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REGULAR ARTICLE

Molecular characterisation of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum* sp.)

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Abstract To identify active diazotrophs in sugarcane, 16S rRNA and *nifH* transcript analyses were applied. This should help to better understand the basis of the biological nitrogen fixation (BNF) activity of a high nitrogen fixing sugarcane variety. A field experiment using the sugarcane variety RB 867515 was conducted in Seropédica, RJ, Brazil, receiving the following treatments: unfertilised and fertilised controls without inoculation, unfertilised with inoculation. The five-strain mixture developed by EMBRAPA-CNPAB was used as inoculum. Root and leaf sheath samples were harvested in the third year of cultivation to analyse the 16S rRNA and *nifH* transcript diversity. In addition to *nifH* expression from *Gluconacetobacter* spp. and *Burkholderia* spp., a wide diversity of *nifH* sequences from previously uncharacterised *Ideonella/Herbaspirillum* related phylotypes in sugarcane shoots as well as *Bradyrhizobium* sp. and *Rhizobium* sp. in roots was found. These results were confirmed using 16S cDNA analysis. From the inoculated bacteria, only *nifH* transcripts from *G. diazotrophicus* and *B. tropica* were detected in leaf sheaths and roots. Known as well as yet uncultivated diazotrophs were found active in sugarcane roots and stems using molecular analyses. Two strains of the inoculum mix were identified at the late summer harvest.

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Introduction

Sugarcane is one of the most important agricultural crops used as source for biofuels and renewable energy. It is grown in over 110 countries worldwide; 50% of the total production occurs in Brazil and India. In Brazil in particular, sugarcane plays a fundamental role in the production of bioethanol, which is used as a substitute for fossil fuels in combustion engines. In 2010, almost 10 million ha of sugarcane were planted in Brazil (IBGE 2010) and approximately half of the cane juice has been fermented to produce alcohol as a biofuel over the years (Boddey et al. 2003). Because waste products such as bagasse are used as an energy source in the production process, bioethanol production from sugarcane has a clear positive energy balance (Boddey et al. 2008). Nevertheless, the balance between energy gain and energy input in sugarcane/bioethanol production needs to be optimised further and aspects of sustainability and environmental protection have to be considered. The use of synthetic nitrogen fertiliser is a huge burden on environmental sustainability because it gives rise to denitrification, which results in the release of the greenhouse gas dinitrogen monoxide. In Brazil, commercial sugarcane crop production uses moderate amounts of nitrogen fertilisation (60–100 kg Nha^{-1} $year^{-1}$), whereas in most other countries, the agricultural practice is to apply 250 kg Nha^{-1} $year^{-1}$ to sugarcane plantations (Fuentes-Ramirez et al. 1999). Despite the fact that low levels of nitrogen fertilisation were used in Brazil, the nitrogen content in the soil was not depleted and consistently high sugarcane yields were obtained. Thus, the occurrence of biological nitrogen fixation (BNF) in sugarcane has been hypothesised already for decades (Doebereiner 1961; Ruschel et al. 1975). Many new diazotrophic bacteria have been isolated using semisolid nitrogen-free media, according to Doebereiner (1995). Using ^{15}N enriched tracing and natural abundance techniques, careful estimates have shown that, in some sugarcane cultivars, BNF is occurring, and up to 70% of plant nitrogen could be derived from BNF (Lima et al. 1987; Urquiaga et al. 1992; Yoneyama et al. 1997; Boddey et al. 2001; Oliveira et al. 2002). However, it remains unclear which bacteria are actively fixing nitrogen in sugarcane. In addition, the efficacy of BNF in sugarcane varies considerable depending on the site of sugarcane cultivation (Yoneyama et al. 1997; Biggs et al. 2002; Hoefsloot et al. 2005), which may be caused by the presence/absence of key N_2-fixing bacteria and their nitrogen-fixing activity *in planta*. In addition, the diazotrophic community may be very different when *Saccharum* sp. is grown in different soils and under different climate conditions (Magnani et al. 2010). *nif* negative mutants of *Gluconacetobacter diazotrophicus* PAL5 have been demonstrated to have a lower stimulatory effect under N-limiting conditions, and *G. diazotrophicus* PAL5 wild type strain inoculation induced ^{15}N fixation in the sugarcane plants (Sevilla et al. 2001). In addition to plant genetic and microbiological factors, the availability of water, phosphate, and molybdenum have been found to have a major influence on BNF in sugarcane (Doebereiner 1997; Boddey et al. 2003). A detailed understanding of the diazotrophic bacteria and the mechanisms they use to transfer nitrogen from the bacterium to the plant in high BNF sugarcane varieties remains a major area of ongoing research.

Many diazotrophic bacteria have been isolated from the sugarcane rhizosphere since Doebereiner et al. (1972) introduced nitrogen-free semisolid enrichment media. *Azospirillum* spp. (including *A. amazonense*), *Enterobacter* spp., *Erwinia* spp., *Beijerinckia* spp., *Azotobacter* spp., and *Derxia* spp. have been isolated and identified from the sugarcane rhizosphere (Boddey et al. 2003). In addition, many unique diazotrophic species, which live endophytically within sugarcane roots, stems and leaves, such as *Gluconacetobacter* spp., *Herbaspirillum* spp. or *Burkholderia* spp., are currently known (James and Olivares 1998; Reis et al. 2004). For example, the mixed inoculum developed by EMBRAPA-CNPAB (Seropédica, RJ, Brazil) consists of the following strains: *A. amazonense* strain BR 11115 (CBAmC), originally isolated from the stems of the sugarcane variety CB45-3, *G. diazotrophicus* strain BR 11281 (PAL5, type strain), isolated from the roots of the commercial sugarcane variety SP77-5181 (Cavalcante and Doebereiner 1988), *Herbaspirillum seropedicae* strain BR 11335 (HRC 54) and *H. rubrisubalbicans* strain BR 11504 (HCC 103), isolated from the roots of the sugarcane variety SP70-1143 and

stems of the variety SP70-1284, respectively, and *Burkholderia tropica* strain BR 11366 (Ppe8), isolated from the buds of the sugarcane variety SP71-1406 (Oliveira et al. 2009).

The initial attempts to characterise the nitrogen-fixing bacteria in crop plants using a cultivation-independent *nifH* gene diversity assessment provided a different insight into the diversity of diazotrophs in sugarcane compared to the enrichment/cultivation approach. Ando et al. (2005) found a high prevalence of *nifH* genes related to *Bradyrhizobium* sp., *Klebsiella* sp. and *Serratia* sp. in field-grown sugarcane in Japan. Reiter et al. (2002) found that more than 50% of the *nifH* genes recovered from sweet potatoes were related to *Rhizobium* spp. The *nifH* gene diversity has also been extensively studied in *Leptochloa fusca* (Kallar grass) (Hurek et al. 2002), sweet potato (Terakado-Tonooka et al. 2008) and maize (Roesch et al. 2008), which have also indicated that there is a large diversity of uncultured endophytic diazotrophs in diverse plants.

