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Full Length Research Paper

Detecting bacterial endophytes in tropical grasses of the *Brachiaria* genus and determining their role in improving plant growth

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Plant-growth-promoting (PGP) bacteria include a diverse group of soil bacteria thought to stimulate plant growth by various mechanisms. *Brachiaria* forage grasses, of African origin, are perennials that often grow under low-input conditions and are likely to harbour unique populations of PGP bacteria. Three bacterial strains that tested positive for nitrogenase reductase gene sequences (*nifH*) were isolated from *Brachiaria* hybrid CIAT 36062 and introduced into *Brachiaria* hybrid cv. Mulato, which also had indigenous endophytic bacteria testing positive for *nifH* gene sequences. Under conditions of nutrient deficiency, inoculated Mulato plants had significantly higher biomass production, chlorophyll and total nitrogen contents in leaves than do control plants and were darker green. Strains of endophytic bacteria were then artificially introduced into *Brachiaria brizantha* CIAT 6294, which does not have indigenous endophytic bacteria. Results were consistent with those obtained with artificially inoculated Mulato plants, suggesting that these endophytic bacteria do benefit plant growth. DNA sequence analysis demonstrated that the *nifH* gene sequences were highly similar to those from *Klebsiella pneumoniae* and other N_2-fixing organisms and that the *nif* genes had consensus sequences identical to those of other N_2-fixing bacteria.

Key words: Bacterial endophytes, green fluorescent protein, nitrogenase reductase, plant growth-promoting bacteria, *Brachiaria.*

INTRODUCTION

Endophytic bacteria are known to reside in tissues of their plant hosts without causing them harm. These bacteria are often isolated from surface-sterilized tissues or extracted from internal plant parts. In general, many of the entry points for pathogenic bacteria also serve for endophytic ones. Several different endophytic bacteria may reside within a single plant (Kobayashi and Palumbo, 2000). These endophytes either remain localized at their entry points or spread to other parts of the plant (Hallmannet al., 1997). Various bacterial endophytes have been reported to live within cells, intercellular spaces, or the vascular system of a diversity of plants (Hallmann et al., 1997; James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998b; Sturz et al., 2000; Rosenblueth and Martínez-Romero, 2006). Although endophyte populations vary in different plants according to many factors, bacterial populations are generally larger in roots and smaller in stems and leaves (Lamb et al., 1996).

Plant-associated bacteria play key roles in their hosts' adaptation to changing environments in various ecosystems. These interactions between plants and beneficial bacteria can significantly affect general plant health and soil quality. Associative nitrogen (N_2) fixing bacteria may benefit their hosts by acting as N_2 biofertilizers and plant

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Abbreviations: PGPB, Plant-growth-promoting bacteria; **GFP,** green fluorescent protein.

growth promoters. Several endophytic bacteria enhance growth and improve general plant health (Stoltzfus et al., 1997; Sharma and Nowak, 1998). Many plant-growth-promoting bacteria (PGPB), including a diverse group of soil bacteria, are thought to stimulate plant growth by various mechanisms such as protecting plants against pathogens, providing plants with fixed N_2 (Hurek et al., 2002; Iniguez et al., 2004), producing plant hormones, or enhancing mineral availability in the soil (Sessitsch et al., 2002).

Brachiaria grasses of African savannahs have supported millions of African herbivores over thousands of years. Some of these grasses have many desirable agronomic traits for improving animal production in the tropics (Miles et al., 2004). For example, they are persistent and can grow in a variety of habitats ranging from humid waterlogged areas to semi-arid conditions. These grasses are likely to harbour unique populations of N_2-fixing or plant-growth-promoting bacteria, because they are very well adapted to acid soils and often grow under low-input conditions.

Our study is aimed to determine the existence of endophytic bacteria in association with *Brachiaria* species and examines the effects of these endophytic bacteria on *Brachiaria* plant growth and development. We report here the isolation, characterization and effect of endophytic bacteria on the growth and development of *Brachiaria* plants. A preliminary report of parts of this work has already been published in Kelemu et al. (2006).

MATERIALS AND METHODS

Bacterial isolates

Isolates were obtained from leaf, stem, and root tissues of seedlings of *Brachiaria* hybrids CIAT 36062 (BRNO93/1371) and CIAT 36061 (cv. Mulato) generated from embryogenic calluses. Each tissue was sliced into pieces of about 1 cm^2 in size and surface-sterilized with 1% NaOCl solution for 2 min, then with 70% ethanol for 1 min and rinsed three times with sterilized distilled water. Each sample was then macerated with a mortar and pestle in 1 ml of sterilized distilled water. Of this sample, 100 µl were taken and a dilution series conducted. A total of 100 µl of each dilution was spread on plates containing nutrient agar medium (Difco Laboratories, Detroit, MI.). The plates were incubated at 28°C for 24 h. Independent bacterial colonies were counted. A bacterial strain, isolated from roots of CIAT 36062 and designated 01-36062-R2, was selected and used for this study.

