study are shown in bold: Numbers at branches represent bootstrap values >50% from 1,000 replicates. The scale bar shows the number of nucleotide substitutions per site

(Döbereiner 1995) has been successfully applied to enumerate the culturable diazotrophic populations present in the tissues of many *Poaceae* species, but most particularly those of agricultural importance (Baldani and Baldani 2005). Furthermore, the pellicle formed within the semisolid media allows for the direct isolation of the predominant micro-aerophilic diazotrophic bacteria growing within them. Our results showed that the diazotrophic population associated with these two elephant grass genotypes varied from 10^2 to 10^6 bacteria g-1 of fresh weight. Similar results were observed by Reis et al. (2000), Kirchhof et al. (1997a, b) and Reis et al. (2001) when they analyzed the stems and roots of 14 elephant grass genotypes cultivated in Brazil and much lower population sizes generally in leaves. Earlier studies also showed this effect for other *Poaceae,* such as maize (*Zea mays*) and sugarcane (*Saccharum* sp.) (Roesch et al. 2006; Pariona-Llanos et al. 2010) as well as in elephant grass genotypes (Kirchhof et al. 1997a, b; Reis et al. 2001). It should be mentioned that in our study the leaves and stems were surface disinfested and the stems were also

Table 3 Ability of the diazotrophic bacteria originated from elephant grass to fix nitrogen, produce indole compounds and solubilize phosphate

Isolate	Nase Activity[1]	Indol Prod. [2]	PO_4^- Solub.
JN314	+++	+	–
JN315	+++	+	–
JN316	+++	+	–
JN317	++	+	–
JN318	+++	+	–
JN353	+	+	–
JN357	++	+	–
JN358	+++	++	–
JN360	+++	+++	–
JN361	++	+	–
JN365	+++	+	–
JN375	+	+	–
JN384	+	+	–
JN402	++	+	–
JN404	+++	+	–
JN405	+++	+	–
JN406	+++	+	–
JN407	++	+	–
JN410	+++	+++	–
JN450	++	+++	–
JN451	+++	+	–
JN452	+++	+	–
JN453	+++	+	–
JN454	++	+	–
JN455	++	+++	–
JN494	+	+	–
JN496	+++	+	–
JN499	++	+	–
JN500	+++	+	–
JN123	nd[3]	++	–
JN125	+++	+	–
JN126	+++	+	–
JN127	+++	+	–
JN128	nd	+	–
JN129	+	+	–
JN13	+++	++	–
JN130	+	+	–
JN131	+++	nd	–
JN134	++	+	–
JN135	++	+	–
JN15	++	++	–
JN17	+++	+	–
JN18	+	+	–
JN184	+++	+++	–
JN185	++	+	–
JN186	++	+	–
JN188	++	+	–
JN189	+++	+	–
JN19	++	+	–
JN191	+++	+	–
JN192	+++	+	–
JN194	nd	+	–
JN20	+	+++	–
JN21	+++	+	–
JN22	nd	++	–
JN23	+++	+	–
JN245	+++	+	–
JN246	+	++	–
JN247	+++	++	–
JN249	+++	+	–
JN254	+++	++	–
JN313	+++	+	–
JN70	+	+	–
JN72	+++	+	–
JN73	+++	+	–
JN76	+++	+	–
NF326	+	+	–
NF374	+++	+	–
NF503	++	+++	–
NF504	++	+++	–
NF506	+++	nd	–
NF79	+++	++	–
LP342	+	+++	–
LP343	+++	++	+
LP344	++	+	–
LP345	+	+++	+
LP346	nd	+++	–
LP384	nd	+++	–
LP386	+	+++	+
LP389	nd	nd	–
LP391	+	++	–
LP392	nd	+++	+
LP429	nd	+++	+
LP431	+	+++	+
LP432	+++	+++	–
LP433	nd	+++	+
LP436	nd	nd	+

Table 3 (continued)