Currently, it is not well understood, how bacteria-mediated plant growth promotion takes place in sugarcane. The bacteria used in inoculations to date have the ability to fix molecular nitrogen under laboratory conditions, but it has not been definitely proven, how BNF and plant growth promotion occurs *in planta*. These bacteria are also able to promote plant growth by producing and secreting phytohormones, reducing ethylene content, increasing the availability of rare nutrients or inhibiting phytopathogens. Thus, many plant-associated diazotrophic bacteria may have the general ability to promote plant growth. Analysing the transcription of the *nifH* gene, which encodes for nitrogenase reductase, in different plant tissues will certainly provide new insights into the active nitrogen-fixing bacteria that are associated with sugarcane. Furthermore, high-resolution cloning and sequencing techniques are needed to trace diazotrophs that are inoculated into plant tissues because they may be very similar to indigenous endophytic strains that already reside within the plant, and with which they have to compete. In addition, the important question of whether or not the inoculated strains contribute to the BNF of the plant can only be answered by using transcriptional fusions with a visible or fluorescent reporter proteins in model experiments or by analysing *nifH* transcripts in field grown plants.

Methods

Field experiment, sugarcane inoculation and reference bacteria

The field trial was located at the EMBRAPA Agrobiologia research farm (22° 45′S, 43° 40′W and 26 m above sea level), Seropédica, Rio de Janeiro, Brazil. The soil (Itaguaí series) at this site is classified as an Planosol (FAO) or Typic Fragiaquult (USDA, Soil Taxonomy). Soil characteristics tested in accordance with EMBRAPA (in the year 1997) of the 0–20 cm layer: pH in H_2O 5.4; 1.1 $cmol_c$ Ca^{2+} dm^{-3}; 0.2 $cmol_c$ Mg^{2+} dm^{-3}; 0.1 $cmol_c$ Al^{3+} dm^{-3}; 26.1 mg P dm^{-3}; 27.0 mg K dm^{-3}, 0.48% organic carbon, 0.83% organic matter, and 0.043% N. The experiment was conducted in 26.4 m^2-randomised plots, which were separated by a distance of 1.1 m between plots. Two nitrogen treatments were applied: unfertilised and nitrogen fertilised with 120 kg N (urea) ha^{-1}. The unfertilised plots were either left uninoculated (control) or inoculated with a polymeric liquid inoculant (IPC 0.8) or, alternatively, with a polymeric gel inoculant (IPC 2.2). Carboxymethyl cellulose and starch polymers, mixed at a ratio of 3:2, were used as the inoculation vehicle at 0.8 and 2.2 gl^{-1} as described by da Silva et al. (2009). The mixed inoculum of diazotrophs was developed by EMBRAPA-CNPAB (Seropédica, RJ, Brazil) and contained *Gluconacetobacter diazotrophicus*, *Azospirillum amazonense*, *Burkholderia tropica*, *Herbaspirillum seropedicae* and *H. rubisubalbicans* (Oliveira et al. 2006). The sugarcane variety RB867515 was planted in a long-term trial in 2007. The field plots were inoculated at planting and inoculation was repeated every year after harvesting. In March 2009 (end of summer season, 6 months after last inoculation), selected plants from each treatment were dug out and samples of roots and leaf sheaths were cut, washed twice with tap water and were immediately frozen in liquid nitrogen in the field. Combined samples from the same treatments were analysed.

Several *Bradyrhizobium* strains from the Center for Genomic Sciences, Universidad Nacional Autónoma de México culture collection were used for comparative phylogenetic analysis. They were isolated in the laboratory from soils of the Los Tuxtlas rainforest in Veracruz, Mexico, using the legumes *Macroptilium atropupureum* (siratro) or *Vigna unguiculata* (cowpea) as trap plants (Ormeño-Orrillo et al. 2009).

DNA/RNA extraction and PCR approaches

RNA and DNA extractions were performed using a classical phenol-chloroform extraction method combined with the column-based AllPrep DNA/RNA Mini Kit (Qiagen, Germany) to purify and isolate RNA and DNA from 400 mg plant material as described by Toewe et al. (2011). Contaminating DNA in the RNA extractions was detected using a 16S rRNA coding gene targeted PCR with the primers 341F and 907R (Muyzer et al. 1996). Contaminating DNA was removed using RQ1 RNA-free DNase (Promega, USA). cDNA was synthesised with an Omniscript RT Kit (Qiagen, Germany) using random primers (Promega, USA).

All PCR reactions were performed using a TopTaq Kit (Qiagen, Germany) in a PEQStar 96 Universal thermocycler (Peqlab, Germany). 16S rRNA cDNA was amplified using the primers 341F and 907R (length about 550 bp, Muyzer et al. 1996). The annealing temperature for the PCR reaction was 56.5°C and had 30 cycles. *nifH* mRNA was amplified using nested PCR

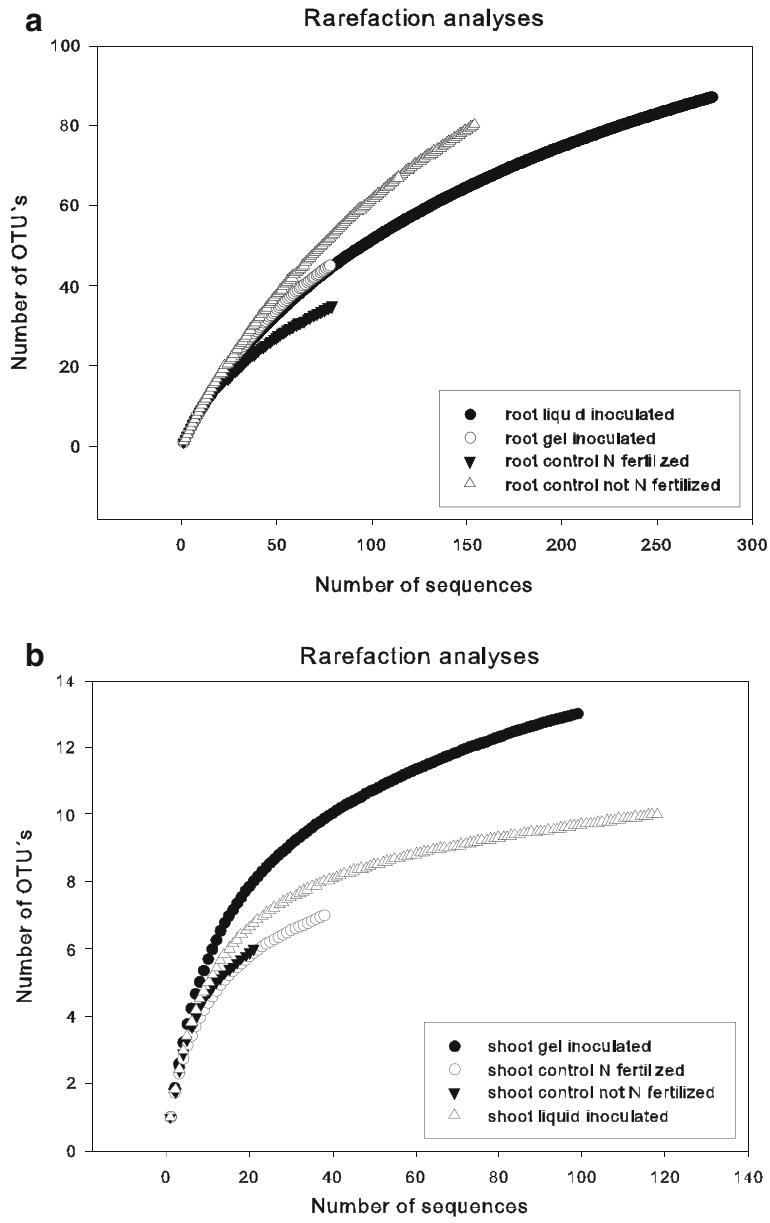

Fig. 1 Rarefaction analyses of 16S-rRNA clone libraries of the root samples (**a**) and leaf sheath samples (**b**). Matrix was calculated with Dotur (97% value)

with the primers PolF and PolR for the initial amplification (Poly et al. 2001) and *nifH*For and *nifH*Rev (length about 314–317 bp) for the second amplification step as described in Roesch et al. (2006), in which the annealing temperature was 55°C, and there were 33 cycles, respectively. Amplicons were verified using agarose gel electrophoresis. Genomic DNA from pure cultures of *H. seropedicae* HCC103, *H. rubrisubalbicans* HRC54, *A. amazonense* CBAmC, *B. tropica* Ppe8, and *G. diazotrophicus* PAL5 (diazotrophs in the inoculation mix) were used as a positive *nifH*-amplification control using this nested PCR approach.

