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ARTICLE

Expression of the *nifH* gene in diazotrophic bacteria in *Eucalyptus urograndis* plantations

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Abstract: A large proportion of eucalypt plantations in Brazil are located in areas with low soil fertility. The actions of microorganisms are of great importance for the cycling of nutrients, including nitrogen (N), that are essential for plant metabolism. Denaturing gradient gel electrophoresis (DGGE) was used to monitor and identify the total and active microorganisms involved in the N cycle in both the soil and root systems of a forest of *Eucalyptus urograndis* with sections that were fertilized with N or unfertilized. Quantitative real-time PCR was used to examine the expression of the *nifH* gene in N-fixing bacteria present in both the soil and root systems. According to the DGGE analysis, in the total and active populations of N-fixing bacteria, the presence and expression of the *nifH* gene were influenced by the winter and summer seasons and (or) N fertilization, respectively. DGGE band sequencing from total DNA samples showed that the most abundant group of diazotrophic bacteria belonged to Alphaproteobacteria in both the soil and root systems. Quantitative real-time PCR revealed that *nifH* expression was higher in the soil samples, especially in those that did not receive N fertilization. The differences in the composition of the total and active diazotrophic populations highlight the importance of evaluating the active populations, because they are effectively responsible for the biogeochemical transformation of N and also control its' availability to plants.

Key words: nifH gene, nested PCR-DGGE, transcriptase reverse, qPCR, Alphaproteobacteria.

Résumé : Une grande partie des plantations brésiliennes d'eucalyptus sont situées sur des sites peu fertiles. L'action des microorganismes est d'une grande importance dans le recyclage des nutriments, incluant l'azote (N) qui est essentiel au métabolisme des plantes. La technique de DGGE a été employée pour effectuer le suivi et identifier la quantité de microorganismes totaux et actifs impliqués dans le recyclage de N à la fois dans le sol et les systèmes racinaires d'une forêt d'*Eucalyptus urograndis* comprenant des sections fertilisées à l'azote et d'autres pas. La PCR quantitative en temps réel a été utilisée pour examiner l'expression du gène *nifH* dans les bactéries fixatrices d'azote présentes à la fois dans le sol et les systèmes racinaires. Selon l'analyse DGGE, chez les populations totales et actives de bactéries fixatrices d'azote, la présence et l'expression du gène *nifH* étaient respectivement influencées par les saisons hivernale et estivale et/ou par la fertilisation azotée. Le séquençage des bandes de DGGE à partir d'échantillons d'ADN total a démontré que le groupe le plus abondant de bactéries diazotrophiques appartenait aux Alphaprotéobactéries, à la fois dans le sol et les systèmes racinaires. Le PCR quantitatif en temps réel a révélé que l'expression des gènes *nifH* était plus élevée dans les échantillons de sol, et spécialement dans ceux qui n'avaient pas été fertilisés à l'azote. Les différences dans la composition des populations diazotrophiques totales et actives soulignent l'importance d'évaluer les populations actives puisqu'elles sont effectivement responsables de la transformation biogéochimique de l'azote et qu'elles contrôlent sa disponibilité pour les plantes. [Traduit par la Rédaction]

Mots-clés : gène *nifH*, PCR-DGGE nichée, transcriptase inverse, qPCR, Alphaprotéobactéries.

Introduction

In Brazil, the demand for forest products such as cellulose, charcoal, paper, sanitizers, and wood has led to an increase in planted forests, which now encompass 7 million hectares, of which 74.8% are eucalyptus plantations and 25.2% are pine plantations. Approximately 54.2% of the land planted with eucalypts (4 873 952 ha) is concentrated in the southeastern region of Brazil, and the state of Minas Gerais is one of the major producers (Associação Brasileira de Produtores de Florestas Plantadas (ABRAF) 2012). Most eucalypt plantations are located in low-fertility soils, where fertilization and nutrient cycling are fundamental to increase and maintain forest production (Gama-Rodrigues et al. 2005).

The productivity gains in eucalypt plantations in response to nitrogen (N) application have been relatively low, especially in areas under tropical and subtropical conditions. This fact indicates that the soil has been able to supply much of the N demand of plants (Pulito 2009). The cycling of the organic pool present in the litter fall and in the microbial biomass can contribute to the N supply at different levels. Together, these natural sources are sufficient to meet the N demand of eucalyptus plantations for several rotations, especially in low-fertility soils (Barreto et al. 2008, 2012).

The N from microbial biomass is recognized as a readily available fraction, but little is known about how biotic and abiotic factors affect N dynamics in Brazilian eucalyptus plantations

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Table 1. Climate, rainfall, and physicochemical characteristics from the eastern region of the state of Minas Gerais, Brazil.

	Mean temperature (°C)		Relative humidity (%)		Mean rainfall (mm)							
Treatment	January	June	January	June	January	June	pH (H_2O)	Organic matter ($dag \cdot dm^{-3}$)	Ca ($mg \cdot dm^{-3}$)	Mg ($mg \cdot dm^{-3}$)	K ($mg \cdot dm^{-3}$)	P ($mg \cdot dm^{-3}$)
WNF	22.40	20.90	77.80	76.10	169.70	2.50	4.24	1.71	18.00	7.30	22.99	1.59
NF							4.11	1.84	52.00	12.15	27.07	3.02

Note: WNF, without nitrogen fertilization; NF, with nitrogen fertilization; Ca, calcium; Mg, magnesium; K, potassium; P, phosphorus.

(Gama-Rodrigues et al. 2005). Moreover, the microorganisms involved in N mineralization control the N availability by regulating the immobilization and mineralization processes and are considered to be a primary N storage compartment in the soil (Gama-Rodrigues et al. 2005).

Some microorganisms, known as diazotrophic micro-organisms, are capable of performing biological nitrogen fixation (BNF) and can transfer much of the N in the soil to the rhizosphere (Wartiainen et al. 2008). BNF is a highly energetic process performed by nitrogenase enzymes encoded by *nif* genes, which are sensitive to the presence of oxygen and highly regulated at both transcriptional and post-transcriptional levels (Jenkins and Zehr 2008).