To locate bacterial cells within the plants and confirm whether they constituted 'true' endophytic bacteria as according to the criteria of Reinhold-Hurek and Hurek (1998a), isolate 01-36062-R2 was transformed with the green fluorescent protein (GFP) gene (Miller et al., 2000) and artificially introduced into CIAT 36061 (cv. Mulato). Root and leaf tissues were then examined under the microscope at 1, 2, 3 and 5 months after inoculation.

To evaluate various primers, we used two strains (designated 02-36062-H4 and 03-36062-V2) isolated from stems and leaves of the same *Brachiaria* genotype, *Bradyrhizobium* species CIAT 2469 that had been isolated from the legume *Desmodium* species and a pathogenic bacterium *Xanthomonas campestris* pv. *graminis* (included as a negative control).

Bacterial DNA extractions

DNA extraction was conducted using a modified protocol based on combinations of standard methods, including growing bacterial cells in liquid LB medium (in 1litre of distilled water, 10 g tryptone, 5 g yeast extract, 10 g NaCl and 10 ml 20% glucose), treating cells with a lysozyme mixture (10 mg ml^{-1} in 25 mM Tris-HCl and pH 8.0) and RNase A solution and extracting DNA with STEP (0.5% SDS, 50 mM Tris-HCl, pH 7.5, 40 mM EDTA, proteinase K to a final concentration of 2 mg ml^{-1} and added just before use). The protocol also involved cleaning with phenol/chloroform and chloroform/ isoamyl alcohol and precipitation with ethanol. DNA quality was checked on 1% agarose gels.

Nested-polymerase chain reaction (PCR) amplification

Three primers, originally designed by Zehr and McReynolds (1989) and Ueda et al. (1995) were used to amplify fragments of *nifH* genes. Amplification steps described by Widmer et al. (1999) were adopted. The final product of the nested-PCR amplification was about 370 bp in size.

Cloning the amplified DNA fragments

The amplified products were eluted from agarose gel, using Wizard® PCR Preps DNA Purification System according to instructtions supplied by the manufacturer (Promega Corporation, Madison, WI). The purified fragments were ligated to the cloning vector (pGEM®-T Easy Vector System, Promega Corporation) and used to transform *Escherichia coli* DH5-α, using standard procedures (Sambrook et al., 1989).

Plasmid extraction

Plasmids were extracted from transformed *E. coli* DH5-α cells, using a Wizard® *Plus* Minipreps DNA Purification System according to the protocol supplied by the manufacturer (Promega Corporation). To confirm whether the transformants contained the desired size of insert (about 370 bp), the plasmid DNA was digested to completion with the restriction enzyme *Eco*RI. The digested products were separated by electrophoresis on a 1% agarose gel (Bio-Rad Laboratories, Hercules, CA), stained with ethidium bromide and photographed under UV light.

Amplification of DNA inserts for sequencing

PCR reactions (25 µl) contained 20 ng $µl^{-1}$ plasmid DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, primers T7 (5'-GTAATACGA CTCACTATAGGGC-3') and Sp6 (5'-TATTTAGGTGACACTATAG-3'); each at 0.1 µM concentration and 0 to 5 U *Taq* polymerase. The reactions were amplified in a programmable thermal controller (MJ Research, Waltham, MA) programmed with 35 cycles of a denaturation step for 30 s (2 min for the first cycle) at 94°C, annealing for 30 s at 50°C and primer extension for 1 min (4 min in the final cycle) at 72°C. To further confirm the expected size insert, samples of amplified products were separated on a 2% agarose gel by electrophoresis.

The ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit

(Applied Biosystems, Foster City, CA) was used to further prepare the samples for sequencing. Sequencing was conducted, using ABI PRISM® 377 DNA Sequencer (Applied Biosystems). The sequence data were compared with sequences in different databases, using the program WU BLAST version 2.0 or 2.1 (http://www.ncbi.nlm.nih.gov/BLAST/-). The program compares nucleotide sequences across databases and calculates the statistical significance of matches found.

Specific primer construction

Based on the sequence data, primers were designed using the program DNAMAN, version 4:0 (Lynnon Biosoftware, Vaudreuil, Quebec, Canada) and synthesized by Integrated DNA Technologies (Coralville, IA). These primers were tested on bacteria that were confirmed positive or negative controls: strains 01-36062-R2, 02-36062-H4 and 03-36062-V2, *Bradyrhizobium* and *X. campestris* pv. *graminis.*

Bacterial inoculum preparation

Three endophytic bacterial isolates (01-36062-R2, 02-36062-H4 and 03-36062-V2), originally isolated from *Brachiaria* CIAT 36062 roots, leaves and stems, respectively, tested positive for sequences of the *nifH* gene (which encodes nitrogenase reductase). The isolates were maintained at -80°C in 20% glycerol. Bacterial cells were removed from each of the stored samples, plated on nutrient agar medium (Difco Laboratories, Detroit, MI) and incubated for 24 h at 28°C. The cells from each bacterial strain were collected from the plates, suspended in sterilized distilled water and adjusted to a concentration of optical density (OD_{600}) = 1.0 with a spectrophotometer.