16S rDNA and *nifH* gene-fragments from cultured bradyrhizobia were amplified with primers fD1 and rD1, and nifHF and nifHI, respectively, as described (Laguerre et al. 2001; Weisburg et al. 1991).

Cloning and sequencing

Amplicons were purified using a NucleoSpin® Extract II kit (Macherey-Nagel, Germany) and cloned using a Topo TA Cloning® Kit (Invitrogen, USA) with pCR®2.1-TOPO® vector according to the manufacturer's instructions. All inserts were verified by PCR upon their correct size using multiple cloning site flanking M13F and R promotor sequences. Plasmids were isolated using a NucleoSpin®Plasmid Kit (Macherey-Nagel, Düren, Germany). The quantity and purity of the plasmid extracts were spectrophotometrically measured at 260 nm (NanoDrop Technologies, USA) (Miller 2001).

Sequencing reactions were performed using a BigDye Terminator (BDT) v3.1 Sequencing Kit with the primers M13F, M13R and 341F on an ABI 3730 sequencer (Applied Biosystems, Germany).

Sequences were verified using Finch TV 4.1 (Geospiza, USA) and assembled using Lasergene® software (DNASTAR, USA). Chimera check of 16S rRNA coding gene sequences was performed using the chimera test programs Bellerophon (http://foo.maths.uq.edu.au/~huber/bellerophon.pl, Huber et al. 2004), Pintail and Mallard (www.bioinformaticstoolkit.org, Ashelford et al. 2005) and Chimera Check from RDP (www.rdp.cme.msu.edu, Cole et al. 2009). Potential chimeric sequences were excluded from subsequent analyses. 16S rRNA sequences were aligned with the help of the SILVA comprehensive ribosomal RNA databases (www.arb-silva.de, Pruesse et al. 2007). Phylogenetic analyses were performed using the ARB software package (www.arb-home.de, Ludwig et al. 2004). For this purpose, we used the updated SILVA small-subunit rRNA gene database (SSU 102) as a basis for the sequence analysis. For the phylogenetic analyses of the *nifH* transcripts, we used the updated *nifH* database from Zehr et al. (http://www.es.ucsc.edu/~wwwzehr/research/database/). Sequences were imported into the databases, and the alignments were checked and manually corrected. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura et al. 2007). Phylogenetic trees were constructed by applying neighbour-joining (Saitou and Nei 1987) and max-

Table 1 Phylogenetic affiliation (phylum and class level) of the 16S-rRNA cDNA sequences (% of total clones analysed per sample type)

	Root liquid inoculated	Root gel inoculated	Root control not Nitrogen fertilised	Root Nitrogen fertilised	Leaf sheath liquid inoculated	Leaf sheath gel incoulated	Leaf sheath control not Nitrogen fertilised	Leaf sheath Nitrogen fertilised
Alpha-*Proteobacteria*	32	50	42	29	100	98	81	97
Beta-*Proteobacteria*	3	2	3	1.5	0	2	19	0
Gamma-*Proteobacteria*	2	4	0	1.5	0	0	0	0
Delta-*Proteobacteria*	5	5	11	11	0	0	0	0
Actinobacteria	20	14	17	7	0	0	0	0
Firmicutes	1	0	0	0	0	0	0	0
Bacteroidetes	0	0	0	0	0	0	0	3
Acidobacteria	1	2	2	0	0	0	0	0
Verrucomicrobia	0	0	1	0	0	0	0	0
Planctomycetes	36	23	25	50	0	0	0	0

imum parsimony (Fitch 1966) methods. Rarefaction curves were generated using the software Dotur (Schloss and Handelsman 2005).

Results

Community analysis of sugarcane associated bacteria

A diversity analysis of active diazotrophic bacterial communities in roots and shoots of sugarcane plants (variety RB867515) growing at the EMBRAPA-CNPAB field station in Seropedica, RJ in March 2009 was conducted by constructing and analysing 16S rRNA cDNA and *nifH* cDNA clone libraries. In this study, we investigated the diversity of diazotrophs in uninoculated and inoculated plants and in unfertilised vs. N-fertilised plots. The main focus was on liquid-inoculated plants grown on unfertilised plots; 21 to 279 16S rRNA cDNA sequences and 15–48 *nifH* cDNA sequences per tissue and treatment were obtained (Table S1).