The diazotrophic bacteria (DB) community was recently described in eucalypt plantations (Castellanos et al. 2010; da Silva et al. 2014). da Silva et al. (2014) identified the main groups of DB in *E. globulus* but did not evaluate the gene expression responsible for BNF. Therefore, considering the presence of the DB community in eucalypts and the lack of response to N fertilization in tropical soils, our hypothesis is that these bacteria, also found in Brazilian eucalypt plantations, contribute to BNF.

The aims of this study were to evaluate the DB communities and the *nifH* gene expression in *E. urograndis* plantations influenced by the season and by N fertilization.

Materials and methods

Study location

The soil and root system samples were obtained from *E. urograndis* plantations owned by the Japanese–Brazilian cellulose production company CENIBRA (Celulose Nipo-Brasileira S.A., Minas Gerais) in a plot of renewed forest in the eastern region of Minas Gerais, Brazil. The plants used were 4 years old and were sampled from two plots. The first plot was planted on 21 February 2008, and it received N fertilization during the rainy season after planting (November 2008, 300 $kg \cdot ha^{-1}$). The second plot was planted on 24 July 2008 and did not receive fertilization. The soil in the fertilized plot was classified as a cambic dystrophic Oxisol, which has a prominent clayey texture and is alic, kaolinitic, and hypoferric. The soil in the nonfertilized plot was classified as a typical dystrophic Oxisol, which also has a prominent clayey texture and is alic, kaolinitic, and mesoferric. The climate, rainfall, and physicochemical properties of the study region are shown in Table 1. The information in this table was obtained from CENIBRA, i.e., the company where the samples were collected. The main difference observed was the amount of rainfall in different sampling periods (Table 1). Trees were randomly selected in the planted area (1666 $plants \cdot ha^{-1}$), and the spacing between the trees was 2 m × 3 m. Samples were collected in January and June of 2012, corresponding to the summer and winter, respectively.

Letter of authorization

In 2012, a cooperation protocol between the private company CENIBRA and the Universidade Federal de Viçosa (UFV) was established within the research project "Diazotrophic bacteria and arbuscular mycorrhizal fungi in eucalypt plantation" as part of the Doctor of Science thesis of Marliane de Cássia Soares da Silva. CENIBRA pledged to cede an area for the collection of soil and root system samples, which were necessary for the development of the study. Samples were carried out according to the methodology of CENIBRA with supervision of the responsible authority, Dr. Guilherme Luiz de Jesus (email: guilherme.jesus@cenibra.com.br).

Sampling

Three composite samples were collected from each plot. Each composite sample consisted of soil or roots collected from three neighboring trees. These trees were randomly selected within the area. For each tree, 10 random points were sampled in a radius of 40 cm around the trunk at a depth of 0–10 cm. The fragments of roots were composed primarily of 5 g of fine roots (0–1 mm in diameter). The samples were placed in plastic bags, stored on ice, transported to the laboratory, and stored at –20 °C for up to 2 months before analysis.

Moisture content

Soil samples from each of the replicates and from each plot were weighed and placed in a drying oven at 60 °C for 48 h to determine the moisture content.

Isolation and quantification of DB

Quantification of the number of DB was performed using the plate count method with an LGI culture medium (Döbereiner 1988). Ten grams of soil were added to 90 mL of saline solution (0.9% *w*/*v* of sodium chlorate) in an Erlenmeyer flask and kept under constant agitation at 760 rpm for 1 h. Then, serial dilutions were performed using a saline solution up to 10^{-6}, and 0.1 mL of each dilution was plated on the LGI medium. Analyses were performed in triplicate.

Obtaining total DNA from environmental samples

Total DNA was extracted from 250 mg of each root system and soil sample using an Ultra Clean Power Soil kit (Mobio Laboratories, Solana Beach, California, USA) according to the manufacturer's instructions. Prior to DNA extraction, the roots were rinsed four times with distilled water to remove impurities, homogenized in liquid nitrogen, and ground using a porcelain mortar and pestle.

Obtaining cDNA from environmental samples

Total RNA was extracted from 2 g of each root system and soil sample using a PowerSoil Total RNA Isolation Kit according to the manufacturer's instructions. Prior to RNA extraction, the roots were rinsed four times with distilled water to remove impurities, homogenized in liquid nitrogen, and ground using a porcelain mortar and pestle.

The cDNA was obtained from 2 μg of the total RNA using the ImProm-II Reverse Transcription System (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions.

Nested PCR of *nifH* genes

To assess the DB community, the PCR technique was used for the *nifH* gene of the DB community from root endophytes and from the surface of the roots. The total DNA and cDNA were used as a template in a PCR reaction to amplify the *nifH* gene, which is

involved in the N-fixation process. The primers used to amplify *nifH* were 19F and 407R, producing a 390 basepair (bp) fragment (Ueda et al. 1995). This step was followed by a nested PCR using the oligonucleotides 19F-C (with addition of the GC clamp) and the primer 278R (Direito and Teixeira 2002), which yields a 260 bp fragment. The PCR mixture consisted of 20 ng of total DNA, 0.2 μmol·L^{-1} of each oligonucleotide, 200 μmol·L^{-1} dNTP, 2 mmol·L^{-1} $MgCl_2$, 0.5 mg·mL^{-1} bovine serum albumin (BSA), and 1.25 units of GO Taq DNA polymerase (Promega) in a total reaction volume of 50 μL. The PCR reaction cycling conditions used were those described by Direito and Teixeira (2002).

