# Chapter 94

# Culture-Independent Assessment of Diazotrophic Bacteria in Sugarcane and Isolation of *Bradyrhizobium* spp. from Field-Grown Sugarcane Plants Using Legume Trap Plants

**Luc Felicianus Marie Rouws***
*Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil*

**Doreen Fischer* and Michael Schmid**
*Department of Environmental Sciences, Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany*

**Veronica Massena Reis and José Ivo Baldani**
*Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil*

**Anton Hartmann**
*Department of Environmental Sciences, Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany*

## 94.1 INTRODUCTION

The development and extension of biological based economy, the so-called "sustainable bioeconomy," is a major focus of scientific research and technological development in many countries, including Germany and Brazil. The German government recently launched a 6 billion Euro developmental program to develop a biological based economy. In Brazil, an important focus on development and support is on bioenergy crops such as sugarcane, which have ideal climatic conditions for high biomass development with high sugar content. The global goal for energy crops is to

*These authors contributed equally.

*Biological Nitrogen Fixation, Volume 2*, First Edition. Edited by Frans J. de Bruijn.
© 2015 John Wiley & Sons, Inc. Published 2015 by John Wiley & Sons, Inc.



REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 956



reduce the dependence on fossil energy sources – which are limited, which increase the price, and which, most importantly, cause major climatic problems for Planet Earth. The challenge is to grow energy crops, such as sugarcane, with the lowest inputs of fossil energy that is required to drive the industrial Haber–Bosch process of reducing atmospheric dinitrogen to ammonia, the basis for plant nitrogen fertilizer. 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 was planted in Brazil (IBGE, 2010) and approximately half of the cane juice has been fermented to produce alcohol as a biofuel. In contrast to many other energy crop systems, the overall energy balance in Brazilian sugarcane/bioethanol production is clearly positive (Boddey et al., 2008). For most other bioenergy processes, the overall energy balance is around one or even below. Since the energy to be spent for the synthesis of industrial nitrogen fertilizer is a major input, the reduction of fertilizer application would make the energy production even more attractive and efficient in terms of global climate change impact.

The biological nitrogen fixation (BNF) potential of sugarcane with its associated bacteria had come into focus already some decades ago (Döbereiner, 1961; Ruschel et al., 1975). Using $^{15}$N-isotope enrichment tracing and natural abundance techniques, it was demonstrated that some sugarcane cultivars could derive up to 70% of plant nitrogen from BNF (Lima et al., 1987; Urquiaga et al., 1992; Boddey et al., 2001; see Chapter 108). A major breakthrough in the characterization of the diazotrophic bacteria, especially in plant associations, was the use of nitrogen-free semisolid media, such as NfB, JNfB, or LGI (Döbereiner, 1995), to efficiently enrich microaerobic nitrogen-fixing bacteria. This resulted in the characterization of many new diazotrophic species, such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia tropica*, *Azospirillum amazonense,* and others (Cavalcante and Döbereiner, 1988; Reis et al., 2004; Perin et al., 2006; Baldani and Baldani, 2005; see Chapter 88). However, it could not yet be determined which of these bacteria contributed to the plant nitrogen fixation. It became clear that a diversity of diazotrophic bacteria are distributed in the whole plant, colonizing roots, stem, and leaves endophytically (James et al., 1994). Inoculation experiments with single or mixtures of diazotrophic bacteria were performed, which finally led to the development of the five-strain inoculum introduced by Embrapa Agrobiologia, Seropédica (Oliveira et al., 2003; Oliveira et al., 2006; Oliveira et al., 2009). These inoculations are presently being performed at different places in Brazil. Significant plant growth promotion effects have been found in production-scale field trials, but the level of BNF could not be increased significantly (Schultz et al., 2012). It also became apparent that the diversity of diazotrophic bacterial community of sugarcane plants is quite variable in different regions and cultivars (Asis et al., 2000; Asis et al., 2002; Magnani et al., 2010; Beneduzi et al., 2013; Suman et al., 2001). While a diversity of *Gluconacetobacter diazotrophicus* was found in many studies on different continents using LGI-semisolid media as an enrichment approach, in some other trials it was almost completely missing. In general, within the bacterial diversity retrieved from sugarcane by regular cultivation techniques without enrichment for diazotrophs, the diazotroph community was only found as a minor fraction (Magnani et al., 2010).

An alternative to the cultivation approach is the application of cultivation-independent molecular approaches, using 16S rDNA and *nifH*- or *nifD*-DNA probes, (see Chapters 3, 8, 20, 77) which have the advantage of covering also organisms that are slow growing or generally difficult to grow in pure culture. Several other plants have been investigated in detail with this approach, such as potato, maize, or rice (Roesch et al., 2006; Sessitsch et al., 2002, 2012), which has led to new insights in the diversity of diazotrophs in these systems and the overall bacterial community. Concerning sugarcane, Ando et al. (2005) retrieved for the first time *nifH*-sequences from sugarcane. More recently, Thaweenut et al. (2011), Burbano et al. (2011), and Fischer et al. (2012) studied *nifH*-transcripts in different sugarcane cultivars under different growth conditions. They found, besides *nifH*-sequences of other diazotrophs, also *nif*-transcripts of *Bradyrhizobium* and *Rhizobium* spp. In this chapter, the characterization of the diversity of active bacteria (16S cDNA) and active diazotrophs (*nifH* cDNA) in the sugarcane cultivar RB867515 is described, and the cultivation of *Bradyrhizbium* spp. from this plant using the legume trap plant *Vigna unguiculate* (cowpea) and direct cultivation on plates is presented.

## 94.2 MATERIALS AND METHODS

### 94.2.1 Greenhouse and Field Experiments

Greenhouse and field experiments were conducted at the Embrapa Agrobiologia CNPAB institute and experimental field station (22°45′S, 43°40′W and 26 m above sea level) in Seropédica, Rio de Janeiro, Brazil. The soil (Itaguai series) is classified as a Planosol (FAO) or Typic Fragiaquult (USDA, Soil Taxonomy). The soil characteristics in the 0–20 cm layer were 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. In the greenhouse experiment the cultivar RB867515 was tested in a Sand-Teosint Mix with six plants per pot; after 4 weeks the plants were harvested.

REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 957



In the field experiments, the plants (cultivar RB867515) were grown for 2 years; the sugarcane plants were analyzed in late summer (March) of the first and second year. While there was no N-fertilization in the greenhouse experiment, there were plots in the field experiments that received urea (120 kg/ha). When the sugarcane plants were inoculated with the five-strain Embrapa-inoculum, the plots received no N-fertilization; more details are presented in Fischer et al. (2012).

### 94.2.2 DNA/RNA Extraction and PCR Approaches Characterizing the Bacterial Diversity *in planta*

RNA and DNA extractions of plant material were performed using the 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 (polymerase chain reaction) using the primers 341F and 907R (Muyzer et al., 1996). Contaminating DNA was removed using RQ1 RNA-free DNase (Promega, USA). An important step in this reverse transcription polymerase chain reaction (RT-PCR) protocol was that the cDNA was synthesized with the Omniscript RT Kit (Qiagen, Germany) using random primers (Promega, USA) to achieve a mostly unbiased reverse transcription. All PCR reactions were performed using the 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 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 the second PCR, the annealing temperature was 55 °C, and 33 cycles were used. Amplicons were verified using agarose gel electrophoresis. Genomic DNA from pure cultures of *Herbaspirillum seropedicae* HCC103, *H. rubrisubalbicans* HRC54, *Azospirillum amazonense* CBAmC, *Burkholderia tropica* Ppe8, and *Gluconacetobacter diazotrophicus* PAL5 (diazotrophs in the inoculation mix) was used as positive *nifH*-amplification controls using this nested PCR approach.

Amplicons of the 16S- and *nifH*-cDNA were purified using NucleoSpin® Extract II Kit (Macherey-Nagel, Germany) and cloned using Topo TA Cloning® Kit (Invitrogen, USA) according to the manufacturer′s instructions. Sequencing was performed using BigDye® Terminator (BDT) v3.1 Sequencing Kit (Invitrogen, USA) on an ABI 3730 sequencer (Applied Biosystems, Germany). See details of sequence analysis in Fischer et al. (2012). Evolutionary relationships in the phylogenetic tree were conducted using ARB software (Ludwig et al., 2004) with a neighbor-joining algorithm (Saitou and Nei, 1987).

### 94.2.3 Preparations for the Isolation of Endophytic Bacteria from Sugarcane

Unlignified white shoot roots from 5-month-old sugarcane plants (cv. RB867515) growing at the Embrapa Agrobiologia experimental field station were collected in June 2012. The roots were thoroughly washed with tap water and surface-disinfected by sequential immersion in 70% ethanol for 30 s, and in undiluted commercial bleach (2.5% hypochlorite) under gentle agitation for 8 min. Then the roots were washed six times with sterile water and the disinfection procedure was evaluated by plating 100 µl of the last washing water on plates containing yeast mannitol agar (YMA) (Vincent, 1970). In addition, the disinfected roots were placed on YMA plates for 10 s before continuing the isolation procedure. These plates were incubated for at least 10 days to permit bacterial growth.

The disinfected roots (1.8 g) were macerated in a sterile mortar and suspended in 17 ml saline solution (NaCl 0.85%). Seeds of *V. unguiculata* (cowpea) were surface-disinfected by immersion in ethanol (70%) and then in undiluted commercial bleach for 5 min. After washing six times with sterile water, the seeds were germinated on water agar (0.8%) for 3 days at 28 °C in the dark.

### 94.2.4 Isolation of Rhizobia from Sugarcane Tissues Using In Vitro Grown *V. unguiculata* Plants

*S*urface-disinfected *V. unguiculata* seedlings were transferred to 150 ml tubes with slopes of agarized Norris solution and 15 tubes were inoculated with 1 ml of sugarcane root suspension each. Negative controls (three plants) received 1 ml sterile saline solution and positive controls were inoculated with 1 ml of a suspension of strain BR3299 ($OD_{600} = 0.1$) (Radl et al., 2014). The plants were grown in a growth chamber at 25 °C with 12 h photoperiod under artificial light. Sterile water was added when necessary. Thirty days after inoculation, the occurrence of nodulation was evaluated. Nodules were collected and superficially disinfected by immersion in ethanol (70%) for 2 min, followed by treatment with undiluted commercial bleach for 3 min. After washing six times with sterile water, nodules were crushed on YMA plates, which were incubated at 28 °C for 7 days. Purity of bacterial isolates was checked by repeated streaking on fresh plates. Trap-plant isolates received names based on the number of the trap plant they were isolated

REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 958



from, followed by a sequential number, as follows: P (# *V. unguiculata* plant - # isolate). Representative isolates were individually inoculated on *V. unguiculata* plants to check the nodulation capability, using the same methodology.

### 94.2.5 Isolation of Rhizobia from Sugarcane Tissues by Direct Plate Cultivation

Aliquots of 100 µl of $10^{-1}$ and $10^{-2}$-fold diluted root extracts were dispersed on plates with modified YMA, containing as the carbon source mannitol (1 g/l) or a combination of the *Bradyrhizobium* spp. preferential carbon sources arabinose (0.5 g/l) and sodium gluconate (0.5 g/l) (Tong and Sadowsky, 1994). Incubation was performed at 28 °C for up to 21 days and slow-growing isolates were selected. Isolates were named as M or AG (mannitol or arabinose/sodium gluconate, respectively) followed by sequential numbers.

### 94.2.6 Genotypic Grouping of the Isolates by ERIC-PCR

ERIC-PCR was carried out with cell suspensions of the isolates under study as template in 20 µl volumes containing GoTaq® DNA Polymerase (Promega) and primers ERIC 1 (5′ATGTAAGCTCCTGGGGATTCAC 3′) and ERIC 2 (5′AAGTAAGTGACTGGGGTGAGCG 3′), basically as described previously (Woods et al., 1993). After PCR cycling, aliquots of 10 µl were loaded on 2% agarose TAE gels and electrophoresed for 4 h at 80 V. The molecular weight marker 1 kb plus ladder (Invitrogen) was used as reference. To determine genetic relatedness of isolates, a dendrogram was constructed based on the gel images that were analyzed using BioNumerics software version 6.6 (Applied Maths, Belgium), as described by Rademaker et al. (2000) with the DICE index, by the unweighted pair-group method of arithmetic averages (UPGMA). Optimization and tolerance levels were adjusted to 1.75.