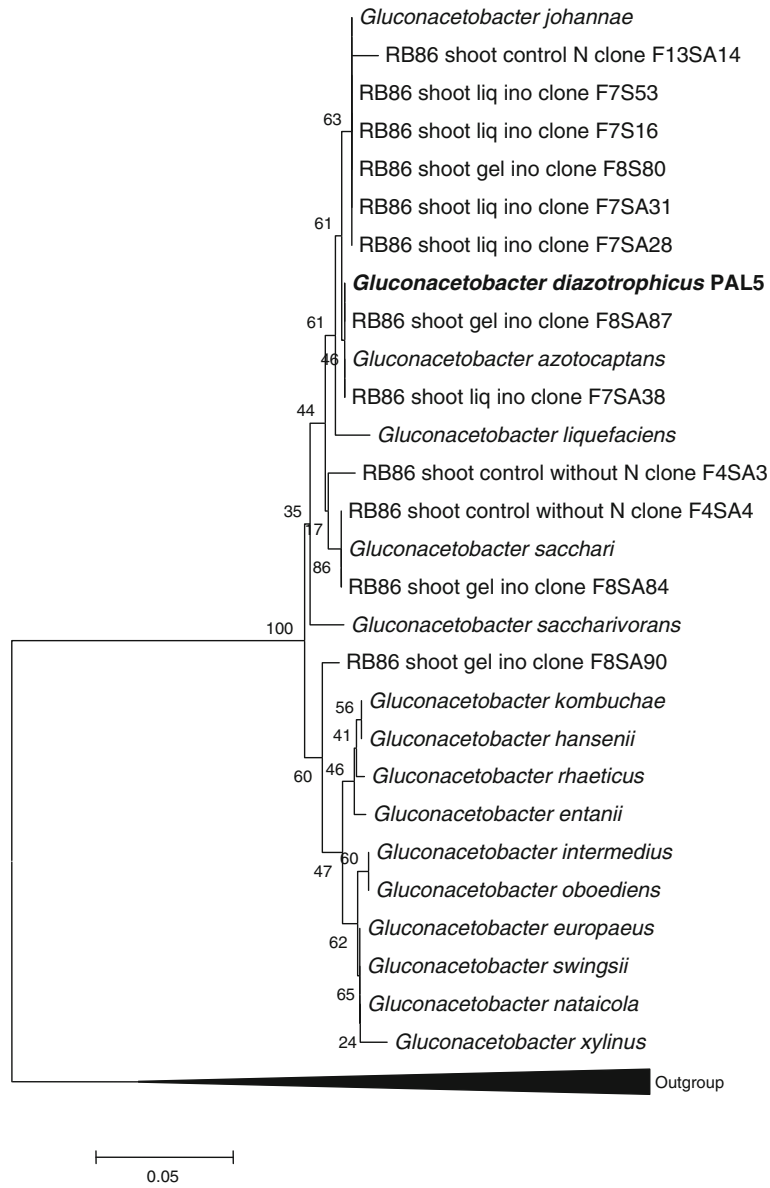

Fig. 2 16S rRNA cDNA *Gluconacetobacter*. Evolutionary relationships of 16S rRNA sequences achieved in this study to sequences of type strains (accession numbers in Table S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

Although the rarefaction analyses of 16S rRNA sequences from root samples showed no saturation (Fig. 1a), the shoot samples showed a considerable degree of diversity saturation (Fig. 1b). Rarefaction analyses of *nifH* cDNA clone libraries showed saturation in each tissue and treatment with 1 to 8 OTUs per treatment (data not shown).

Diversity of 16S rRNA cDNA clone libraries

Clones harbouring sequences of Alpha-*Proteobacteria* were the most abundant in all root and shoot samples, which reached 80–100% in the shoots (Table 1). They mostly belong to the families *Acetobacteraceae*, *Bradyrhizobiaceae*, *Rhizobiaceae* and *Rhodobactera-*

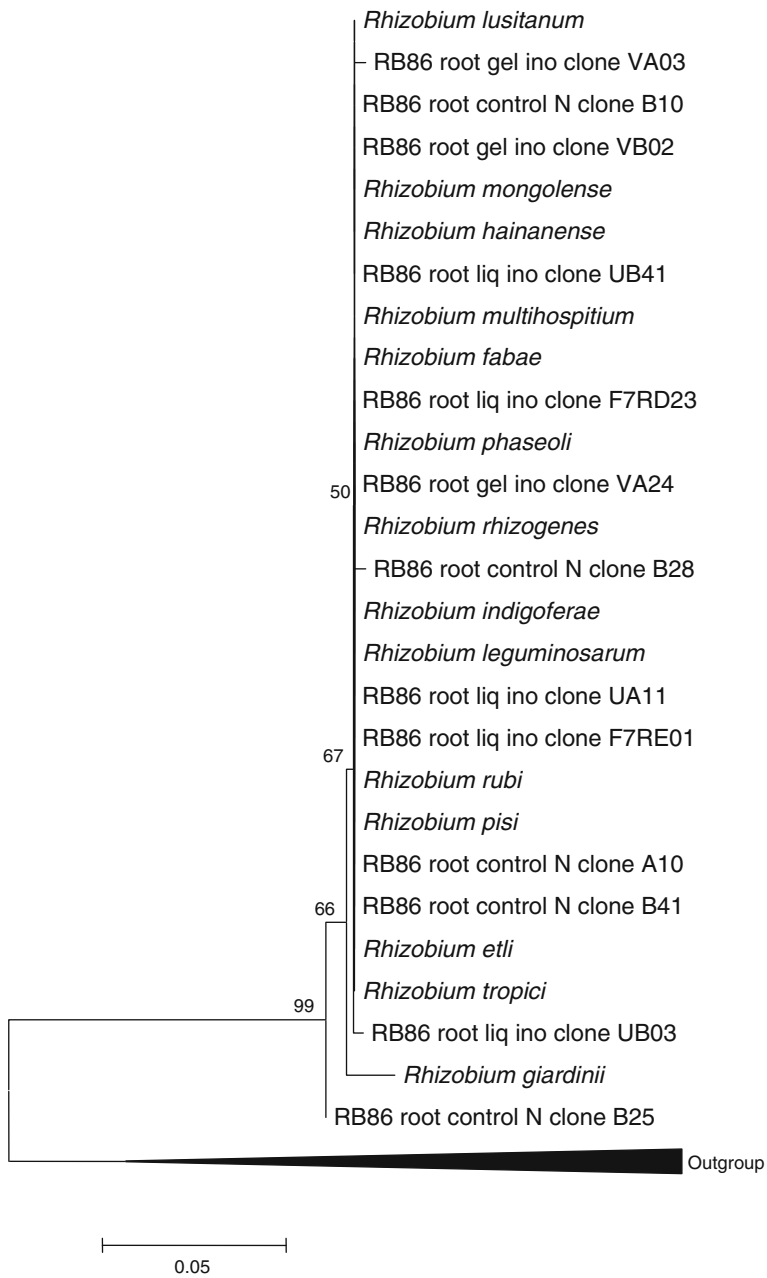

Fig. 3 16S rRNA cDNA *Rhizobium.* Evolutionary relationships of 16S rRNA sequences achieved in this study to sequences of type strains (accession numbers in Table S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st +2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

cea. In leaf sheath tissues from all the treatments, we found mostly sequences that are closely related to the genus *Gluconacetobacter* (Fig. 2). Sequences from the family *Acetobacteraceae* were found exclusively in the leaf sheaths of plants from all the treatments, whereas *Rhizobiaceae* sequences were only found in the roots. While 16S rRNA sequences related to *Rhizobium* spp. (Fig. 3) were detected in the roots from all the treatments, sequences related to *Bradyrhizobium* spp. were detected only in the roots from unfertilised plants (Fig. 4). Sequences related to Beta-*Proteobacteria* sequences were found in each root sample and in two leaf sheath samples (Table 1); the 16S rRNA sequences were related to *Burkholderiaceae* (Fig. 5) and *Oxalobacteraceae* (Fig. 6). 16S rRNA sequences related to Gamma- and Delta-*Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes* were found in low numbers and only in the root samples, whereas *Planctomycetes* and *Actinobacteria* showed high relative abundances in the root samples, where they probably resided on soil particles still associated with the roots (Table 1).