Denaturing gradient gel electrophoresis (DGGE)

The DNA and cDNA amplicons obtained by the nested PCR of the soil and root samples were analyzed by DGGE (DCode Universal Mutation Detection System, Bio-Rad Laboratories, Inc., California, USA). The external markers used were obtained from the DNA extracted from pure cultures, which belong to collection of the Department of Microbiology of Universidade Federal de Viçosa–BIOAGRO, Minas Gerais, Brazil, from the following DB: *Pseudomonas fluorescens*, *Bradyrhizobium* sp., *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum*, *Rhizobium tropici*, *Burkholderia brasiliensis*, *Burkholderia sabiae*, *Stenotrophomonas maltrophila*, and *Acinetobacter calcoaceticus*. A sample of approximately 150–200 ng of DNA from each nested PCR reaction was applied to an 8% (*w*/*v*) polyacrylamide gel in a 1× TAE buffer. These gels were prepared and subjected to vertical electrophoresis, as described by da Silva et al. (2014). The bands of interest were excised, eluted, and subjected to a new PCR reaction with the same primers but without the addition of the GC clamp. The PCR products were then sequenced by Macrogen, Inc. (Korea). All sequences were edited using Sequencher software (version 4.1, Gene Codes Corporation, Ann Arbor, Michigan, USA). The results were analyzed by a comparison with the sequences deposited in NCBI GenBank using the BLASTx tool (Altschul et al. 1997).

Quantitative real-time PCR

The quantification of the *nifH* gene expression in the DB present in the soil and root system samples was performed by real-time qPCR using RNA samples. The following two primer pairs were used for the amplification of the cDNA: 19F and 278R for the *nifH* gene and 530F and 1100R for the 16S rDNA. This latter primer pair was used as an endogenous control, and both sets of primers were used at a concentration of 100 nmol·L^{-1}. The quantification cycle (Cq) obtained was used to quantify the relative expression of the *nifH* gene using the following formula: Cq (2 − ΔΔCq). Real-time qPCR reactions were performed in a volume of 20 μL using the Fast EvaGreen qPCR Master Mix in the Eco Real-Time PCR System (Illumina, California, USA). DNA amplification was then performed with an initial denaturation cycle at 95 °C for 30 s followed by 45 amplification cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 3 min.

Statistical analyses

Statistical analyses of cultured DB counts were performed using an analysis of variance (ANOVA) with Tukey's post-hoc tests, and the level of significance was set at $p < 0.05$.

DGGE profiles were analyzed and compared using BioNumerics software (version 5.1), which considers both the number and the intensity of the bands to perform the dendrogram, and aligned according to the external markers, thus allowing for normalization and subsequent comparisons between samples.

Statistical analyses of variations in the *nifH* gene expression were determined by ANOVA at a 0.5% probability level followed by Bonferroni's post-hoc tests to compare all pairs of columns.

Table 2. Influence of nitrogen fertilization and season on counts of colony forming units (CFU) of diazotrophic bacteria from soil samples in planted forests of Eucalypt.

	Log (CFU)·g^{-1} dry soil	
Treatment	Winter	Summer
WNF	5.95±0.17	6.07±0.08
NF	5.99±0.41	6.07±0.07

Note: WNF, without nitrogen fertilization; NF, with nitrogen fertilization.

Results

Quantification of the moisture content and DB counts

The moisture content in the samples ranged from 10% to 13% in both sites, regardless of the season. The levels of N-fixing bacteria in the soil from forest plantations of *E. urograndis* were not different ($p > 0.05$) in the winter and summer seasons and were unaffected by N fertilization (Table 2).

Analysis of DB communities

The amplification of the *nifH* gene was performed successfully, both from total DNA and from cDNA samples from the soil and root systems, which allowed for the analysis of amplicons by DGGE. This technique allowed the culture-independent analysis of DB from the soil and roots of the sampled plots (da Silva et al. 2014).

The total DB population was evaluated by PCR-DGGE (Figs. 1A and 2A), and the populations actively involved in N fixation at the time of sampling were evaluated by RT-PCR-DGGE (Figs. 1B and 2B).

DGGE revealed significant differences between the total DNA and cDNA samples from the soil and the root system (Figs. 1 and 2). The band profiles obtained from the DNA samples from both the soil and the root system were characterized by a larger number of bands, representing all present populations of DB, regardless of their activity.

The dendrograms from the DGGE gels revealed that samples from the soil were grouped; both the total diazotrophic and the metabolically active populations formed large groups according to their N fertilization status, with 62% similarity (Figs. 1C and 1D). However, when these groups were analyzed, they formed subgroups according to the season of the year and were characterized by approximately 75% similarity (Figs. 1C and 1D).