### 94.2.7 PCR, Sequencing of 16S rRNA and *nifH* Genes, and Phylogenetic Analysis

PCR reactions were conducted using cell suspensions in sterile ultrapure water as template with GoTaq® DNA Polymerase (Promega, USA). 16S rRNA genes were amplified using the primers 27F and 1492R (Lane, 1991). The *nifH* gene was amplified with primers nifHF and nifHI (Laguerre et al., 2001). Reactions of isolates that did not produce amplicons using primers nifHF and nifHR were submitted to PCR with primers PolF and PolR (Poly et al., 2001). The PCR products were partially sequenced using primer 1492R for 16S rRNA and primer nifHI for the *nifH* gene using an ABI 3730xl DNA analyzer (Applied Biosystems, Germany). The 16S rRNA sequences (accession numbers KF113075-KF113106) and the *nifH* sequences (accession numbers KF113052-KF113074) have been deposited in the NCBI GenBank.

All sequences were submitted to blast analyses (Altschul et al., 1997), and for phylogenetic analyses, the sequences from reference strains and sequences obtained in the present study were aligned and analyzed using the ARB software (Ludwig et al., 2004).

### 94.2.8 Acetylene Reduction Assay (ARA)

For ARA, JMV medium (Reis et al., 2004) with some modifications was used, containing (in g/l) 5.0 mannitol, 0.6 $K_2HPO_4$, 1.8 $KH_2PO_4$, 0.2 $MgSO_4$, 0.1 NaCl, 0.02 $CaCl_2{\cdot}2H_2O$, 0.002 $Na_2MoO_4{\cdot}2H_2O$, 0.00235 $MnSO_4{\cdot}H_2O$, 0.0028 $H_3BO_3$, 0.00008 $CuSO_4{\cdot}5H_2O$, 0.00024 $ZnSO_4{\cdot}7H_2O$, 0.0001 Biotin, 0.0002 pyridoxol-HCl, 0.0656 Fe-EDTA, 1.6 agar, pH 6.5. Three dosages of nitrogen source were tested: 0, 0.04, and 0.4 g/l yeast extract. Serum vials (20 ml) were filled with 10 ml semisolid media, inoculated with 20 µl cell suspensions in sterile water and closed with cotton plugs. After 5 days incubation at 30 °C, vials were sealed with rubber stoppers and 10% of the air space was replaced with acetylene gas. After 3 h incubation at 30 °C, 0.5 ml aliquots of the gas phase were analyzed by gas chromatography with flame ionization, using Perkin Elmer F11 equipment with a 50 cm Poropak N column at 70 °C. Nitrogenase activity was determined, after comparison to a standard curve, as the quantity (nmol) of ethylene produced per vial during the 3 h incubation period.

## 94.3 RESULTS

### 94.3.1 Cultivation-Independent Assessment of Diazotrophs in Sugarcane Cultivar RB867515

The characterization of the bacterial community colonizing sugarcane roots and leaf sheaths was first investigated using general primers for 16S rRNA-gene to obtain an overview about the entire communities in the greenhouse experiment. Four weeks after inoculation of the sugarcane plants (cultivar RB867515) with the 5-strain inoculum of Embrapa Agrobiologia, RNA was extracted from roots and leaf sheaths. The composition of bacteria in the samples is presented in Figure 94.1.

The predominance of alpha-proteobacteria is up to 70% especially in the inoculated roots, followed by beta- and delta-proteobacteria. Among the alpha-proteobacteria, sequences of *Acetobacteriaceae*, *Rhodospirillaceae,* and *Bradyrhizobiaceae* were identified. In the leaf sheaths also

REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 959



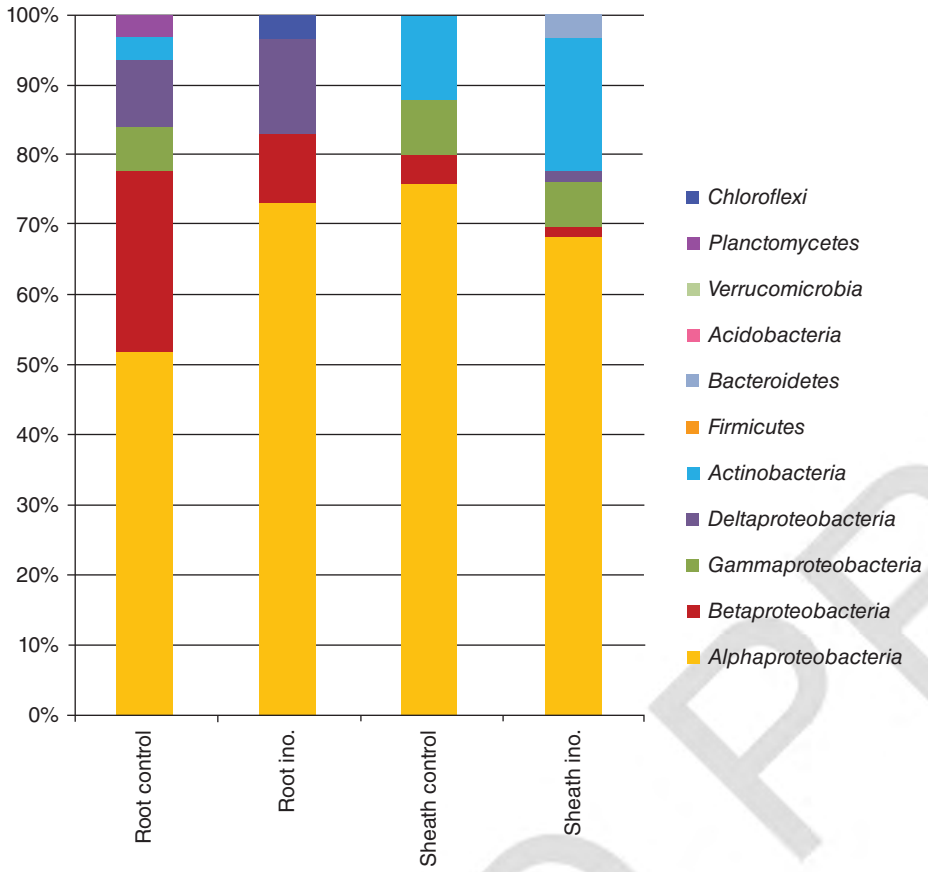


**Figure 94.1** Bacterial composition (based on 16S rRNA cDNA) in root and leaf sheath samples after 4 weeks under greenhouse conditions.

alpha-proteobacteria dominated; after inoculation with the bacterial mixture, *Herbaspirillum* sp. *Gluconacetobacter* and *Bradyrhizobium* were found. Thus, there were indications that the inoculum may have been established, but obviously there were other bacteria, such as bradyrhizobia, regularly present, which may have come from the plant material.