Plant inoculation

Twenty tillers of about 1 month old were prepared from a single mother plant of *Brachiaria* hybrid CIAT 36061 (cv. Mulato). Their roots were washed with sterilized distilled water and prepared for inoculation. In a separate experiment, 12 *B. brizantha* CIAT 6294 (cv. Marandu) plants that were about 1 month old were also used for inoculation. These plants were selected after examining with nested PCR, which showed no amplified products for sequences of nifH gene, indicating the absence of endophytic bacteria containing these sequences.

The roots of half of the selected tillers were immersed in a beaker containing a mixture of equal volumes (50 ml each) of the three strains of endophytic bacterial suspensions described above. The remaining plants were immersed in a beaker containing the same volume of sterilized distilled water as control plants. All plants were kept immersed for 48 h, after which they were removed and rinsed three times with sterilized distilled water.

Each plant was then transplanted to pots containing sterilized sand (95%) and clay loam soil (5%) and left to grow in the greenhouse under natural daylight with a maximum photon flux density of 1100 µmol m^{-2} s^{-1} and at temperatures between 19°C (during night) and 30°C (during day). No nutrients were applied to the pots.

Plant growth and development attributes

On day 65 after inoculation, the following parameters of plant growth and development were measured on both control and treated plants. Plant height from stem base to the highest part of the plant, number of tillers, number of leaves and leaf area, using a LI-300 leaf area meter (LI-COR Biosciences, Lincoln, NE) were measured. Leaf chlorophyll content was measured, using a Minolta SPAD 502 Chlorophyll Meter (Spectrum Technologies, East-Plainfield, IL) by measuring across the third fully developed leaf. An average of six measurements was taken. Total N content in leaves and stems was measured according to the methods described by Salinas and García (1985). Total N uptake in leaves and stems was determined based on biomass dry weight and N content per unit dry weight. Soluble protein content in leaves was measured as described by Rao and Terry (1989). Dry matter distribution among leaves, stems and roots were measured by drying each tissue type separately in an oven at 70°C for 48 h and by recording the dry weights.

Bacterial populations in roots

About 1 g of root sample was taken from each individual plant, surface-sterilized (in 1% NaOCl solution for 2 min, 70% ethanol for 1 min and rinsed three times in sterilized distilled water) and macerated in a mortar and pestle in 1 ml of sterilized distilled water. Of this macerated sample, 100 µl was taken and a dilution series performed. These were plated on nutrient agar medium and incubated for 24 h at 28°C to determine bacterial colony growth and calculate the number of bacterial cells per gram of root weight.

Phylogenetic analysis

Phylogenetic analysis of the nucleotide sequences was conducted, using the neighbour-joining (NJ) method and applying the parameters as described in the MEGA 3.1 program (Kumar et al., 2004). The bootstrap resampling test with 1000 replications was also applied.

Experimental design and statistical analysis

The experiment had two treatments (with and without artificial inoculation), each with 10 plants and arranged in a completely randomized design. Analysis of variance was determined, using statistical analysis software, version 9.1.3 (SAS Institute, Cary, NC). A *t*-test was also conducted.

RESULTS AND DISCUSSION

Characterization of endophytic bacteria

Endophytic bacteria that reside in plant tissues without causing visible harm to the plant were successfully isolated from surface-sterilized *Brachiaria* tissues. Three bacterial isolates designated 01-36062-R2, 02-36062-H4, and 03-36062-V2 were isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively. They tested positive for sequences of the *nifH* gene, which encodes nitrogenase reductase (Figure 1).

The root and leaf tissues from *Brachiaria* plants that were inoculated with the endophytic bacterial cells transformed with GFP were found to have bacterial cells located in intercellular spaces, thus further fulfilling the criteria, published by Reinhold-Hurek and Hurek (1998a),

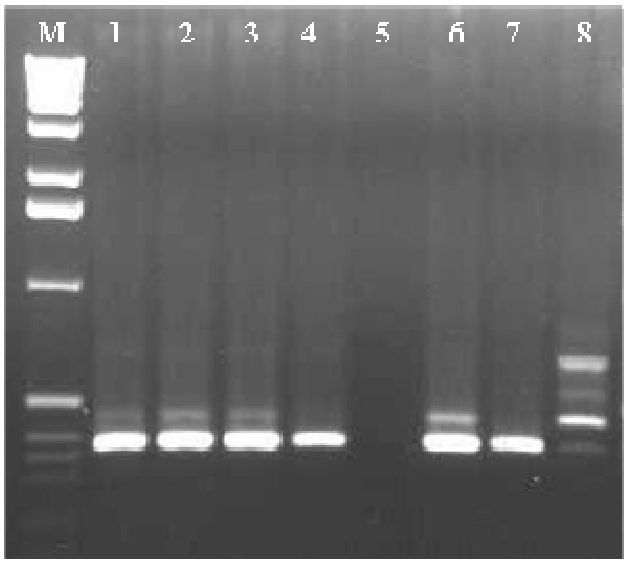

Figure 1. Nested PCR analysis of endophytic bacterial colonies isolated from *Brachiaria* hybrid CIAT 36061 (cv. Mulato) plants, for *nif*H gene sequences. Lanes 1-4 are independent bacterial colonies isolated from Mulato plants (lanes 1 and 2, DNA of bacteria isolated from leaves; lanes 3 and 4 isolated from roots). Lane 5 is negative control *Xanthomonas campestris* isolate 1015; lanes 6 and 7, are positive control *Bradyrhizobium* isolate 2469 and DNA from positive endophytic bacterium 01-36062-R2 isolated from *Brachiaria* hybrid 36062, respectively. Lane 8 is a randomly picked bacterium among bacterial colonies on culture medium. Lane M is size marker.