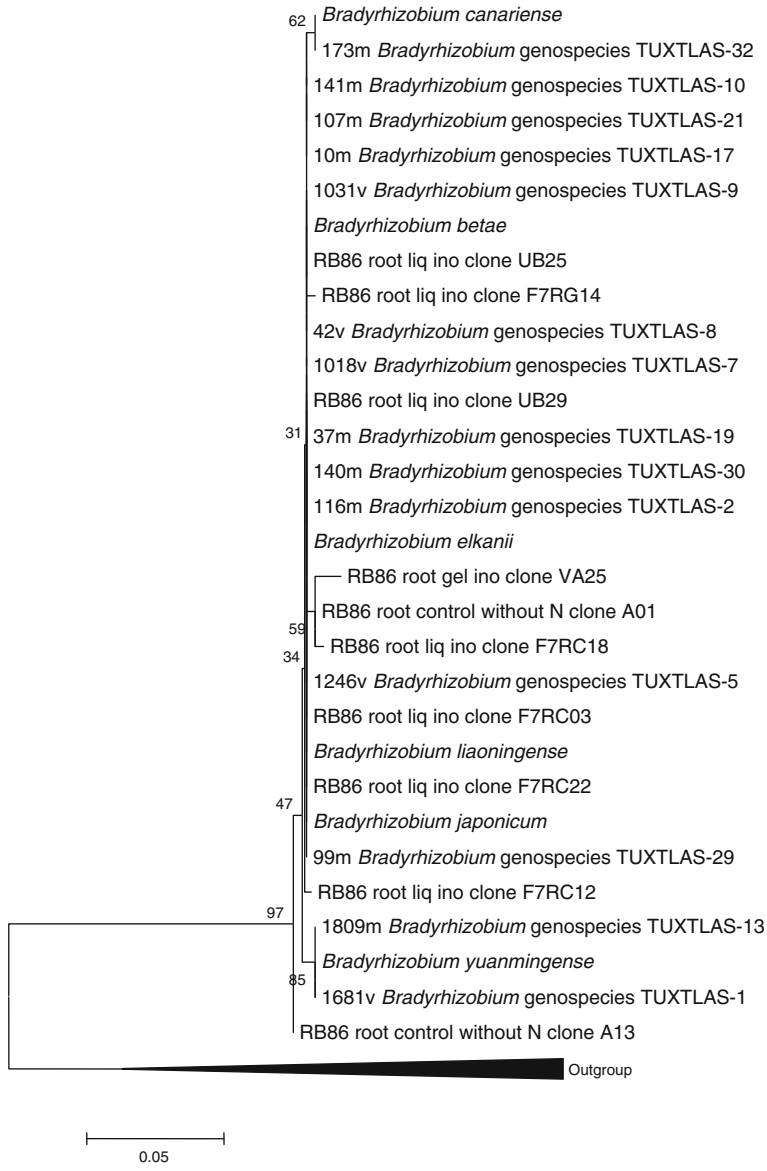

Fig. 4 16S rRNA cDNA *Bradyrhizobium* with sequences from Mexican Tuxtlas forest soil isolates. Evolutionary relationships of 16S rRNA sequences achieved in this study to sequences of type strains (accession numbers in Table S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

Diversity of *nifH* cDNA clone libraries

Frequently, *nifH* cDNA sequences affiliated to Alpha-*Proteobacteria* were found in the roots (*Bradyrhizobium* spp.) and leaf sheaths (*Gluconacetobacter diazotrophicus*). *nifH* cDNA sequences related to *Rhizobium* spp. were found exclusively in the leaves from unfertilised sugarcane (Table 2). In addition, *Azospirillum brasilense* related sequences were found in the roots (Table 2). Phylogenetic analyses of the *nifH* sequences related to

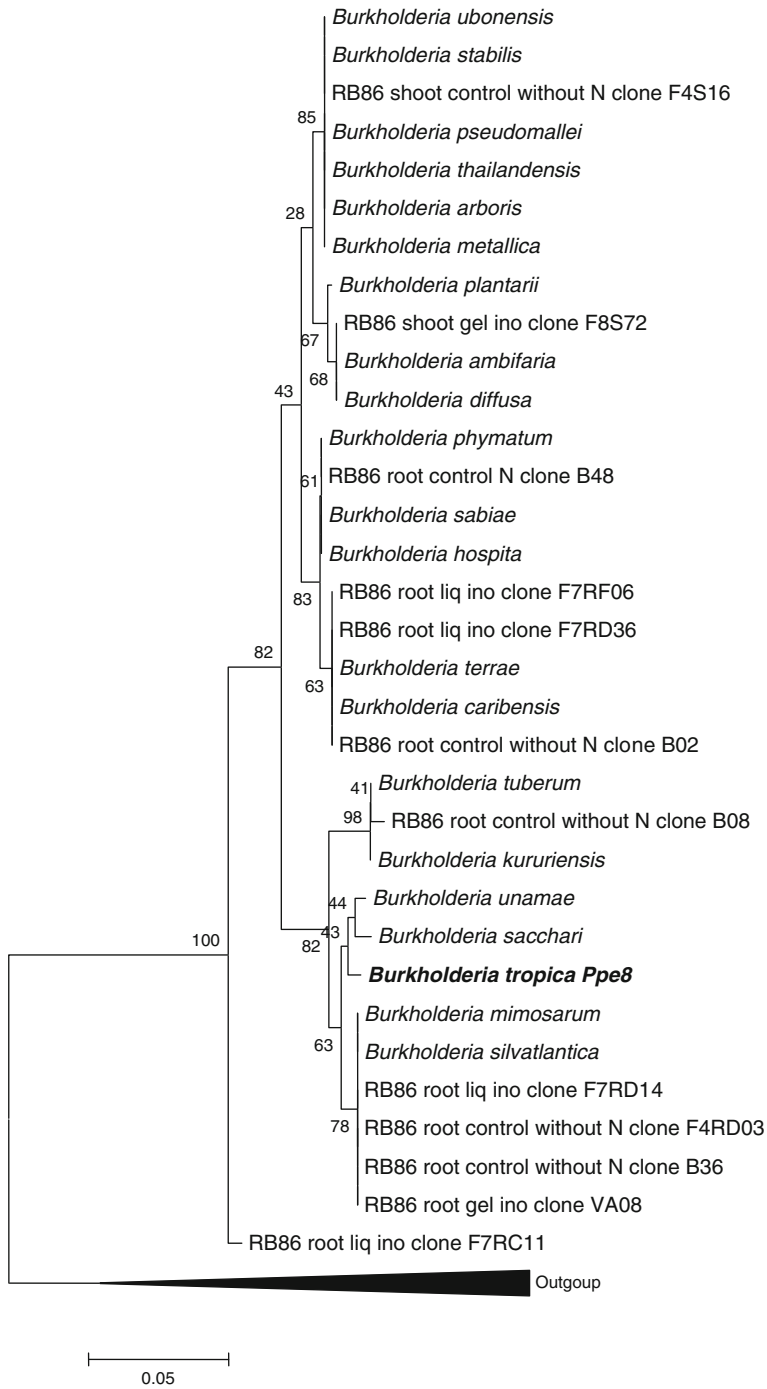

Fig. 5 16S rRNA cDNA *Burkholderia*. Evolutionary relationships of 16S rRNA sequences achieved in this study to sequences of type strains (accession numbers in Table S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st +2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

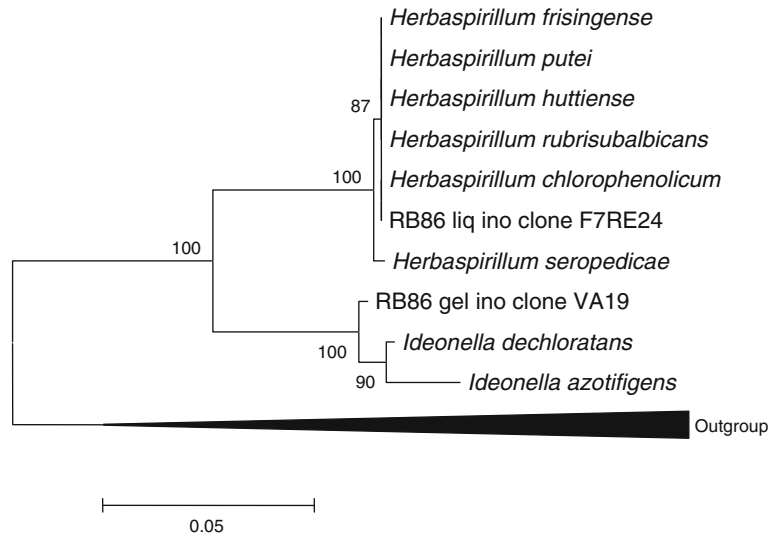

Fig. 6 16S rRNA cDNA *Oxalobacteraceae*. Evolutionary relationships of 16S rRNA sequences achieved in this study to sequences of type strains (accession numbers in Table S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

Rhizobium spp. showed a high similarity to *nifH* sequences from *Rhizobium tropici* and *Rhizobium multihospitum* (Fig. 7).