In the field experiment (Fig. 94.2) performed at the field station of Embrapa-Agrobiologia in Seropédica, the effect of N-fertilization and inoculation with the 5-strain bacterial mix was investigated (Fig. 94.3).

At the harvest in March 2008, the difference between 16S rRNA DNA, representing the bacterial community, and cDNA, representing the active bacterial community, is only visible in some subpopulations. Within the alpha-proteobacteria, no sequences could be found that were related to *Gluconacetobacter diazotrophicus* or *Azospirllum amazonense*, which were part of the inoculum. Also, neither *Herbaspirillum seropedica* nor *Burkholderia tropica*-related 16S rRNA DNA-sequences could be identified, while other *Burkholderia*-, *Herbaspirillum*- and *Ralstonia*-related sequences were found (data not shown). Apparently, the strains from the inoculum were not part of the dominating community at the time of sampling (March, late summer).

**Figure 94.2** Field trial at Embrapa Agrobiologia, Seropedica, 2008.

However, there were a number of clone sequences identified related to *Bradyrhizobium* spp. (Fig. 94.4) and other Rhizobiales (not shown).

16S cDNA and the *nifH*-transcripts in sugarcane roots and leaf sheaths of the same fields were investigated in March 2009. Reinoculation with the 5-strain inoculum was performed after the first cut in 2008. A high diversity of 16S cDNA and *nifH*-transcripts could

REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 960



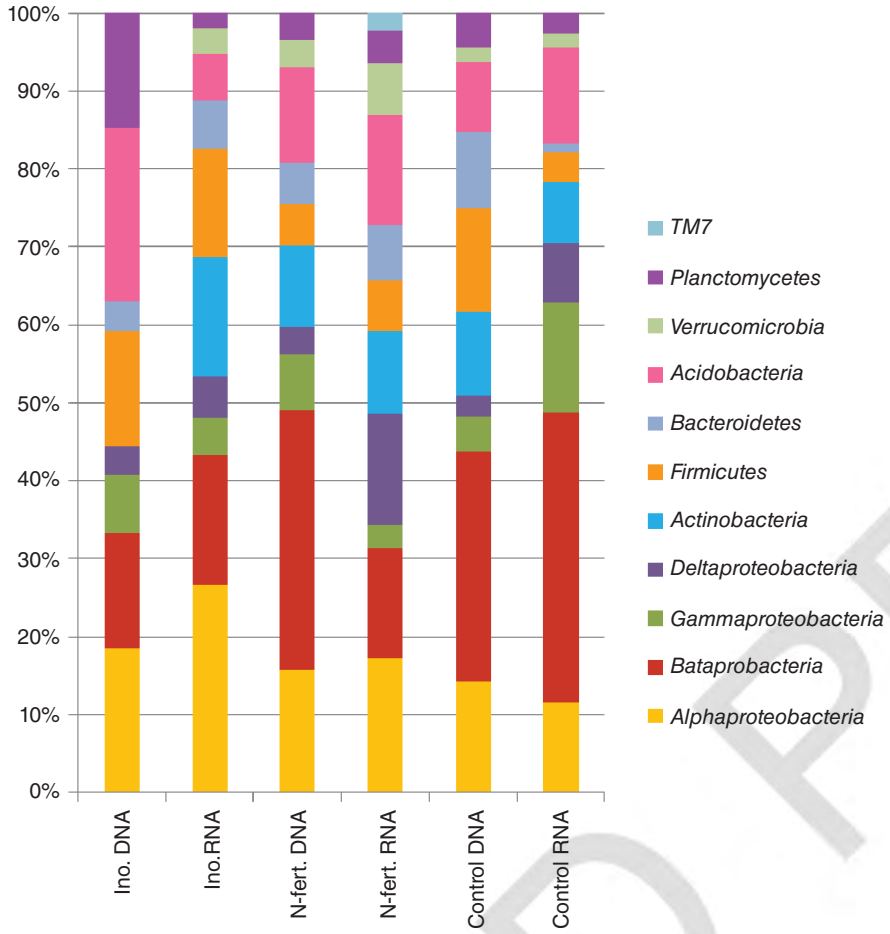


**Figure 94.3** Bacterial composition (16S rRNA sequence diversity from RNA and DNA) from root samples of the field trial 2008.

be retrieved from these sugarcane plants (Fischer et al., 2012). *Gluconacetobacter diazotrophicus nifH*-cDNA, as well as 16S cDNA-sequences identical to the inoculated strain were found in ample amount in leaf sheaths of inoculated sugarcane plants (Fischer et al., 2012). However, 16S cDNA and *nifH*-cDNA of the other inoculant strains could not be found. A detailed sequence analysis of the *nifH*-sequences of each of the inoculation strain revealed that *Azospirillum amazonense* CBAmC as well as *A. amazonense* DSM2787 harbor a *Bradyrhizobium*-like *nifH*-sequence. However, this sequence could also not be found within the *nifH* cDNA-sequences retrieved from the sugarcane plants. However, 16S cDNA- and *nifH* cDNA sequences of a new type of bacterial species within the *Ideonella–Azohydromonas–Herbasprillum* cluster was found very frequently mostly in leaf sheaths (not shown). Besides other not yet cultured bacteria from the *Burkholderiaceae*, many 16S cDNA and *nifH* cDNA-sequences from *Bradyrhizobium* were found in roots (Fig. 94.5). *Rhizobium* spp. sequences were retrieved mostly from leaf sheaths (Fischer et al., 2012).

## 94.3.2 Isolation and Characterization of *Bradyrhizobium* sp. from Sugarcane Cultivar RB867515 by Direct Plate Cultivation and by Using *V. unguiculata* Trap Plants

On the basis of the results obtained with culture-independent approaches, we conducted experiments aiming to isolate bacteria from the genus *Bradyrhizobium* from sugarcane tissues, by inoculating extracts of surface-disinfected sugarcane roots directly on plates or on *V. unguiculata* (cowpea) trap plants. After the disinfection procedure of sugarcane roots, no bacteria grew when water of the final washing step was spread on YMA plates or when disinfected and washed root fragments were placed on YMA plates. This suggested that the procedure was effective in removing the root surface-associated microbial community.