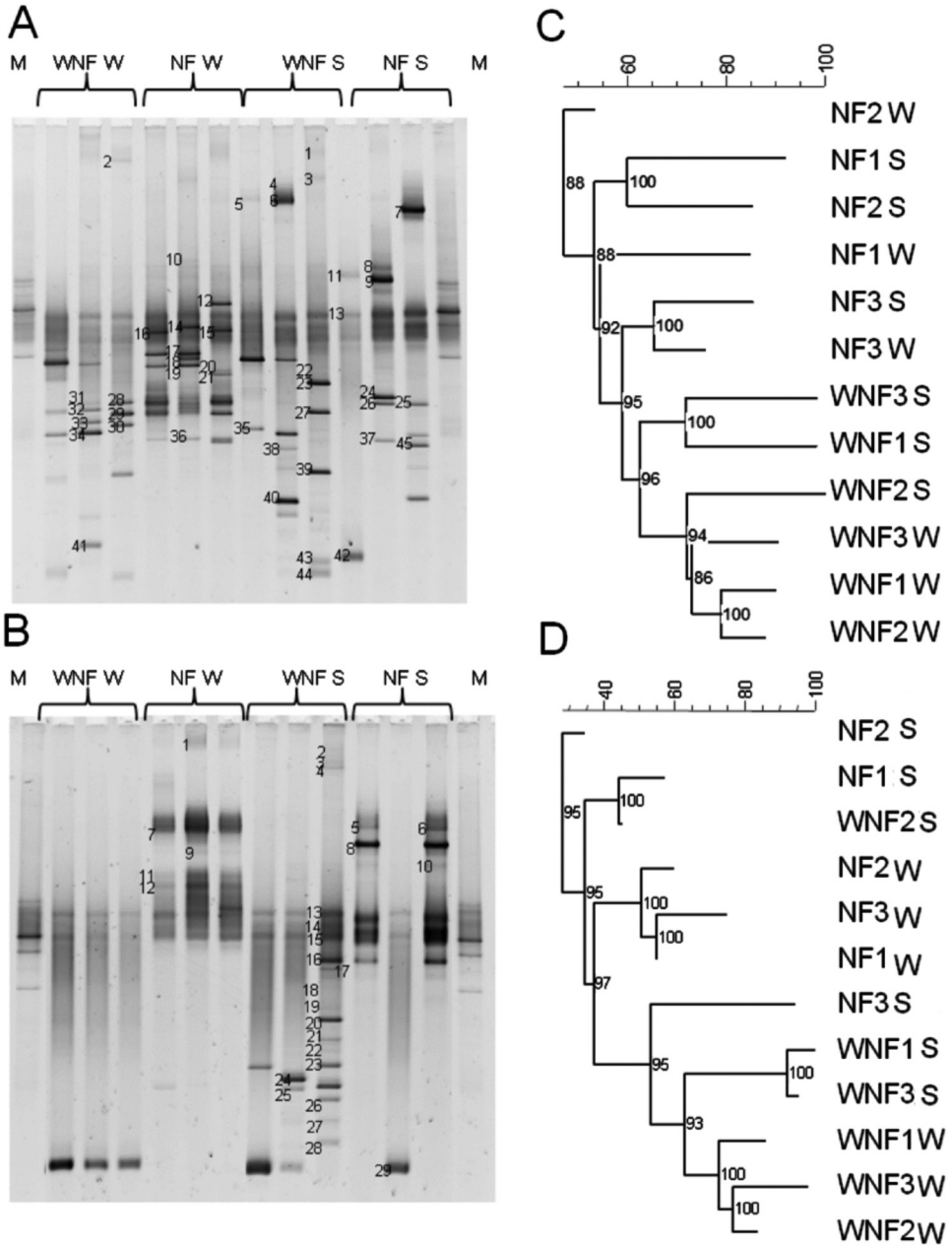
This pattern was not observed in samples taken from the root system. Rather, root system samples were grouped together according to the winter and summer seasons (Figs. 2C and 2D). Two major groups with 75% similarity were observed in the sample profile corresponding to the total DB of the root system, and the main group was found for the summer and had 82% similarity (Fig. 2C). For the cDNA, the samples were more similar to the two main groups, exhibiting 92% similarity. The first group consisted of the winter samples, and the second group consisted of the summer samples (Fig. 2D).

Identification of DB populations

The bands corresponding to the communities of DB obtained by DGGE were excised, sequenced, and analyzed using the BLASTx algorithm (NCBI). In both the DNA and cDNA samples obtained from the soil and root system, the bacteria group found most frequently corresponded to the class Alphaproteobacteria, followed by the phylum Cyanobacteria (Fig. 3). Overall, Alphaproteobacteria were more abundant in most samples; however, Cyanobacteria were predominant in the cDNA samples from the root system.

Representatives of the phylum Firmicutes and the class Deltaproteobacteria were observed in all samples except for the cDNA

Fig. 1. Cluster analysis obtained from the DGGE banding pattern of the microbial communities using the *nifH* gene in soil samples from *E. urograndis* plantations in the Minas Gerais region, Brazil. (A) and (B) denaturing gradient gel electrophoresis (DGGE) band patterns from samples of DNA and cDNA, respectively. (C) and (D) Dendrograms of the neighbor joining method (DNA samples) and the unweighted pair group method with arithmetic mean (UPGMA; cDNA samples), respectively. NF, with nitrogen fertilization; WNF, without nitrogen fertilization; W, winter; S, summer; M, marker.



samples obtained from the root system. The class Gammaproteobacteria was identified only in total DNA samples, and the class Betaproteobacteria was found in both DNA and cDNA samples obtained from the soil (Fig. 3; Appendix Tables A1 and A2).

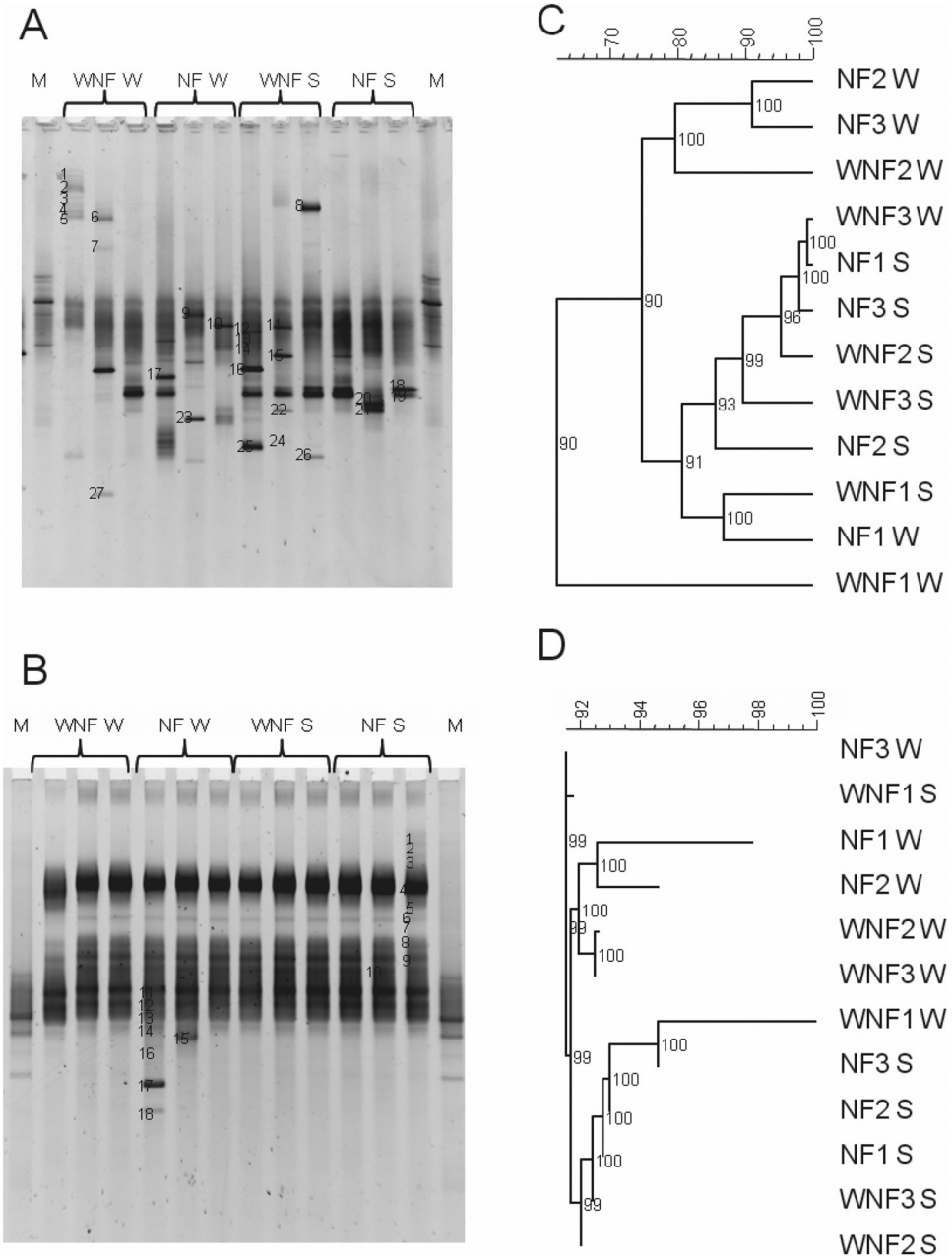
The total DNA samples extracted from the soil and the root system had a higher richness of DB species when compared with the cDNA samples. However, the species found in soil samples were largely different from those identified in the root system samples (Fig. 4; Appendix Tables A1 and A2). For example, the genus *Bradyrhizobium* was predominant in the soil samples, whereas the genus *Hyphomicrobium* was most common in the root system samples.