Phylogenetic analyses of the *nifH*-sequences related to *Bradyrhizobium* spp. showed a close relationship to different validly described (geno)species from *Bradyrhizobium* (Fig. 8). Minor differences between the sequences of known strains themselves and between the sequences in this study do not allow for a phylogenetic classification based on *nifH* sequences. Phylogenetic analyses of the 16S rRNA sequences closely related to *Bradyrhizobium* spp. showed different phylotypes in the genus *Bradyrhizobium* (Fig. 4). These sequences could be detected exclusively in the root samples from unfertilised sugarcane. The highest similarities were found to the species *B. elkanii*. A comparison with the sequences obtained from bacterial isolates of Mexican (Tuxtlas) forest soil

Table 2 Phylogenetic affiliation (genus and species level) of the *nifH*-cDNA sequences (% of total clones analysed per sample type)

	Root liquid inoculated	Root gel inoculated	Root control not Nitrogen fertilised	Root control Nitrogen fertilised	Leaf sheath liquid inoculated	Leaf sheath gel inoculated	Leaf sheath control not Nitrogen fertilised	Leaf sheath Nitrogen fertilised
Azospirillum brasilense	0	8	8	0	0	0	0	0
Gluconacetobacter diazotrophicus	0	0	0	0	39	0	0	0
Bradyrhizobium spp.	69	75	20	67	0	2.5	0	0
Rhizobium spp.	0	0	0	0	18	2.5	10	0
Methylocapsa spp.	0	0	36	6	0	0	0	0
Burkholderia tropica	28	0	24	0	0	0	6	0
Ideonella/*Herbaspirillum* like sequences	3	17	12	27	43	95	84	100

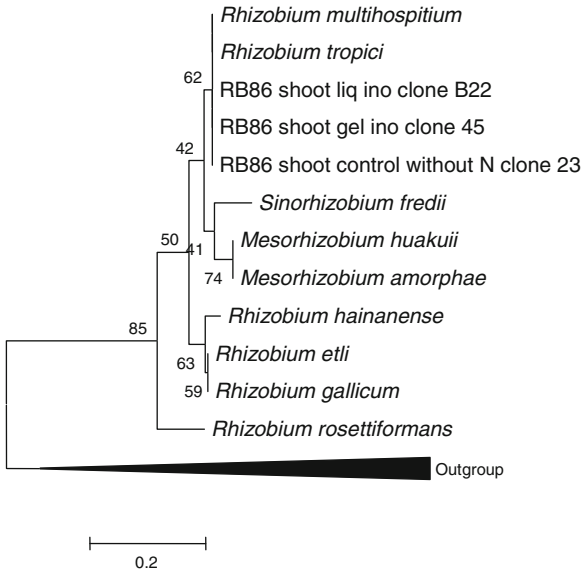

Fig. 7 *nifH* cDNA *Rhizobium*. Evolutionary relationships of *nifH* cDNA sequences achieved in this study to sequences of known diazotrophs (accession numbers in Tables S2 and S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

samples via trap plants showed high similarities to some of our sequences (Fig. 8). Two phylotypes of *Bradyrhizobium* were distinguishable, including *nifH* sequences found in sugarcane roots of all the treatments that showed a high similarity to the *A. amazonense nifH* sequence, which surprisingly resemble the *Bradyrhizobium* phylotype (Fig. 8), as well as to *Bradyrhizobium* sp. isolates from tropical soils that used *Macroptilium atropurpureum* or *Vigna unguiculata* as trap plants.

Sequences affiliated to Beta-*Proteobacteria*, primarily *Herbaspirillum-Ideonella* related sequences, were more abundant in leaf sheaths than in roots (Table 2). Figure 9 shows a close relationship between cloned *nifH* cDNA sequences and *Ideonella azotifigens/ dechloratans*. This group of sequences could be found in the roots and leaf sheaths of all the samples tested. *nifH* cDNA sequences closely related to *Burkholderia tropica* could be detected in the roots and shoots of mostly unfertilised plants (Fig. 10). This finding closely reflected the distribution of related diazotrophic bacteria based on the 16S rRNA cDNA analyses.

Colonization of sugarcane by the inoculated bacteria

A clear PGPR-effect was detected in several field experiments due to the mixed EMBRAPA inoculation (Reis et al. 2008; da Silva et al. 2009). Despite the fact that the rarefaction analysis of 16S rRNA cDNA clone libraries from root and shoot samples are not saturated, we obtained information about the establishment of at least part of the inoculated bacteria. Several sequences of the inoculated bacterial strain *G. diazotrophicus* PAL 5 (Fig. 2) were detected in the leaf sheath tissues from inoculated plants. However, the less frequent detection of the same sequences in uninoculated plants suggests that resident strains of *G. diazotrophicus* are also present in uninoculated plants. 16S rRNA cDNA - sequences related to several *Burkholderia* spp. were found, which were—however—not identical to the inoculant strain Ppe8 (Fig. 5). In the 16S rRNA cDNAclone libraries, none of the other bacterial inoculants were found. This finding may be interpreted to mean that the inoculants are only a very minor part of the bacterial community at the time of sampling.

The analyses of *nifH* cDNA clone libraries showed high abundances of *nifH* cDNA from *G. diazotrophicus* in the leaf sheaths from liquid inoculated plants. Eight *nifH* cDNA sequences obtained from leaf sheaths of liquid inoculated samples showed high similarities >99% to the *nifH* sequence achieved of *G. diazotrophicus* PAL 5, ten other *nifH* cDNA sequences from the same sample showed >85% similarity to the *nifH* gene sequence of *G. diazotrophicus* PAL 5. In addition, five *nifH* cDNA sequences identical to *Burkholderia tropica* Ppe8 were detected in the root and shoots of inoculated and uninoculated plants (Fig. 10). This suggests that the *B. tropica* Ppe8 *nifH* sequence type is part of the resident N_2-fixing bacteria. Alternatively, it may have been distributed into the field from the inoculated plots. *nifH* sequences identical to *A. amazonense* CBAMC (Fig. 8), as well as to *Herbaspirillum seropedicae* HRC54and *H. rubrisubalbicans* HCC103 (Fig. 9) were not detected at the time of sampling.