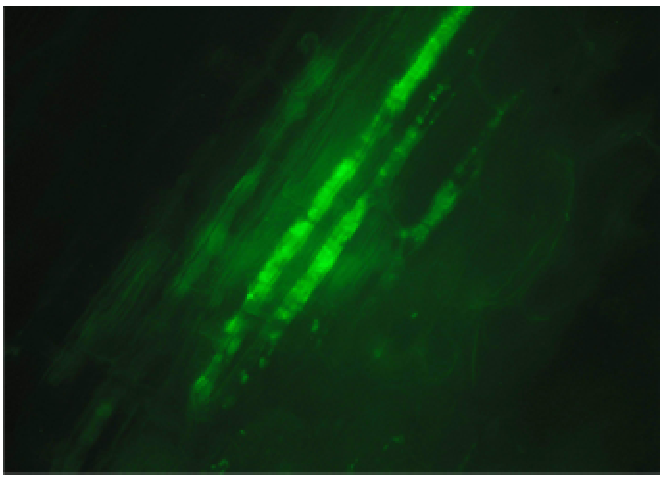

Figure 2. *Brachiaria* hybrid CIAT 36062 root tissues taken from plants artificially inoculated (two months after inoculation) with bacterial endophyte strain 01-36062-R2 transformed with the green fluorescent protein gene, fluorescence emission under UV light, using a Leica H filter, magnified 100X.

on what constitutes 'true' endophytic bacteria (Figure 2).

Because N_2 fixation is performed by diverse groups of prokaryotic organisms, detection of a marker gene that is unique and is required for N_2 fixation may be useful for our studies. Many researchers have used the *nifH* gene with several PCR primers that amplify the gene from

GVIQADSTRLILHAKAQNTIMEMAAEVGSVEDLELEDVLQIGYGGVRCAESGGPEPGVGCAGRG
VITAINLEEEGAYVPDLDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMALYA

Figure 3. Consensus sequences obtained as deduced amino-acid sequences.

5′-TGGGTGTGATCCAAGCCGACTCCACGCGTTTGATCCTGCATGCGAAAGCGCAGAACACC
ATTATGGAGATGGCCGCCGAAGTCGGCTCCGTCGAAGACCTGGAATTAGAAGACGTGCTGC
AAATCGGTTACGGCGGCGTGCGCTGCGCGGAATCCGGTGGCCCGGAGCCAGGTGTGGGC
TGTGCCGGTCGTGGCGTGATCACCGCGATTAACTTCCTCGAAGAAGAAGGCGCTTACGTGC
CGGATCTGGATTTTGTTTTCTACGACGTGCTGGGCGACGTGGTATGCGGTGGTTTCGCCAT
GCCGATTCGTGAAAACAAAGCGCAGGAGATCTACATCGTTTGCTCTGGCGAGATGATGGCC
CTCTACGCA-3′

Figure 4. Consensus sequences obtained as deduced nucleotide sequences.

microbes and other samples (Auman et al., 2001; Lovell et al., 2001; Poly et al., 2001; You et al., 2005). Tropical forage grasses and grasslands may be ideal for studying associations with N_2-fixing bacteria because of their perennial nature and low chemical inputs, including fertilizers. *Brachiaria* hybrid CIAT 36062 was chosen for these studies because it showed greater amounts of total N in leaves than the other genotypes tested and remains green under field conditions in acid soils with low N input (Rao et al., 1998). We have subsequently determined that other genotypes of *Brachiaria* such as CIAT 36061 (cv. Mulato) also contain endophytic bacteria that test positive for *nifH* gene sequences.

Using nested PCR and three primers designed to amplify the *nifH* gene sequences, amplified products were generated with template DNA from these bacterial strains. Fatty acid analysis conducted on these three strains resulted in matching with various bacteria that are known to be N_2 fixers and/or plant growth promoters (Example, *Flavimonas oryzihabitans*, a PGP rhizobacterium that infects grasses).

The fatty acid analysis data of isolate 01-36062-R2 matched it with *Leclercia adecarboxylata*, *Klebsiella pneumoniae* and *Enterobacter cloacae* at the 0.879, 0.841 and 0.820 similarity index, respectively. Of these, *E. cloacae* has been described as being prevalent in citrus plants (Araújo et al., 2002). The match however, was not conclusive.