Thirty days after individually inoculating 15 *in vitro* grown *V. unguiculata* plants with 1 ml of the extract obtained by macerating the disinfected roots, 13 of these plants presented nodules from which bacteria could be isolated.

PROOFS
REVISED

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 961



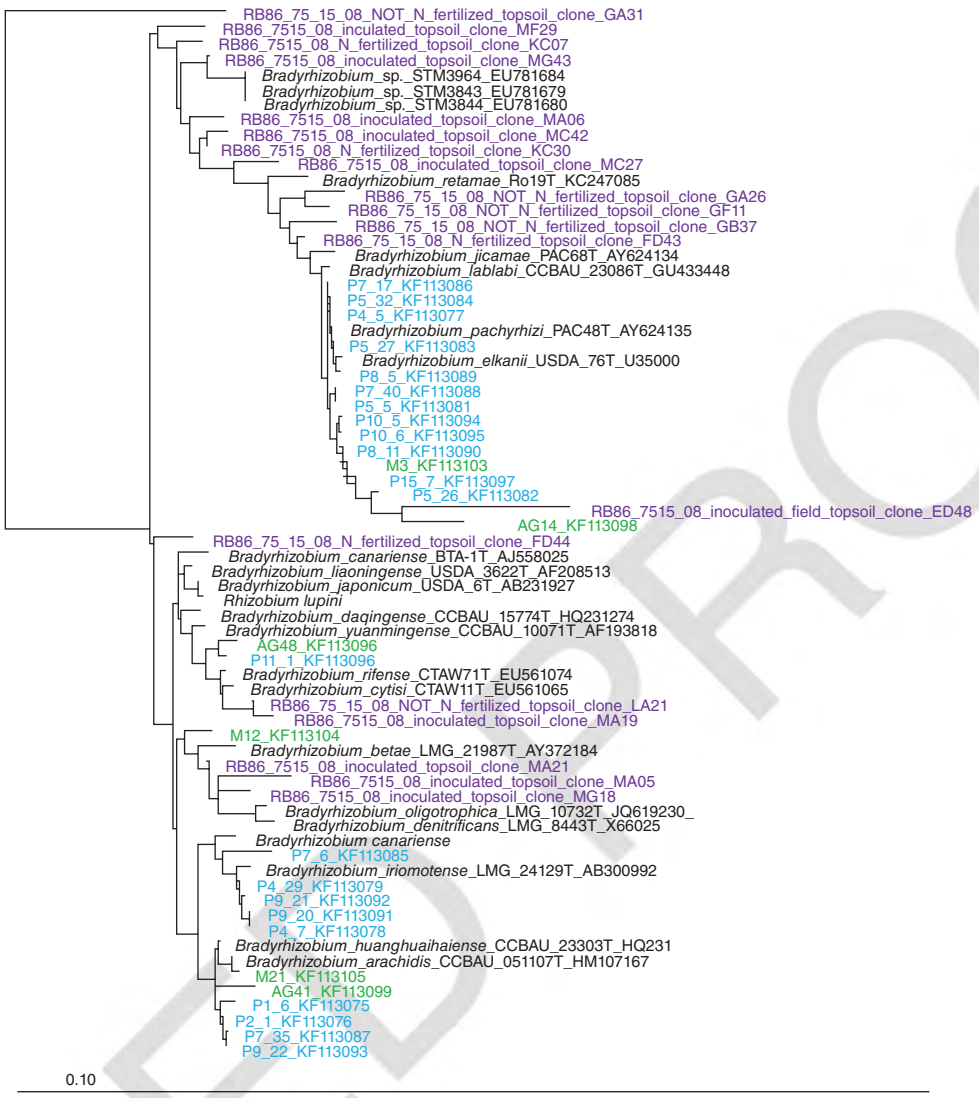


**Figure 94.4** Phylogenetic tree (neigbor-joining) of 16S rRNA gene of *Bradyrhizobium* spp. Sequences were obtained from the field trial 2008 (purple), from plate isolation approach (green), and trap plant approach (blue).

Three un-inoculated control plants did not present nodules, whereas the positive control nodulated abundantly. From the nodules formed on the 13 plants inoculated with sugarcane root extract, a total of 112 bacterial isolates was obtained (Rouws et al., 2014). These isolates received names as follows: P followed by [number of *V. unguiculata* plant]-[isolate number]. The large majority (109 isolates) grew slowly and alkalinized the culture medium, characteristics typical of the genus *Bradyrhizobium* (Kuykendall, 2005). Three isolates (P5-2, P5-21 and P5-25) were fast-growing and showed a neutral/acidic reaction on YMA. These data indicated that *V. unguiculata* nodule-inducing bacteria occur as endophytes in roots of sugarcane cv. RB 867515.

ERIC-PCR fingerprinting was applied to identify genotypic redundancy among the 112 isolates. When all isolates/strains were grouped with a low level of correlation (28% similarity) this analysis revealed 28 distinct ERIC profiles. Some identical fingerprint profiles were shared by up to 36 isolates, showing that some genotypes were redundant in the collection. Isolates sharing identical ERIC-PCR profiles were obtained from nodules of the same plant, but also from nodules of independent *V. unguiculata* plants inoculated with the same aliquot of sugarcane root extract. Some ERIC-PCR profiles (13) represented fewer isolates and several profiles represented unique isolates in the collection. The relative abundance of certain ERIC-PCR profiles may reflect the abundance of the respective bacterial genotype in the sugarcane roots, differences in colonization efficiency of the *V. unguiculata* trap plants by different bacterial genotypes, or differences in bacterial growth rate.

REVISED PROOFS


**Figure 94.5** Phylogenetic tree (neigbor-joining) of *nifH* of *Bradyrhizobium* spp. Sequences were obtained from the greenhouse trial 2009 (pink), from the field trial 2009 (orange, see Fischer et al. (2012)), from plate isolation approach (green), and trap plant approach (blue).

REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 963



Based on the ERIC-PCR profiles, 23 representative isolates were selected for more detailed analyses.