In the cDNA samples taken from the soil, *Hyphomicrobium* sp. were the most common bacteria, followed by *Nostoc* sp. and *Bradyrhizobium* sp. In contrast, *Nostoc* sp. (89% of the total) were most common in the root system samples, in which *Bradyrhizobium* sp. (11%) were also present (Fig. 4; Appendix Tables A1 and A2).

Expression of the *nifH* gene

After using real-time qPCR to quantify *nifH* gene expression, the soil samples exhibited increased expression compared with the root system samples. The expression of this gene was higher in all samples without N fertilization when compared with the fertilized samples (Fig. 5). Among the samples that were not fertilized,

Fig. 2. Cluster analysis obtained from the DGGE banding pattern of the microbial communities using the *nifH* gene of samples of root systems from *E. urograndis* plantations in the Minas Gerais region, Brazil. (A) and (B) denaturing gradient gel electrophoresis (DGGE) band patterns from samples of DNA and cDNA, respectively. (C) and (D) Dendrograms of the neighbor joining method (DNA samples) and the unweighted pair group method with arithmetic mean (UPGMA; cDNA samples), respectively. NF, with nitrogen fertilization; WNF, without nitrogen fertilization; W, winter; S, summer; M, marker.



those from the winter season showed a higher expression of the *nifH* gene.

Discussion

Soils contain a large diversity of mostly nonculturable bacteria. In this study, no significant differences in the DB culturable community were observed between fertilized and nonfertilized samples or between samples obtained during the winter and samples obtained during the summer. Other studies have similarly reported no differences in the number of DB in the soils of plantations of *Eucalyptus* spp. during the summer and winter seasons (Castellanos et al. 2010; da Silva et al. 2014). Additionally, no differences were observed in the number of DB in the summer and winter seasons in a study on the secondary plant cover of a biological reserve (Silva and Melloni 2011).

The lack of access to most of the DB present in these environmental samples suggests that nonculturable DB can play an important role in N fixation and may even be primarily responsible for this function. In a study by Meng et al. (2012), many sequences of nonculturable DB were observed, indicating that only a small fraction of these bacteria can actually be cultured.

Molecular biology techniques can provide a method to identify microorganisms present within environmental samples that cannot be cultured (Poly et al. 2001; Buckley et al. 2007). Indeed, the number of bands observed in the DGGE profiles was greater than the total number of distinct colonies observed in the culture

Fig. 3. Radar diagram representing the distribution of the predominant groups of bacteria in samples of the soil and root systems from *E. urograndis* plantations in the Minas Gerais region, Brazil, generated by the sequencing of bands obtained by denaturing gradient gel electrophoresis (DGGE) of *nifH*, using the total DNA and cDNA as a template.