Isolate	Nase Activity[1]	Indol Prod. [2]	PO_4^- Solub.
LP474	nd	nd	+
LP475	+	+	−
LP476	nd	+	−
LP478	+	+	+
LP479	++	+	+
LP480	nd	+	−
LP481	+	++	−
LP482	+	+	+
LP490	+	+++	−
LP518	++	+	+
LP520	nd	+	+
LP521	nd	+	−
LP522	+	+	−
LP523	nd	+	−
LP524	+	+++	−
LP526	+	+++	−
LP111	++	+++	−
LP161	++	+++	−
LP163	nd	+	−
LP281	+	++	+
LP285	++	+++	+
LP290	nd	++	−
LP44	++	+++	−
LP45	++	++	−
LP47	+	+++	−
LP43	++	+++	−
JM300	+	+	−
JM302	+	++	−
JM303	nd	++	−
JM308	nd	++	−
JM309	nd	++	−
JM310	+	++	+
JM316	+	−	−
JM348	nd	+++	−
JM349	nd	+	+
JM351	+	+	+
JM353	+	+++	−
JM355	+	+++	−
JM356	+	+++	−
JM387	++	+	−
JM393	+	+	−
JM394	+	+	−
JM395	nd	+++	−
JM397	++	+	−

Table 3 (continued)

Isolate	Nase Activity[1]	Indol Prod. [2]	PO_4^- Solub.
JM399	+	+	−
JM439	nd	+	−
JM442	+	+	−
JM443	nd	++	−
JM445	+	++	−
JM446	nd	++	−
JM448	nd	++	−
JM486	+	++	+
JM489	+	++	+
JM490	nd	+++	+
JM491	nd	++	−
JM1	nd	+	−
JM117	++	+	−
JM119	nd	+	+
JM121	nd	++	−
JM122	nd	+++	−
JM123	+	+	−
JM14	nd	++	−
JM149	+	++	−
JM174	nd	+	−
JM175	nd	++	−
JM178	+	++	+
JM180	++	++	+
JM182	+	+	+
JM2	+	+	+
JM231	nd	+	−
JM232	+	+++	−
JM233	nd	+	−
JM235	+	+++	+
JM240	nd	+	−
JM241	nd	++	−
JM242	+	++	−
JM248	nd		−
JM4	+	+++	+
JM444	+	++	−
JM55	+	++	−
JM57	+	+	−
JM58	++	++	+
JM59	+	+++	+
JM6	+	+++	−
JM60	+	++	+
JM61	nd	++	−
JM63	+	++	−
JM65	+	+	−

Table 3 (continued)

Isolate	Nase Activity[1]	Indol Prod. [2]	PO_4^- Solub.
JM66	nd	+	–
JM7	nd	+	–
LG343	+	++	+
LG378	++	+	–
LG382	+	+	+
LG387	nd	++	+
LG469	nd	++	+
LG516	++	+	–
LG102	++	++	–
LG106	+	+	–
LG151	+	+	–
LG157	nd	+	–
LG166	nd	+	–
LG207	++	+	+
LG208	+	++	+
LG210	++	+++	+
LG211	++	+	–
LG212	+	+	–
LG213	++	++	–
LG214	+	++	+
LG216	nd	+	–
LG218	nd	+++	+
LG222	++	+	+
LG270	nd	+	–
LG277	nd	++	–
LG278	nd	+	–
LG279	+	+	+
LG49	+	++	+
LG91	++	++	+

[1] Acetylene reduction: + <100 nmol ethylene h^{-1} mg $protein^{-1}$; ++ ≥100; +++ ≥ 300;

[2] Indole prod.: + 0,1 – 3 μg indole componds mL^{-1} ; ++ 3 – 10; +++ ≥ 10;

[3] *nd* not detectable

peeled, therefore eliminating possible contamination from surface diazotrophic bacteria. Conversely, the roots were not surface sterilized, and as they are the (1) in closest contact with the soil and its microflora, and (2) the principal entrance for bacterial colonization of the plant (Pariona-Llanos et al. 2010), the highest number of diazotrophic bacteria were expected to be isolated from them.

The BOX-PCR was applied as a first step for clustering closely related isolates amongst the 204 diazotrophs isolated from these two elephant grass genotypes. This technique has been used by many authors evaluating the genetic diversity of bacterial strains and communities associated with plants (Hurek et al. 1997; Albino et al. 2006; Couillerot et al. 2010). In addition, BOX-PCR has been suggested as a good way to discriminate taxonomically between strains/isolates within a species (Lerner et al. 2010; Nayak et al. 2011). Analyzes of the 204 diazotrophic bacteria using this technique revealed that the growth medium influences the diversity of nitrogen-fixing bacteria with the highest genetic diversity obtained from the JMV medium followed by the LGIP medium. In addition, cluster analysis within dendrograms suggested that several isolates did not possess high homology to the diazotrophic reference strains commonly found associated with these plants, therefore indicating the possible occurrence of intra-specific diversity within species. However, the cluster analysis did not indicate either an effect of the plant tissues or the plant genotype on the diversity of the diazotrophic isolates. Indeed, isolates from both genotypes (CNPGL F06-3 and Cameroon) were distributed randomly within the dendrograms.