In addition to these trap plant isolates, the direct plate cultivation strategy permitted the selection of eight slow-growing and one fast-growing *nifH*-containing isolates (determined by PCR analysis). Sequence analyses of the 16S rRNA gene of these 32 isolates confirmed that 28 belong to the genus *Bradyrhizobium*, 1 isolate (AG46) was most closely related to the genus *Methylobacterium*, 1 isolate to the genus *Herbaspirillum* (AG47), and the 2 fast-growing isolates (M30 and P5-2) grouped together with species from the genus *Rhizobium* (Fig. 94.4).

When individually inoculated on *in vitro* grown *V. unguiculata* plants, all but one (the fast-growing isolate P5-2) trap plant isolates were able to nodulate. However, among the direct plate isolates only M3 and AG14 were able to induce nodules, whereas the other *Bradyrhizobium* spp. isolates (M12, M21, AG41 and AG48) were not. This observation demonstrated that the culture strategy had a significant influence on the isolation of specific groups of bacteria. It also showed that sugarcane plants harbor besides nodulating *Bradyrhizobium* spp. also Bradyrhzobia that are unable to nodulate a promiscuous legume such as *V. unguiculata*.

For 21 of these selected isolates, the partial *nifH* gene was sequenced and the sequences were aligned with *nifH* genes from reference strains and with the *nifH* cDNA sequences described in the present study (Fig. 94.5). These analyses showed that de *nifH* gene of most isolates, including the nodule-inducing direct plate isolates M3 and AG14, clustered together in a branch with the *B. elkanii* strain USDA $76^T$. It was interesting to note that the *nifH* gene of the other two *Bradyrhizobium* spp. direct plate isolates M12 and AG48 were quite dissimilar from this cluster and were more similar to some *nifH* cDNAs obtained in this study. This emphasized that the culture strategy may greatly influence the diversity of bacteria recovered from an environmental sample.

Twelve representative isolates were tested for *in vitro* nitrogenase activity by ARA. The majority (M3, AG14, P5-2, P8-11, P10-5, P11-1 and P15-7) did not present any detectable activity (data not shown). However, four isolates presented significant nitrogenase activity in a semisolid medium containing 0.4 g/l yeast extract, as determined by the quantity of ethylene produced: M12 (7 nmol), AG48 (51.7 nmol), P9-20 (17.7 nmol), and P9-21 (3.5 nmol). Therefore, some sugarcane endophytic *Bradyrhizobium* spp. could fix nitrogen without being associated to a nodule environment, a prerequisite for sugarcane-associated BNF.

## 94.4 DISCUSSION

The important contribution of microbes to overall plant performance was revived in recent years when the term "plant microbiome" became popular (Turner et al., 2013). Metagenomic approaches enabled the analysis of plant associated microbes, including the endophytic microbial community, with high throughput genome sequencing approaches.

In energy crops, the awareness of the importance of bacterial impacts on plant nutrition and health goes back much longer. Fostered by the successful discoveries by the group of Johanna Doebereiner of several new diazotrophic bacterial genera and species several decades ago, energy plants such as sugarcane were also investigated in detail (Döbereiner, 1961; Döbereiner, 1995; see Chapter 88). In *Pennisetum purpureum,* another promising energy plant for the tropics, thorough estimates of $^{15}N$ natural abundance measurements demonstrated similar high rates of BNF in some cultivars as was found in sugarcane (Urquiaga et al., 2012; Morais et al., 2012). Using cultivation-based approaches, a diversity of diazotrophic bacteria were found in several cultivars of field-grown *Pennisetum* (Videira et al., 2012). Based on enrichment and isolation studies on nitrogen-free semisolid media – such as NfB, JNfB, LGI – *Enterobacter cloacae* and other *Enterobacter* spp. were isolated, which were shown to harbor *nifH*-genes. Besides *Gluconacetobacter diazotrophicus*, diazotrophic *G. sacchari* were also characterized for the first time, which apparently had acquired their *nifH*-genes from *Enterobacter* spp. colonizing *P. purpureum.* When the diversity of diazotrophs was studied with a cultivation-independent approach by cloning the 16S rDNA and *nifH*-gene from roots and stems of *P. purpureum*, a much higher diversity of diazotrophic community, became apparent (Videira et al., 2013). After the experience with sugarcane and the demonstration of a considerable occurrence of *Bradyrhizobium* and *Rhizobium* sequences, it was not a surprise to find *nifH*-transcripts from these bacterial genera also in *Pennisetum* cultivars (Videira et al., 2013). Therefore, similar research efforts should also be brought forward in *Pennisetum* to isolate *Bradyrhizobium* spp. using the legume trap plant approach and other more direct plate isolation techniques, as were applied in sugarcane (Rouws et al., 2014). It is quite obvious that the successful approach of enrichment and isolation of diazotrophic bacteria using nitrogen-free semisolid media leads to the isolation of only a subfraction of the whole community of diazotrophic bacteria from non-leguminous plants.

The occurrence of *Rhizobium* and *Bradyrhizobium* sp. in non-leguminous plants had attracted already quite intense attention, especially in cropping systems, where a monocot crop (such as rice or wheat) and legumes (clover or soybean) are used in alternation for hundreds of years, such as in the Nile valley and delta in Egypt (Yanni et al., 2001; see Chapter 111). A diversity of *Rhizobium leguminosarum*

REVISED PROOFS

Trim Size: 8.5in x 11in deBruijn c94.tex V3 - 05/08/2015 9:20 P.M. Page 964



bv.trifolii isolates could be obtained from rice roots, which colonize rice plants endophytically and exert a plant-growth promoting effect. *Rhizobium leguminosarum* was also shown to colonize roots of wheat and other non-leguminous plants (Schloter et al., 1997). While stimulation of growth of rice by selected *R. leguminosarum* bv. trifolii strains could be demonstrated repeatedly (Yanni et al., 2001; see Chapter 111), evidence for *in planta* nitrogen fixation could not be found. Photosynthetic bradyrhizobia have also been isolated from the African wild rice *Oryza breviculata* (Chaintreuil et al., 2000). A recent study reported on the isolation of an endophytic *Bradyrhizobium* sp. isolate (strain AT1) from sweet potato and ARA analyses and $^{15}$N-isotope dilution studies suggested that this strain might increase plant growth through BNF (Terakado-Tonooka et al., 2013).