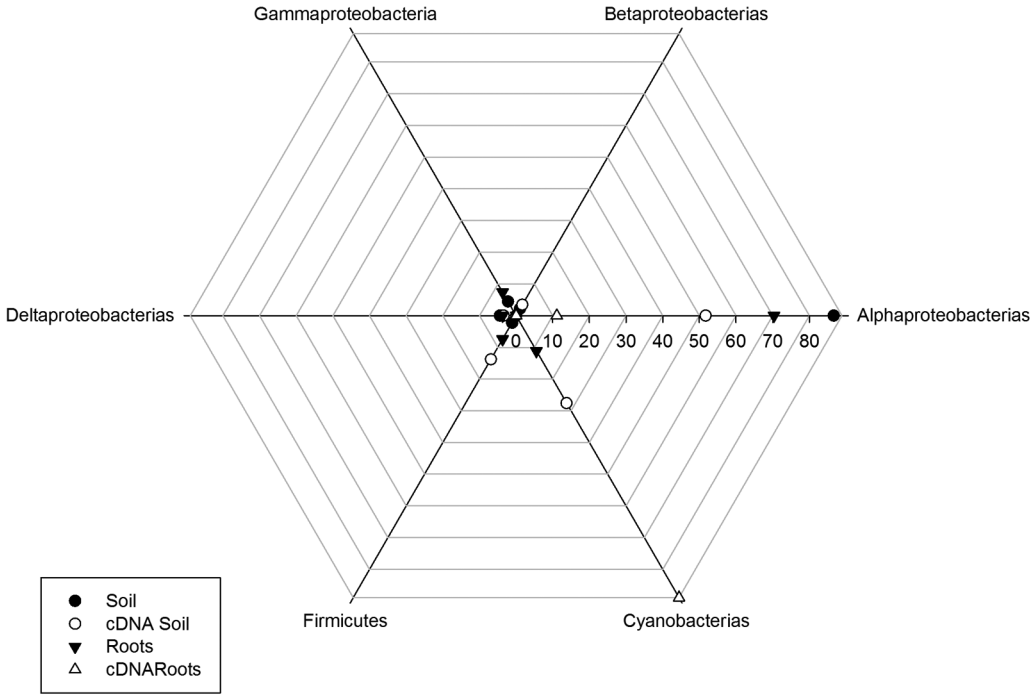


Fig. 4. Radar diagram representing the distribution of the predominant species of bacteria in samples of the soil and root systems from *E. urograndis* plantations in the Minas Gerais region, Brazil, generated by the sequencing of bands obtained by denaturing gradient gel electrophoresis (DGGE) of *nifH*, using the total DNA and cDNA as a template.

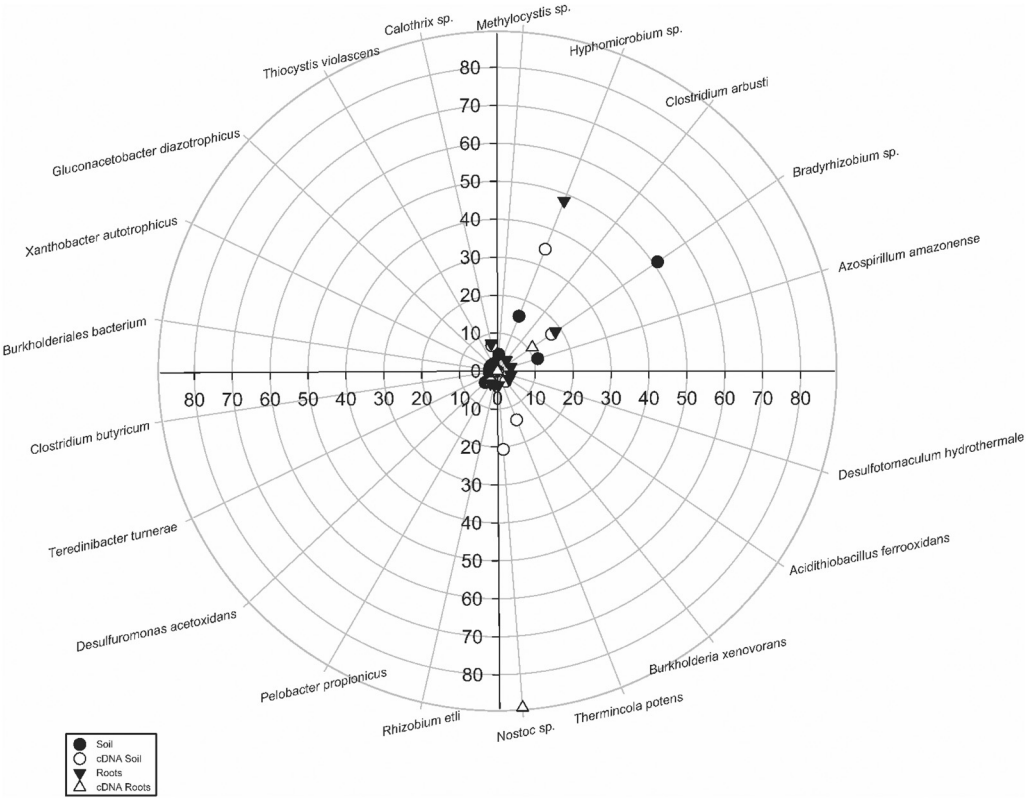
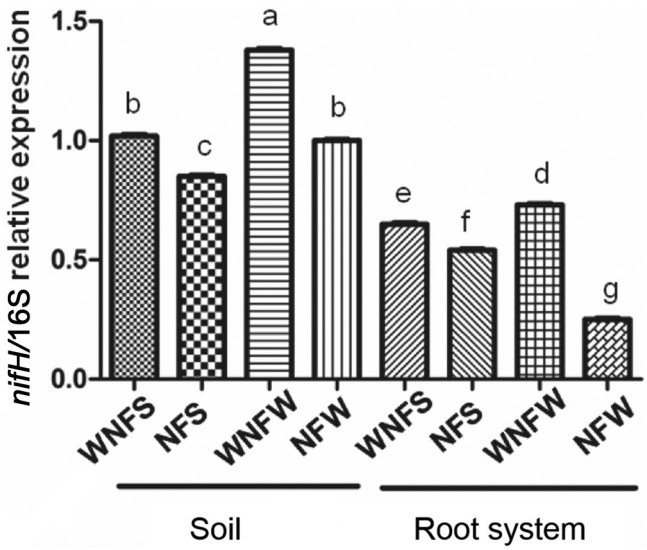


Fig. 5. Relative expression of *nifH*/16S rDNA of the microbial community in samples of the soil and root systems from *E. urograndis* plantations in the Minas Gerais region, Brazil. NF, with nitrogen fertilization; WNF, without nitrogen fertilization; W, winter; S, summer. Columns with the same lowercase letter above them do not differ (Bonferroni's post-hoc tests, 5% probability).



medium. The DGGE profiles also revealed that the microbial populations present in the total DNA samples exhibited a greater number of bands than those present in the cDNA samples. This result was expected because the total DNA samples allow for the amplification of the *nifH* gene from all microorganisms, active or not. The variation between replicates may be attributed to the samples and the sensibility of the PCR technique (Hirsch et al. 2010). The samples are from the same area but are not identical, with 1 g of soil having high microbiological heterogeneity even though it is from the same area. Other studies also have shown these some variation in the soil samples PCR considered as replicates (Wartiainen et al. 2008; da Silva et al. 2014).