Although this study focused on strain 01-36062-R2, the fatty acid analysis also matched isolate 03-36062-V2 with *F. oryzihabitans* at a 0.887 similarity index and isolate 02-36062-H4 with *Agrobacterium rubi* (syn. *Rhizobium rubi*) at a 0.845 similarity index. A N_2-fixing endophytic strain of *K. pneumoniae* (Kp342) has been isolated from a N_2 efficient line of maize (Chelius and Triplett, 2000). This strain was described as having a very broad host range and being able to colonize the internal tissues of many plants, starting with fewer than 10 cells in the inoculum (Dong et al., 2003). Endophytic colonization and N_2 fixation were demonstrated in wheat inoculated with *K.pneumoniae* strain Kp342 (Iniguez et al., 2004).

Cloning and sequence analysis

In this study, we cloned and sequenced nested-PCR-amplified products, using primers derived from *nif* gene sequences. The goal was to develop a specific primer that would allow us to screen, without using nested PCR, bacteria that contain *nif* gene sequences and are associated with *Brachiaria* and other tropical plants. We used template DNA isolated from bacterial strain 01-36062-R2 (which had been isolated from *Brachiaria* CIAT 36062 and tested positive for *nif* gene sequences) and successfully cloned a 371-bp nested-PCR amplification product, using the pGEM®-T Easy Vector System (Promega Corporation).

Analysis demonstrated the presence of *nifH* gene sequences in these clones. The deduced amino-acid sequence showed a 97% similarity with 120 amino acids that correspond to the *nifH* gene sequence of *K. pneumoniae*. These results agree with the fatty acid analysis results of this bacterial strain, which matched it with *K. pneumoniae* at a 0.84 similarity index (Kelemu et al., 2006). *Nif* genes that encode the nitrogenase complex and other enzymes involved in N_2 fixation have consensus sequences identical to those of various N_2-fixing bacteria.

K. pneumoniae belongs to the Enterobacteriaceae family, whose members are able to fix N_2. It possesses a total of 20 *nif* genes that are clustered in a 24 kb region of the chromosome and are responsible for nitrogenase synthesis and regulation (Cannon et al., 1979). Three of these genes, *nifH*, *nifD* and *nifK*, code for the three structural nitrogenase subunits. *K. pneumoniae* is an endophytic bacterium associated with various plants and involved in N_2 fixation, including maize (Chelius and Triplett, 2001), wheat (Iniguez et al., 2004) and rice (Dong et al., 2003). Our results constitute the first such report for *Brachiaria* species. The consensus sequences

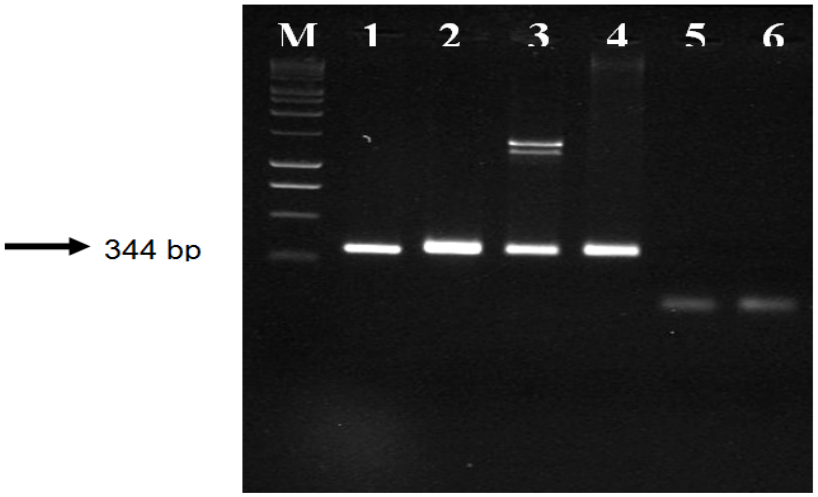

Figure 5. Specific amplifications of template DNA. Lanes 1 to 4, Positive control bacterium *Bradyrhizobium* sp., and strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 isolated from *Brachiaria*, respectively; lane 5 = negative control *Xanthomonas campestris* pv. *graminis*; lane 6 = negative control PCR reaction mixture; lane M = size marker. The primer combination used was 5′-GTTTGATCCTGCATGCAAAAG-3′ and 5′-AGAGCAAACGATGTAGATCTCCTG-3′. The PCR reaction (20 µl) contained 16 ng μl^{-1} template DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 0.2 µM each primer, and 0.2 U *Taq* polymerase. The amplification was carried out in a programmable thermal controller (MJ Research, Waltham, MA) programmed as follows: 94°C for 2 min (94°C for 30 s, 50°C for 45 s, 72°C for 30 s) for 35 cycles; 72°C for 8 min. The products were separated on a 1.2% agarose gel by electrophoresis, stained with ethidium bromide, and photographed under UV light.

obtained in this study as deduced amino-acid sequences and nucleotide sequences are shown in Figures 3 and 4, respectively.