The occurrence of the genus *Azospirillum* was expected considering that species from this genus have already been reported colonizing elephant grass (Reis et al. 2001; Eckert et al. 2001). The BOX-PCR analysis already indicated that most of the isolates from the JNFb and NFb media had very similar profiles, but were different from the type strain, *A. brasilense* Sp7, thereby suggesting that these isolates may belong to the same species, but represent different strains. In fact, analyses based on sequences of full 16S rRNA gene and of *nif*H gene fragments confirmed the presence of the species *A. brasilense*. Indeed, *Azospirillum* species have been repeatedly isolated from the surface and interior of roots and shoots from *Poaceae*, including the C4-grass *Miscanthus* and elephant grass (Hartmann and Bashan 2009; Bashan and Bashan 2010; Eckert et al. 2001). Interestingly, however, no bacteria belonging to the genus *Herbaspirillum* were detected in the JNFb medium, in contrast to earlier studies on elephant grass in Brazil and *Miscanthus* grown in Germany (Reis et al. 2000, 2001; Kirchhof et al. 1997a, b). It is possible that populations of *Herbaspirillum* colonizing plant tissues were very low at the time that the plants were harvested, and thus allowed *Azospirillum* to predominate in these two media; this

phenomenon has been observed previously by other authors, but in other plants (Baldani et al. 2005).

Although the occurrence of the species *G. diazotrophicus* colonizing different tissues of *P. purpureum* genotypes has been previously reported (Kirchhof et al. 1997a, b), the species *G. liquefaciens* and *G. sacchari* are here described for the first time as nitrogen-fixing bacteria colonizing this plant. *Gluconacetobacter liquefaciens*, previously described as a non-N-fixing species, was recently encountered as a diazotroph colonizing the rhizosphere of rice grown in acidic lowland soils of India (Roy et al. 2010), but the diazotrophic *G. sacchari* strains from the present study which expressed nitrogenase activity and harbored the *nif* H gene is the first report of diazotrophy in this species which has hitherto been considered non-fixing (Yamada et al. 1997). Interestingly, *G. sacchari* is very closely related to to *G. diazotrophicus* (Franke-Whittle et al. 1999), and has been detected colonizing sugarcane endophytically (Franke-Whittle et al. 2005). In addition, the phylogenetic analysis based upon *nif* H sequences from representative isolates identified as *G. liquefaciens* and *G. sacchari* clustered them with *nif* H sequences from *Enterobacter*, *Klebsiella* and Bacterium QZ25S, which raises the possibility that the *nif* genes have been transferred between these species via horizontal gene transfer (Raymond et al. 2004; Fischer et al. 2011). However, more data based upon *nif* H and more housekeeping gene sequqnces of *G. liquefaciens* and *G. sacchari* are needed to confirm this hypothesis.

The detection of nitrogen-fixing species of *Burkholderia* colonizing *P. purpureum* has not been previously reported, although this genus has been found associated with other Poaceae, such as sugarcane, maize and rice (Reis et al. 2004; Perin et al. 2006). Phylogenetical analyses based upon 16S rRNA and *nifH* sequences clustered the isolate JM241 within the species *B. silvatlantica*, thereby suggesting the occurrence of this species in these elephant grass genotypes. Similar to that observed for the other bacterial genera in this study, sequencing of almost full length of 16S rRNA from strains within the *Enterobacter* genus confirmed the occurrence of the species *E. cloacae* and *E. oryzae* (as suggested by the partial 16S rRNA sequences). In addition, sequencing of the *nifH* gene fragments showed that these isolates clustered together within a branch of the species *E. cloacae* with high similarity to the Bacterium QZ25S, which was originally isolated from sugarcane tissues (Franke-Whittle et al. 2005). The occurrence of *Enterobacter* as endophytes in this plant has not been reported before, although it has been shown that some *Enterobacter* strains fix nitrogen (acetylene reduction activity) and other diazotrophic species such as *E. cloacae* and *E. oryzae* have also been identified (Peng et al. 2009). Indeed, diazotrophic members of the family Enterobacteriaceae are commonly reported as being endophytic within Poaceae e.g. within rice (Gyaneshwar et al. 2001).