The greater effect of N that was observed in the soil samples is due to the greater impact of fertilizer on the soil relative to the root system. The literature has shown, through the application of molecular techniques, that the structure of the N-fixing microbial population present in the rhizosphere is influenced by N fertilization (Meng et al. 2012; da Silva et al. 2014). In the Portuguese eucalypt, the authors clearly observed the influence of fertilization and seasonality (da Silva et al. 2014). In contrast, the profile of the active population in the root system samples was very similar in the winter and the summer samples regardless of N fertilization. Thus, this population appears to be less sensitive to factors such as fertilization and seasonality.

The diversity of habitats in the soil is important for the maintenance of high bacterial diversity (Blackwood and Paul 2003). This allows for greater environmental variation for different species of free-living DB and thus leads to increased *nifH* gene expression in soil samples. We believe that the root system displays a lower number of DB because bacteria can localize to the root surface or even inside the root itself (endophytic bacteria). In addition, the lower expression of *nifH* gene in the root system could also be due to the availability of root exudates, which may benefit nonDB or even decrease the need for BNF. Root exudates are many and complex, and they not only serve as a carbon source for microbial growth, but also promote the chemotaxis of soil microorganisms to the rhizosphere (Dakora and Phillips 2002). In addition, the presence of endophytic diazotrophic bacteria can result in less diversity of DB in the rhizoplane and rhizosphere compared with the nonrhizospheric soil (Meng et al. 2012).

Expression of the *nifH* gene may also be negatively affected by the availability of N supplied by fertilization, which can diminish the need for BNF, a high-energy process in microorganisms. We observed lower *nifH* gene expression in all fertilization treatments, which is consistent with this hypothesis. Coelho et al. (2009), who used qPCR techniques to demonstrate that *nifH* gene expression decreases with the increased use of N fertilizers, observed a similar result. Wu et al. (2009) also found that the abundance of *nifH* transcripts in N-fixing Alphaproteobacteria decreases with the application of N. In addition, Meng et al. (2012) showed that the diversity of DB also decreases with the addition of high doses of N fertilization, thus suggesting that certain groups of rhizosphere bacteria are sensitive to fertilization.

When assessing the total DB community found in soil samples, the genus *Bradyrhizobium*, order *Rhizobiales*, was the most abundant. This finding may result from the unusual characteristics of this genus, including its slow growth, the fact that it is moderately adapted to semi-arid conditions, and its ability to colonize the roots of many nonleguminous plants and fix N under symbiotic and nonsymbiotic conditions (Kahindi et al. 1997; Coelho et al. 2008). In Portuguese eucalypt plantations, in addition to the genus *Bradyrhizobium*, we also found the genera *Burkholderia* and *Rubrivivax* (da Silva et al. 2014).

However, when the active population found in the soil samples was assessed, the genus *Hyphomicrobium*, order *Rhizobiales*, was predominant. The same result was found when the total DNA samples from the root system were examined. This genus comprises chemo-organotrophic and facultative methylotrophic bacteria; thus, it can be found in multiple different environments, including soil, freshwater, and sewage treatment plants (Gliesche et al. 2005). Species of this genus are also able to use toxic waste compounds not used by other methylotrophic bacteria (Fesefeldt et al. 1997). This metabolic versatility may explain the predominance of the genus *Hyphomicrobium* in the active population of the soil.

In the cDNA samples from the root system, the phylum Cyanobacteria was most prominent, especially the genus *Nostoc*, and the band corresponding to this genus was present in all winter and summer samples analyzed, regardless of N fertilization. Cyanobacteria are widespread photo-autotrophic microorganisms that combine the ability to perform aerobic photosynthesis with prokaryotic characteristics. Some species also have the ability to fix atmospheric N (Pereira et al. 2009; Jensen et al. 2013). Cyanobacteria of the genus *Nostoc* are found in many terrestrial and aquatic ecosystems, and their ability to fix N enhances the quality of nutrient-poor soils (Potts 2000).

Prior work has shown that changes in the community structure of DB have functional significance and has suggested that the rate of N fixation varies as a function of the diversity of the DB community (Hsu and Buckley 2009). In this context, the ecological significance of diversity in the DB community should be investigated with respect to the relationships between bacterial diversity and plant productivity. Therefore, this information can contribute to new discoveries or alternative microorganisms that are able to increase plant nutrition.

Conclusions

The differences found regarding the profiles of the DB communities demonstrate the importance of evaluating the active population. Furthermore, the expression of the *nifH* gene was reduced by N fertilization, demonstrating the influence of fertilization on the DB community in *E. urograndis* plantations.

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Appendix A

Table A1. Identification of diazotrophic bacteria based on the *nifH* gene of bands eluted from DGGE gels of DNA of soil and root system samples of *Eucalyptus urograndis* plantations.