Specific primer development

In this study, we developed specific primers that would allow us to detect endophytic bacteria that are associated with *Brachiaria* species with just one-step PCR instead of nested PCR. Based on the consensus sequence and using the DNAMAN program (Lynnon Biosoftware), nine primers were designed and synthesized. We tested 20 combinations of these primers on selected positive and negative control bacteria. Of these combinations, one pair of primers with sequences 5′-GTTTGATCCTGCA TGCAAAAG-3′ and 5′-AGAGCAAACGATGTAGATCTCC TG-3′ produced only one amplification product of about 344 bp in bacteria used as positive controls, whereas negative controls presented no amplified products (Figure 5).

Nucleotide similarity comparison

The sequences corresponding to the *nifH* gene sequence were edited, cleaned and assembled, using the program Sequencher v 3.0 (Gene Codes Corporation, Ann Arbor, MI). The fragments that showed homology were aligned, using the ClustalW 1.8 program (EMBL-EBI, Cambridge, UK). The sequences that corresponded to strains designated as 01-36062-R2, 02-36062-H4 and 03-36062-V2 (isolated from roots, leaves and stems, respectively, of *Brachiaria* CIAT 36062) were identical to each other.

Analysis demonstrated the presence of *nifH* gene sequences in the sequenced clones, with a similarity of 89% in 283 bp with the *nifH* gene sequence of *K. pneumoniae*, which was designated in GenBank® as AF303353.1. Furthermore, the sequences had an 88% similarity with *Klebsiella* sp. Y83 (DQ821727.1) and *Enterobacter* sp. Y79 (DQ821726.1). The clone from the endophytic bacterium isolated from *Brachiaria* hybrid CIAT 36061 had a 97% sequence similarity in 290 bp with three accessions registered in the GenBank®, designated as DQ982313.1, DQ982300 and DQ982299.1. These sequences corresponded to clones isolated from uncultured diazotrophs (N_2-fixing organisms) isolated from roots and stems of maize plants. *Nif* genes that encode the nitrogenase complex and other enzymes involved in N_2 fixation have consensus sequences identical to those of various N_2-fixing bacteria.