Plant growth promoting traits

According to Döbereiner (1988) bacteria could be considered nitrogen-fixers when their nitrogenase activity, as evaluated by ARA in nitrogen-free semisolid media is at least 50 nmol ethylene h^{-1} per culture. We therefore classified our isolates in three groups based upon their nitrogenase activity (nmol ethylene h^{-1} mg protein^{-1}): low (up to 99.9), medium (from 100 to 300) and high (higher than 300). Of the 204 isolates, 20.6% showed high, 20.6% showed medium and 32.4% exhibited low nitrogenase activity. In addition, about 26.5% were unable to reduce acetylene in the experimental conditions even though a *nif* gene was detected by PCR reaction or by sequencing of the *nifH* fragments. The highest specific nitrogenase activity was observed in cultures grown in the JNFb medium. It has been reported that there is no direct correlation between the presence of the *nif*H gene and the ARA activity of a bacterial strain (Islam et al. 2009), and the fact that a few isolates were unable to reduce acetylene may be due to the growth conditions of the medium, such as an inappropriate carbon source or pH.

Another important growth-promoting trait investigated amongst the 204 isolates was the ability to produce indoles. The literature has reported that diazotrophs produce around 0.1 to 30 μg indole mg protein^{-1} when grown in the presence of 100 μg mL^{-1} L-tryptophan (Rodrigues et al. 2008; Taghavi et al. 2009; Xin et al. 2009). Our results are in agreement with this observation since the isolates and the type strains produced between 0.31 and 19.02 μg indole mg protein^{-1}. Production of IAA in the presence of tryptophan has been reported for several PGPR belonging to the genera *Azospirillum*, *Azotobacter*, *Burkholderia*, *Pantoea*, *Gluconacetobacter* and *Serratia* (Dobbelaere et al. 2003; Pedraza et al. 2004; Dastager et al. 2009). However, it has been reported that the amount of

indole compounds produced in vitro depends on the particular bacterial species, and/or strains, as well as on the culture conditions, such as oxygenation, pH and growth phase (Crozier et al. 2000; Radwan et al. 2002). Nevertheless, it has been demonstrated that the biosynthesis of indole compounds by several plant growth promoting bacteria, including diazotrophs, is, among other growth regulators, responsible for the enhancement of plant root development and the concomitant improvement of mineral and water uptake by roots of many gramineous plants (Spaepen et al. 2009).

We also investigated the ability of the 204 elephant grass diazotrophs to solubilize phosphate, and thus identified bacteria belonging to the genera *Gluconacetobacter*, *Enterobacter* and *Klebsiella* with this ability. The ability of these genera to promote the solubilization of P in vitro has been demonstrated by several authors (Ahemad et al. 2008; Sashidhar and Podile 2010). Although the genus *Burkholderia* is known to solubilize phosphate, this trait was not detected in the strain JM241 (*B. silvatlantica*) isolated in this study, and this result corroborates a previous study involving strains of this species (Perin et al. 2006). It is already known that phosphate is one of the major nutrients limiting root development and consequently plant growth, particularly in tropical soils. However, the efficiency of uptake and use of P following phosphate fertilizer application is low because of the formation of insoluble complexes (Vassilev and Vassileva 2003), and hence searching for phosphate solubilizing bacteria that can enhance the agronomic effectiveness of organic P-containing fertilizer without affecting crop productivity is an alternative strategy that has been pursued by several agricultural microbiologists (Rodríguez and Fraga 1999; Khan et al. 2009). Indeed, such positive attributes, together with other physiological characteristics, such as indole production and nitrogen fixation, make these microorganisms strategically interesting for exploitation in a biofertilizer program to inoculate this C4 plant.

In conclusion, characterization of the diversity and physiology of these diazotrophic isolates is a prerequisite for estimating their potential for application in the field. Similar strategies have been reported by other authors searching for bacteria, including endophytes, with the potential to enhance phytoremediation (Moore et al. 2006), plant growth promoting traits (Jha et al. 2009) and biological nitrogen fixation (Baldani and Baldani 2005). Our study showed a large culturable population of diazotrophic bacteria colonizing roots and stems of both elephant grass genotypes. The molecular analysis of the 204 bacterial strains suggested that there were no significant difference in the genetical diversity amongst the diazotrophic strains colonizing the different tissues. In addition to the already known genera *Gluconacetobacter* and *Azospirillum*, the sequencing of their 16S rRNA and *nifH* sequences revealed the occurrence for the first time of the genera *Burkholderia*, *Klebsiella* and *Enterobacter* colonizing this plant. The plant growth promoting analysis indicated that many of these isolates are potentially good candidates for biotechnological application as biofertilizers for C4 plants, and hopefully will assist in developing ecologically efficient and sustainable agricultural practices for soils low in nitrogen and soluble phosphate.

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