Banding	GenBank access no.	Identification	Identity (%)	Similarity (%)	Class; order
Soil					
1, 6[b], 14[b], 18[b], and 32	ZP_08870627.1	Nitrogenase reductase: *Azospirillum amazonense*	71–100	75–100	Alphaproteobacteria; Rhizobiales
2, 3, 4[b], 5, 10, 11, 17, 19, 20, 22[a], 25[a], 26, 29, 33, 34[a], 35, 40, and 41	ZP_09436926.1	Nitrogenase iron protein, *nifH*: *Bradyrhizobium* sp.	66–100	67–100	Alphaproteobacteria; Rhizobiales
7	YP_003073074.1	Nitrogenase iron protein: *Teredinibacter turnerae*	96	98	Gammaproteobacteria; Alteromonadales
8 and 9	ZP_01312343.1	Nitrogenase iron protein: *Desulfuromonas acetoxidans*	94	100	Deltaproteobacteria; Desulfuromonadales
12[b] and 43	YP_001241772.1	Nitrogenase reductase: *Bradyrhizobium* sp.	88–100	93–100	Alphaproteobacteria; Rhizobiales
13[b]	YP_001600720.1	Nitrogenase reductase: *Gluconacetobacter diazotrophicus*	91	98	Alphaproteobacteria; Rhodospirillales
15, 16, 27[b], 36[b], and 39	YP_004677463.1	Nitrogenase iron protein 2: *Hyphomicrobium* sp.	96–100	97–100	Alphaproteobacteria; Rhizobiales
21	YP_006415625.1	Nitrogenase iron protein: *Thiocystisviolascens*	83	97	Gammaproteobacteria; Chromatiales
28 and 31	YP_006592725.1	Nitrogenase iron protein 2, *nifH*: *Methylocystis* sp.	100	100	Alphaproteobacteria; Rhizobiales;
30 and 38	YP_004677413.1	Nitrogenase iron protein 2: *Hyphomicrobium* sp.	98	100	Alphaproteobacteria; Rhizobiales
37	YP_001415059.1	Nitrogenase reductase: *Xanthobacter autotrophicus*	95	97	Alphaproteobacteria; Rhizobiales
42	ZP_09750647.1	Nitrogenase iron protein: *Burkholderiales bacterium*	99	100	Betaproteobacteria; Burkholderiales
44[b]	YP_005451908.1	Nitrogenase iron protein: *Bradyrhizobium* sp.	98	100	Alphaproteobacteria; Rhizobiales
45[b]	ZP_04525525.1	Nitrogenase iron protein: *Clostridium butyricum*	51	68	Bacilli; Clostridiales
Root system					
1	ZP_10774725.1	Nitrogenase iron protein: *Clostridium arbusti*	96	98	Bacilli; Clostridiales
2 and 4	YP_007135446.1	Nitrogenase: *Calothrix* sp.	98–100	98–100	Cyanobacteria; Nostocales
3[a]	NP_768409.1	Nitrogenase reductase: *Bradyrhizobium japonicum*	90	92	Alphaproteobacteria; Rhizobiales
5	NP_484917.1	Nitrogenase reductase: *Nostoc* sp.	94	98	Cyanobacteria; Nostocales
6	ZP_11409602.1	Nitrogenase iron protein: *Desulfotomaculum hydrothermale*	91	97	Bacilli; Clostridiales
7[a], 8, 11[a], 15, 16, 18[a], 21, and 27	YP_004677463.1	Nitrogenase iron protein 2: *Hyphomicrobium* sp.	87–100	92–100	Alphaproteobacteria; Rhizobiales
9	YP_903117.1	Nitrogenase iron protein: *Pelobacterpropionicus*	96	96	Deltaproteobacteria; Desulfuromonadales
10 and 23[b]	YP_005451908.1	Nitrogenase iron protein: *Bradyrhizobium*	90–93	93–95	Alphaproteobacteria; Rhizobiales
17[b]	YP_002219685.1	Nitrogenase reductase: *Acidithiobacillus ferrooxidans*	85	87	Gammaproteobacteria; Acidithiobacillales
26	ZP_09436926.1	Nitrogenase iron protein, *nifH*: *Bradyrhizobium* sp.	96	97	Alphaproteobacteria; Rhizobiales
22[a]	YP_001241772.1	Nitrogenase reductase: *Bradyrhizobium* sp.	100	100	Alphaproteobacteria; Rhizobiales
24[a]	ZP_08870627.1	Nitrogenase reductase: *Azospirillum amazonense*	100	100	Gammaproteobacteria; Acidithiobacillales;
25	ZP_03506751.1	Nitrogenase reductase: *Rhizobium etli*	94	95	Alphaproteobacteria; Rhizobiales

[a]Only with primer 278R.
[b]Only with primer 19F.

Table A2. Identification of active diazotrophic bacteria based on the *nifH* gene of bands eluted from DGGE gels of cDNA soil and root system samples of *Eucalyptus urograndis* plantations.

Banding	GenBank access no.	Identification	Identity (%)	Similarity (%)	Class; order
Soil					
1, 7, 9, 11, 12, and 25[b]	YP_007048826.1	Mo-nitrogenase iron protein, *nifH*: *Nostoc* sp.	67–100	76–100	Cyanobacteria; Nostocales
2, 3, 4, 20, 21, 22, 23, 24, and 27	YP_004677413.1	Nitrogenase iron protein: *Hyphomicrobium* sp.	94–100	95–100	Alphaproteobacteria; Rhizobiales
5, 6[b], 8, and 10	YP_003639458.1	Nitrogenase iron protein: *Thermincola potens*	90–95	92–100	Bacilli; Clostridiales
13[a]	YP_005451950.1	Nitrogenase iron protein: *Bradyrhizobium* sp.	80	85	Alphaproteobacteria; Rhizobiales
14[b]	YP_903117.1	Nitrogenase iron protein: *Pelobacter propionicus*	77	90	Deltaproteobacteria; Desulfuromonadales
15[b]	YP_004677463.1	Nitrogenase iron protein 2: *Hyphomicrobium* sp.	86	95	Alphaproteobacteria; Rhizobiales
16, 17, 18, and 19	ZP_09436926.1	Nitrogenase iron protein, *nifH*: *Bradyrhizobium* sp.	96	98	Alphaproteobacteria; Rhizobiales
26[b] and 28[b]	YP_007135446.1	Nitrogenase: *Calothrix* sp.	65–87	82–91	Cyanobacteria; Nostocales
29[b]	YP_553849.1	Nitrogenase reductase: *Burkholderia xenovorans*	88	96	Betaproteobacteria; Burkholderiales
Root system					
1–15 and 18	YP_007048826.1	Mo-nitrogenase iron protein, *nifH*: *Nostoc* sp.	97–100	96–100	Cyanobacteria; Nostocales
16 and 17	ZP_09436926.1	Nitrogenase iron protein, *nifH*: *Bradyrhizobium* sp.	93–97	97–100	Alphaproteobacteria; Rhizobiales

[a]Only with primer 278R.
[b]Only with primer 19F.