As demonstrated in our study, a high diversity of *Bradyrhizobium* and *Rhizobium* bacteria was found active as nitrogen-fixing bacteria in sugarcane, because a large number of transcripts of the *nifH*- and also 16S rRNA genes were found in roots and stems of field grown sugarcane variety RB867515. This constitutes evidence for an important role of these bacteria under natural conditions. Therefore, attempts are now under way to inoculate axenically grown sugarcane plantlets with the *Bradyrhizobium* spp. isolates originating from the same sugarcane cultivar and to demonstrate not only efficient endophytic colonization but also *in situ* active nitrogen fixation. Detailed laboratory studies of these bacteria may find specific adaptations to the metabolic scenario of sugarcane to finally establish an efficiently nitrogen-fixing symbiotic system. A final proof of *in planta* nitrogen fixation would be to demonstrate fixation of $^{15}N_2$-gas by sugarcane plants inoculated with *Bradyrhizobium* spp. isolates originating from sugarcane.

## ACKNOWLEDGMENTS

This contribution is written in memory of Dr. Johanna Döbereiner, who pioneered research on diazotrophic bacteria and nitrogen fixation in energy crops already several decades ago. Financial support has been from CNPq (Instituto Nacional de Ciência e Tecnologia FBN and projeto universal process 477231/2012-8), Faperj (process E-12/111.398/2012), and the Deutsche Forschungsgemeinschaft (Grant Ha 1708/9). Additional support from the Helmholtz Zentrum München and Embrapa is greatly acknowledged.

## REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402.

Ando S, Goto M, Meunchang S, Thongra-Ar P, Fujiwara T, Hayashi H, Yoneyana T. 2005. Detection of *nifH* sequences in sugarcane (*Saccharum officinarum* L.) and pineapple (*Ananas comosus* [L.] Merr.). J. Soil Sci. Plant Nutr. 51: 303–308.

Asis CA, Kubota M, Ohta H, Arima Y, Ohwaki Y, Chebotar VK, et al. 2000. Isolation and partial characterization of endophytic diazotrophs associated with Japanese sugarcane cultivar. J. Soil Sci. Plant Nutr. 46: 759–765.

Asis CA, Kubota M, Ohta H, Arima Y, Ohwaki Y, Yoneyama T, et al. 2002. Estimation of the nitrogen fixation by sugarcane cultivar NiF-8 using $^{15}$N diluation and natural $^{15}$N abundance techniques. J. Soil Sci. Plant Nutr. 48: 283–285.

Baldani JI, Baldani VLD. 2005 History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. An. Acad. Bras. Cienc. 77: 549–579.

Beneduzi A, Moreira F, Costa PB, Vargas LK, Lisboa BB, Favreto R, et al. 2013. Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil. Appl. Soil Ecol. 63: 94–104.

Boddey RM, Polidoro JC, Resende AS, Alves BJ, Urquiaga S. 2001. Use of the $^{15}$N natural abundance technique for the quantification of the contribution of $N_2$ fixation to sugarcane and other grasses. Aust. J. Plant Physiol. 28: 889–895.

Boddey RM, Soares LHB, Alves BJR, Urquiaga S. 2008. Bioethanol production in Brazil. In Pimentel D, editor. Biofuels, Solar and Wind as Renewable Energy Systems. Berkeley: Springer, p. 504.

Burbano CS, Liu Y, Rösner KL, Reis VM, Caballero-Mellado J, Reinhold-Hurek B, Hurek T. 2011. Predominant *nifH* transcript phylotypes related *to Rhizobium rosettiformans* in field-grown sugarcane plants and in Norway spruce. Environ. Microbiol. Rep. 3: 383–389.

Cavalcante VA, Döbereiner J. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant Soil 108: 23–31.

Chaintreuil C, Giraud E, Prin Y, Lorquin J, Bá A, Gillis M, et al. 2000. Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. Appl. Environ. Microbiol. 66: 5437–5447.

Döbereiner J. 1961. Nitrogen-fixing bacteria of the genus *Beijerinckia derx* in the rhizosphere of sugar cane. Plant Soil 15: 211–216.

Döbereiner J. 1995. Isolation and identification of aerobic nitrogen fixing bacteria. In Alef K, Nannipieri P, editors. Methods in Applied Soil Microbiology and Biochemistry. London: Academic Press, pp. 134–141.

Fischer D, Pfitzner B, Schmid M, Simões-Araújo J, Reis VM, Pereira W, et al. 2012. Molecular characterization of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum* sp.). Plant Soil 356: 83–99.

IBGE. 2010. Culturas temporárias e permanentes. Prod. Agríc. Munic., Rio de Janeiro 37: 1–91.

James EK, Reis VM, Olivares FL, Baldani JI, Doebereiner J. 1994. Infection of sugarcane by the nitrogen fixing bacterium *Acetobacter diazotrophicus*, J. Exp. Bot. 45: 757–766.

Kuykendall LD. 2005. Genus *Bradyrhizobium*, family Bradyrhizobiaceae. In Garrity G, editor. Bergey's Manual of Systematic Bacteriology. v.2., 2nd ed. New York: Springer–Verlag, pp. 438–443.

Laguerre G, Nour SM, Macheret V, Sanjuan J, Drouin P, Amarger N. 2001. Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiology 147: 981–993.

Lane DJ. 1991. 16S/23S rRNA sequencing. In Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in Bacterial Systematics. New York: John Wiley and Sons, pp. 115–175.

Lima E, Boddey RM, Döbereiner J. 1987. Quantification of biological nitrogen fixation associated with sugarcane using a $^{15}$N aided nitrogen balance. Soil Biol. Biochem. 19: 165–170.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, et al. 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32(4): 1363–1371.

REVISED PROOFS

Magnani GS, Didonet CM, Cruz LM, Picheth CF, Pedrosa FO, Souza EM. 2010. Diversity of endophytic bacteria in brazilian sugarcane. Genet. Mol. Res. 9: 250–258.

Morais RF, Quesada DM, Reis VM, Urquiaga S, Alves BJR, Boddey RM. 2012. Contribution of biological nitrogen fixation to Elephant grass (Pennisetum purpureum Schum.) Plant Soil 356: 23–34.