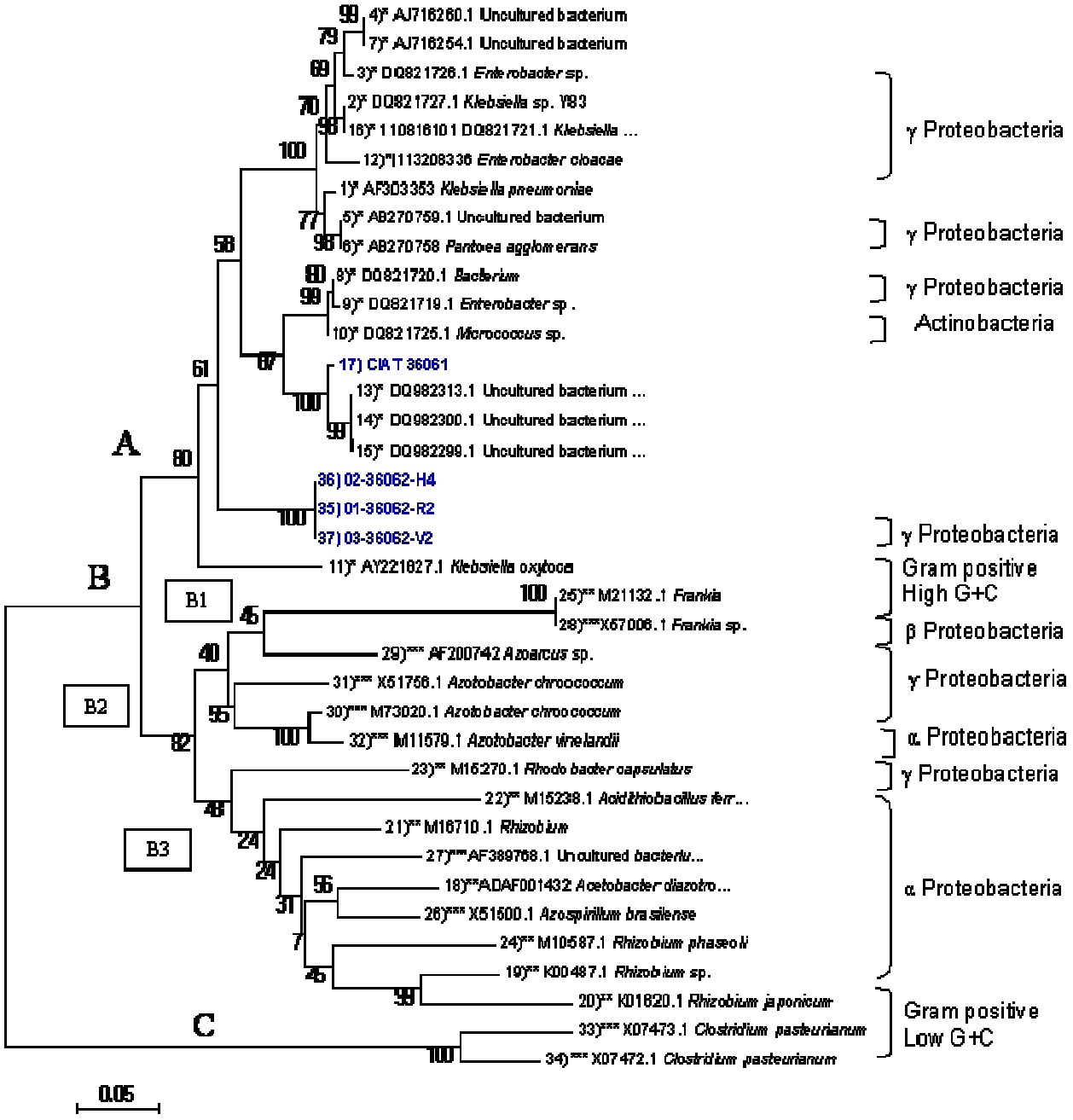

Figure 6. Phylogenetic tree generated from nucleotide sequence analysis of DNA fragments of endophytic bacteria associated with *Brachiaria*, after comparing with sequences that were selected for maximum identity (%), scores, and E-values, and with nucleotide sequences of N_2-fixing organisms used in studies by Franke et al. (1998). The values represent 1000 replications in the bootstrap method.

Sequence comparison

Strains 01-36062-R2, 02-36062-H4, 03-36062-V2 and 36061 (so named for its isolation from the *Brachiaria* grass of the same name) were compared with (a) 16 nucleotide sequences that were selected for having a maximum identity, score, and E-value, and were Registered in the GenBank® (data not shown); (b) 18 nucleotide sequences of N_2-fixing organisms used in studies by Franke et al. (1998); and (c) bacteria with other characteristics.

Figure 6 shows that the sequences analysed were phylogenetically clustered into three groups, A, B, and C, with high bootstrap values of 80, 82 and 100%, respect-

Figure 7. *Brachiaria* hybrid CIAT 36061 plants with indigenous endophytic bacteria (treatment 1) and inoculated with a mixture of three bacterial strains (01-36062-R2, 02-36062-H4, and 03-36062-V2, which were originally isolated from *Brachiaria* CIAT 36062) (treatment 2), 65 days after inoculation. Plants were maintained under greenhouse conditions and given no nutrients.

tively. Group A contained 20 accessions, 8 of which belonged to Gamma Proteobacteria and one to Actinobacteria, one was unidentified, six clones came from uncultured organisms, and four were the endophytic bacterial strains used in this study.

The three sequences from the endophytic bacteria isolated from *Brachiaria* CIAT 36062 and designated 01-36062-R2, 02-36062-H4 and 03-36062-V2 were closely related to *Klebsiella, Enterobacter* and *Micrococcus* spp. Micro-organisms in these three genera are known to be N_2 fixers. These results agree with the biochemical analysis (fatty acid analysis) of isolate 01-36062-R2 (conducted in earlier studies), which showed a match with *Leclercia adecarboxylata*, *K. pneumoniae* and *E. cloacae* at the 0.879, 0.841 and 0.820 similarity index, respectively. The sequence of the endophytic bacterial strain isolated from *Brachiaria* hybrid CIAT 36061 clustered 100% with three clones designated in GenBank® as DQ982313.1, DQ982300, DQ982299.1 and 87% with the rest in Group A (Figure 6).

Group B consisted of 15 accessions subdivided into three subgroups B-1, B-2 and B-3. Subgroup B-1 contained two species of *Frankia* (a soil-inhabiting N_2-fixing bacterium) and one Beta Proteobacteria. Subgroup B-2 consisted of three accessions that belonged to the *Azotobacter* genus. Subgroup B-3 consisted of nine accessions. Group C consisted of two species from the *Clostridium* genus.

Effect of endophytic bacteria on plant attributes that influenced growth and development

Brachiaria hybrid CIAT 36061 carries indigenous endophytic bacteria that cannot be eliminated with certainty. Despite their presence, we introduced three further strains of bacteria, originally isolated from *Brachiaria* hybrid CIAT 36062, into CIAT 36061. In general, these introduced bacteria had a positive effect on the recipient plants' growth and development (Figure 7). Figure 8 shows greater tiller and root development in artificially inoculated CIAT 36061 plants than in those containing only indigenous endophytic bacteria.

Under conditions of deficiencies of N and other nutrients, plants of *Brachiaria* CIAT 36061 inoculated with the three bacterial strains had significantly higher means for all evaluated parameters (except for soluble proteins in leaves) than control plants containing only indigenous bacteria (Table 1).

Analysis of variance showed that the total biomass production (leaf, stem and root) from control plants of *Brachiaria* CIAT 36061 was significantly ($P < 0.05$) less than that from inoculated ones (Figure 9). The data indicated that a close and beneficial interaction existed between the introduced (and indigenous) endophytic bacteria and *Brachiaria* hybrid CIAT 36061, possibly resulting in N_2 fixation and enhanced plant growth. Had we managed to eliminate the indigenous endophytic

Figure 8. *Brachiaria* hybrid CIAT 36061 with indigenous endophytic bacteria (treatment 1) and inoculated with a mixture of three bacterial strains (01-36062-R2, 02-36062-H4, and 03-36062-V2, which were originally isolated from *Brachiaria* CIAT 36062) (treatment 2), 65 days after inoculation. Plants were maintained under greenhouse conditions and given no nutrients. Note the difference in the number of tillers, root growth, and overall development between the artificially inoculated plant and the untreated one.

bacteria from the control plants of CIAT 36061, the difference between bacteria-containing and control plants would probably have been even more dramatic.