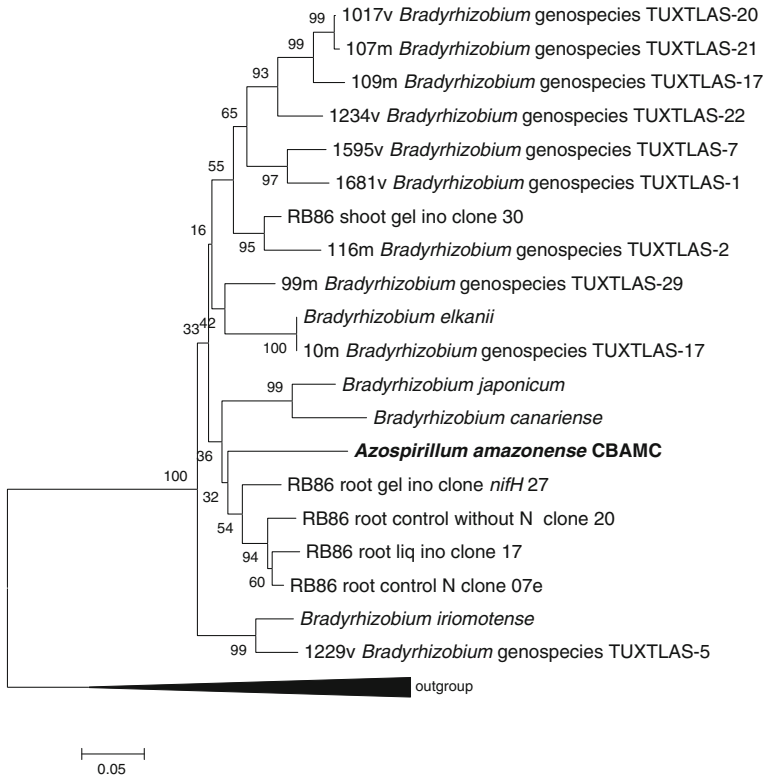

Fig. 8 *nifH* cDNA *Bradyrhizobium* with sequences from Mexican Tuxtlas forest soil isolates. Evolutionary relationships of *nifH* cDNA sequences achieved in this study to sequences of known diazotrophs (accession numbers in Tables S2 and S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

Discussion

Diversity of diazotrophic bacteria associated with sugarcane

Sugarcane is one of the most important crops used in bioethanol production in several countries. Because the energy balance for biofuels needs to be as high as possible, there is a need to reduce high-energy-demanding nitrogen fertiliser, which is usually dependent on fossil fuel sources. Therefore, it is of the utmost importance to increase BNF in the high nitrogen-fixing sugarcane cultivars (Boddey et al. 2003). Endophytic diazotrophic bacteria in sugarcane roots, stems and leaves are commonly accepted as the biological agents of BNF in sugarcane, and thus, a mixed inoculum has been developed by EMBRAPA-CNPAB (Seropédica, RJ, Brazil) that is in the first phase of field testing. However, the diazotrophic bacteria truly fixing nitrogen *in planta* are not known yet. Although many diazotrophs have been described, it is quite possible, that the bacteria responsible for high nitrogen fixation activity are not cultivated yet. Hurek et al. (2002) has shown that the endophytic diazotroph *Azoarcus* sp. BH72 enters a non-culturable phase upon inoculation into plants. Therefore, culture independent experimental approaches are important in discovering uncultured diazotrophs. However, adult field-grown sugarcane plants are difficult to work with and create many experimental challenges. All the DNA/RNA extraction and cleaning methods had to be redesigned and carefully optimised.

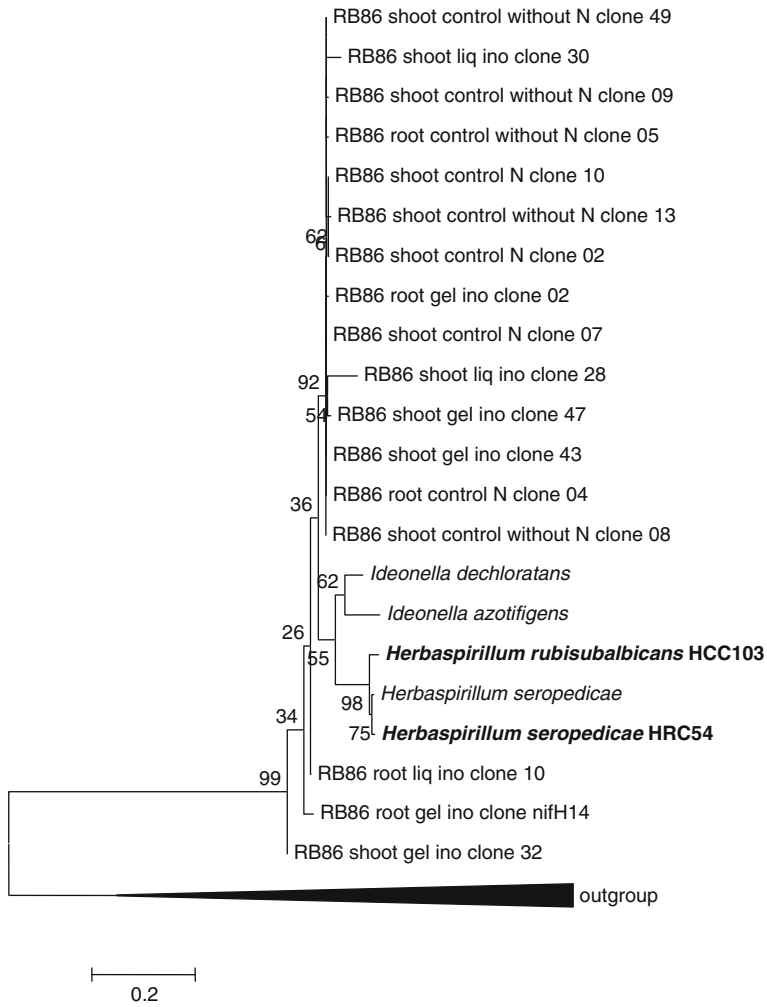

Fig. 9 *nifH* cDNA *Ideonella*/*Herbaspirillum*. Evolutionary relationships of *nifH* cDNA sequences achieved in this study to sequences of known diazotrophs (accession numbers in Tables S2 and S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

The 16S rRNA cDNA and *nifH* cDNA transcript analysis was performed after immediately freezing the root and shoot samples in liquid nitrogen in the field which took maximally 5 min. DNA/RNA extractions were performed from the frozen material in a rapid and efficient extraction procedure, while avoiding all possible conditions where RNAases may be active. Total RNA was first transcribed to cDNA using random primers (Abu Kwaik and Pederson 1996) and the Qiagen Omniscript-RT-kit that had been shown to be non-selective in this reverse transcription step. Specific PCR-systems were then used to amplify 16S rRNA and *nifH* cDNAs. Certainly, the application of different approaches, including varying PCR primer systems, creates differences in the final 16S rRNA cDNA and *nifH* cDNA diversity. In particular, our approach carefully considered that the *nifH* cDNA from the five diazotrophs in the inoculation mix could be detected. Interestingly, the *nifH* cDNA sequence from *A. amazonense* CBAmC clustered within the *Bradyrhizobium* sp. *nifH* genes. This result provides another example that the phylogenetic interpretation of the *nifH* gene cannot be used as phylogenetic marker of the bacterium (like e. g. by 16S rRNA phylogenetic marker gene) because of the possibility of horizontal gene transfer.