Muyzer G, Hottentraeger S, Teske A, Wawer C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA – a new molecular approach to analyze the genetic diversity of mixed microbial communities. In Akkermans ADL, van Elsas JD, De Bruijn F, editors. Molecular Microbial Ecology Manual. Chapter 3.4.4.1. Dordrecht: Kluwer Academic, pp. 1–23.

Oliveira ALM, Canuto EL, Reis VM, Baldani JI. 2003. Response of micropropagated sugarcane varieties to inoculation with endophytic diazotrophic bactéria. Braz. J. Microbiol. 34: 59–61.

Oliveira ALM, Canuto EL, Urquiaga S, Reis VM, Baldani JI. 2006. Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. Plant Soil 284: 23–32.

Oliveira ALM, Stoffels M, Schmid M, Reis VM, Baldani JI, Hartmann A. 2009. Colonization of sugarcane plantlets by mixed inoculations with diazotrophic bacteria. Eur. J. Soil Biol. 45: 106–113.

Perin L, Martínez-Aguilar L, Castro-González R, Estrada-de Los Santos P, Cabellos-Avelar T, Guedes HV, et al. 2006. Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Appl. Environ. Microbiol. 72: 3103–3110.

Poly F, Monrozier LJ, Bally R. 2001. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res. Microbiol. 152: 95–103.

Rademaker JLW, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P, de Bruijn FJ. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA--DNA homology studies: *Xanthomonas* as a model system. Int. J. Syst. Evol. Microbiol. 50: 665–667.

Radl V, Simões-Araújo JL, Leite J, Passos SR, Martins LMV, Xavier GR, et al. 2014. *Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in the semi-arid of Brazil. Int. J. Syst. Evol. Microbiol. 64: 725–730.

Reis VM, Estrada-de los Santos P, Tenorio-Salgado S, Vogel J, Stoffels M, Guyon S, et al. 2004. *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. Int. J. Syst. Evol. Microbiol. 54: 2155–2162.

Roesch LFW, Camargo FAO, Bento FM, Triplett EW. 2006. Biodiversity of diazotrophic bacteria within soil, root and stem of field-grown maize. Plant Soil 302: 91–104.

Rouws LFM, Feitosa G, Zilli JE, Rodrigues Coelho MR, Gustavo Ribeiro X, Hartmann A, et al. 2014. Endophytic *Bradyrhizobium* spp. isolates from sugarcane obtained through differentiated culture strategies. Environ. Microbiol. Rep. 6: 354–363.

Ruschel AP, Henis Y, Salati E. 1975. Nitrogen-15 tracing of $N_2$-fixation with soil-grown sugarcane seedlings. Soil Biol. Biochem. 7: 181–182.

Saitou MH, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Schloter M, Wiehe W, Aßmus B, Steindl H, Becke H, Höflich G. 1997. Root colonization of different plants by plant-growth promoting *Rhizobium leguminosarum bv. trifolii R39* studied with monospecific polyclonal antisera. Appl. Environ. Microbiol. 63: 2038–2046.

Schultz N, Morais RF, Silva JA, Baptista RB, Oliveira RP, et al. 2012. Avaliação agronômica de variedades de cana-de-açúcar inoculadas com bactérias diazotróficas e adubadas com nitrogênio. Pesq. Agropec. Bras. 47: 261–268.

Sessitsch A, Hardoim P, Döring J, Weilharter A, Krause A, Woyke T, et al. 2012. Functional Characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol. Plant-Microbe Interact. 25(1): 28–36.

Sessitsch A, Reiter B, Pfeifer U, Wilhelm E. 2002. Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rRNA genes. FEMS Microbiol. Ecol. 39: 23–32.

Suman A, Shasany AK, Singh M, Shahi HN, Gaur A, Khanuja SPS. 2001. Molecular assessment of diversity among endophytic diazotrophic isolated from subtropical Indian sugarcane. World J. Microbiol. Biotechnol. 17: 39–45.

Terakado-Tonooka J, Fujihara, S, Ohwaki Y. 2013. Possible contribution of *Bradyrhizobium* on nitrogen fixation to sweet potatoes. Plant Soil 367: 639–650.

Thaweenut N, Hachisuka Y, Ando S, Yanagisawa S, Yoneyama T. 2011. Two seasons' study of *nifH* gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of *nifH* genes similar to those of rhizobia. Plant Soil 338: 435–449.

Toewe S, Wallisch S, Bannert A, Fischer D, Hai B, Haesler F, Kleineidam K, Schloter S. 2011. Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. J. Microbiol. Methods 84: 406–412.

Tong Z, Sadowsky MJ. 1994. A selective medium for the isolation and quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains from soils and inoculants. Appl. Environ. Microbiol. 60: 581–586.

Turner TR, James EK, Poole PS. 2013. The plant microbiome. Genome Biol. 14: 209–219.

Urquiaga S, Cruz KHS, Boddey RM. 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. Soil Sci. Soc. Am. J. 56: 105–114.

Urquiaga S, Xavier RP, Morais RF, Batista RB, Schultz N, Leite JM, et al. 2012. Evidence from field nitrogen balance and $^{15}N$ natural abundance data for the contribution of biological $N_2$ fixation to Brazilian sugarcane varieties. Plant Soil 356: 5–21.

Videira SS, Oliveira DM, Morais RF, Borges WI, Baldani VLD, Baldani JI. 2012. Genetic diversity and plant growth promoting traits of diazotrophic bacteria isolated from two *Pennisetum purpureum* Schum. genotypes grown in the field. Plant Soil 356: 51–66.

Videira SS, Pereira e Silva MdC, de Souza Galisa P, Dias ACF, Nissinen R, Baldani VLD, et al. 2013. Culture-independent molecular approaches reveal a mostly unknown high diversity of active nitrogen-fixing bacteria associated with *Pennisetum purpureum* – a bioenergy crop. Plant Soil doi: 10.1007/s11104-0133.1828-4.

Vincent JM. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No 15. Oxford and Edinburgh, UK: Blackwell scientific publications.

Woods CR, Versalovic J, Koeuth T, Lupski JR. 1993. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J. Clin. Microbiol. 31: 1927–1931.

Yanni YG, et al. 2001. The beneficial plant-growth promoting association of *Rhizobium leguminosarum* bv. *trifolii* with roce roots. Aust. J. Plant Physiol. 28: 845–870.

REVISED PROOFS

REVISED PROOFS