Burbano et al. (2011) recently investigated the diversity of *nifH* transcripts in field-grown sugarcane and other plants from three continents. In addition to a very predominant *nifH* transcript phylotype related to *Rhizobium rosettiformans*, which was found in roots, other *nifH* phylotypes were predominant in shoot

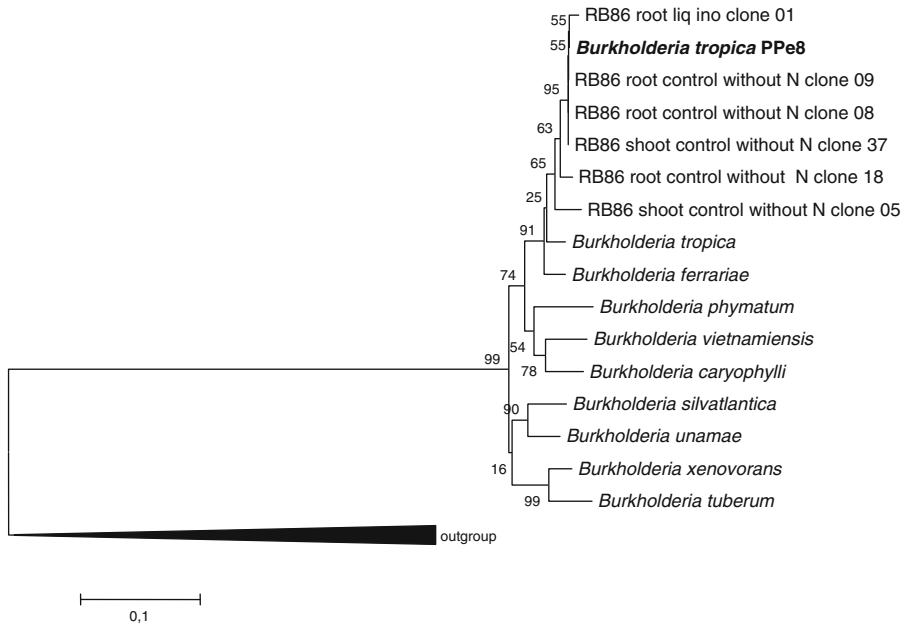

Fig. 10 *nifH* cDNA *Burkholderia.* Evolutionary relationships of *nifH* cDNA sequences achieved in this study to sequences of known diazotrophs (accession numbers in Tables S2 and S3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1981). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

samples and distantly related to *Bradyrhizobium elkanii* or *Sulfurospirillum multivorans.* However, these authors did not detect any of the well-known diazotrophs that have been detected using N-free enrichment approaches, such as *Gluconacetobacter*, *Herbaspirillum* or *Burkholderia*. This could be due to different experimental protocols used for RNA/DNA extraction and cDNA amplification. A similar finding was also recently reported by Thaweenut et al. (2011), who found a predominance of *nifH* sequences related to *Bradyrhizobium* sp., *Azorhizobium caulidonans*, photosynthetic rhizobia and *Rhizobium daejeonese*. In the roots of the Brazilian cultivar RB867515 grown in the Seropédica field station, we found a diversity of *Rhizobium nifH* sequences related to *R. tropici* (Fig. 7). In addition, we have discovered a rich diversity of *nifH* sequences that have some similarity to *B. japonicum nifH* sequences, which are present in isolates from tropical soil that have been enriched using two legume trap plants (Fig. 8). We also found previously unknown *Ideonella/Herbaspirillum nifH* cDNA sequences. Roesch et al. (2008) also found *Ideonella*-like sequences in *nifH* clone libraries from maize plants. Of the previously cultured diazotrophs, only *nifH* transcripts closely related to *G. diazotrophicus* PAL5 were found in the leaf sheaths, and only *nifH* transcripts related to *B. tropica* Ppe8 were found in the roots. These *nifH*-based findings were corroborated by our 16S rRNA cDNA results (Figs. 2 and 5). In addition, we retrieved *nifH* sequences from *Rhizobium* spp. and *Bradyrhizobium* spp. in shoots and roots (Figs. 7 and 8). Therefore, we provide further evidence that, in addition to other previously cultured and uncultured diazotrophs, *Bradyrhizobium* and *Rhizobium* spp. may be contributing to nitrogen fixation in sugarcane. While for *Rhizobium* spp., this is a novel finding, some Bradyrhizobia are known to fix nitrogen in a free-living state or within non-legumes (Kuykendall 2005). These bacteria may have adapted to the endophytic environment of sugar cane in a hitherto unknown way, to fix nitrogen also in a non-leguminous plant. Attempts to localise endophytic bacteria in sugarcane

tissues using fluorescence in situ hybridisation were not successful (data not shown) because of the high level of bacterium-sized auto-fluorescent background particles in the images.

Colonization of inoculated bacteria

Although the rarefaction of 16S rRNA cDNA clone libraries from root samples is not saturated, we could obtain information about the establishment of the bacteria that originated from the inoculation mix and those already residing within the plants. Several sequences identical to the inoculated bacterial strain *G. diazotrophicus* PAL 5 were detected in the leaf sheath tissues from inoculated plants. The detection of identical or very similar sequences in the uninoculated plants suggests that resident *G. diazotrophicus* are present there as well. In the 16S rRNA cDNA clone libraries retrieved from the field experiment in March 2009 (late summer samples), none of the other bacterial inoculants were found. They may have colonised the plants only transiently, which is well known for many bacterial inoculants, such as *Pseudomonas* sp. (Buddrus-Schiemann et al. 2010), but are unable to maintain a constantly high level of colonisation because they are outcompeted. Another possibility could be the lower survival ability of these bacteria in soil (Olivares et al. 1996; Oliveira et al. 2004) which may have affected the initial stages of root colonization.

The analyses of the *nifH* cDNA clone libraries showed high abundances of *nifH* cDNA from *G. diazotrophicus* in the leaf sheaths (not shown) from liquid inoculated plants and *Burkholderia tropica nifH* phylotype (Fig. 10) in the roots from inoculated plants as well as in the root and shoot tissues from uninoculated and unfertilised plants. *Burkholderia tropica nifH* sequences from uninoculated plants suggest that these phylotypes are also present in resident bacteria. Since *nifH*-transcripts of *A. amazonense*, *H. seropedicae* and *H. rubrisubalbicans* sequences were not detected, they apparently do not contribute to nitrogen fixation of the tested sugarcane cultivar at the time of harvest.

Outlook

Our results for the yet uncultured diazotrophs, which belong to the *Bradyrhizobium* spp., *Rhizobium* spp. and *Ideonella/Herbaspirillum* cluster, from the sugarcane cultivar RB867515 and the partial establishment of mostly *G. diazotrophicus* from a mixed inoculum were based on clone library studies. However, the diversity of bacteria detected using a cloning approach is very limited and high-throughput amplicon pyro-sequencing, either focusing on 16S rRNA cDNA or *nifH* cDNA, would certainly allow for a more thorough analysis. This would also enable time course studies of the plant-associated bacterial community in different plant parts during the whole plant development cycle. The diversity of endophytic diazotrophs in sugarcane may be quite versatile depending on the cultivar, soil and climate type. The combined transcript approaches provide a very valuable insight into the active diazotrophic populations in plants. Based on our findings of uncultured diazotrophs in sugarcane, new attempts to enrich and isolate these bacteria in a "molecular guided" enrichment design are now feasible. For example, the use of leguminous trap plants may yield hitherto uncultured *Bradyrhizobium* and *Rhizobium* isolates from sugarcane.

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