A highly significant positive association ($r = 0.89$; $P < 0.01$) was observed between leaf chlorophyll content and total N content in leaves. Inoculated plants maintained a deeper green colour and higher N content in their leaves than control plants. As expected, bacterial cells were isolated from both control plants containing indigenous bacteria and those inoculated with the three bacterial

Table 1. Parameters for plant growth and development in the grass *Brachiaria* hybrid CIAT 36061, evaluated for untreated (control) and endophyte-inoculated plants.

Parameter	Control	Inoculated
Plant height (cm)	104 b	116 a**
Number of leaves per plant	22.5 b	36.9 a***
Number of tillers per plant	4.1 b	7.4 a***
Leaf area (cm^2 per plant)	994 b	1430 a***
Chlorophyll (SPAD units)	34.0 b	43.4 a***
Soluble protein (µg cm^{-2} fresh leaf)	929 a	1095 a
Stem N content (%)	0.51 b	0.67 a**
Green leaf N content (%)	1.0 b	1.3 a**
Dry leaf N content (%)	0.44 b	0.66 a**

Each value is the mean of 10 plants.
*, **, *** = significantly different at the 0.05, 0.01 and 0.001 probability levels, respectively. Data in each row followed by the same letter are not significantly different according to the t-test.

strains with similar values (6.56 $\log_{10}$ cfu g^{-1} of fresh root tissue of inoculated plants versus 6.53 $\log_{10}$ cfu g^{-1} of fresh root tissue of uninoculated control plants). These endophytic bacterial population data are very similar to the natural endophyte concentrations found in alfalfa, maize, sugar beet, squash, cotton and potato, which are reported to vary between 2.0 and 6.0 $\log_{10}$ cfu g^{-1} of tissue (Kobayashi and Palumbo, 2000).

B. brizantha CIAT 6294 (cv. Marandu) had no indigenous endophytic bacteria that had *nifH* gene sequences. We introduced the three strains of bacteria originally isolated from *Brachiaria* hybrid CIAT 36062 into CIAT 6294. The introduction of these bacteria positively affected plant growth and development in the recipient plant CIAT 6294 under nutrient-deficient conditions, with more tiller and root development in the artificially inoculated CIAT 6294 plants than in the control plants.

Analysis of variance showed that the total biomass production (leaf, stem and root) from control plants of *B. brizantha* CIAT 6294 was significantly ($P < 0.05$) less than that from inoculated ones (Figure 10). The data indicated that a close and beneficial interaction existed between the introduced bacteria and *B. brizantha* CIAT 6294, possibly resulting in associative N_2 fixation and enhanced plant growth. These results are consistent with the results for *Brachiaria* hybrid CIAT 36061 (cv. Mulato), which was artificially inoculated with the same three strains of endophytic bacteria.

As with plants of *Brachiaria* hybrid CIAT 36061, under conditions of deficiencies of N and other nutrients, plants of *B. brizantha* CIAT 6294 inoculated with the three bacterial strains had significantly higher means for all evaluated parameters-plant height, number of tillers,

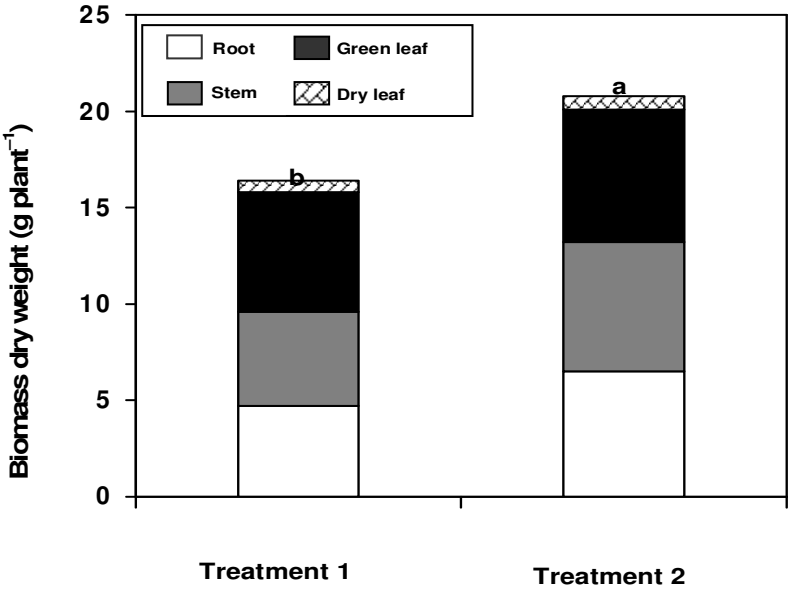

Figure 9. Total biomass production in plants of *Brachiaria* hybrid CIAT 36061 with only indigenous endophytic bacteria (treatment 1; control), and inoculated with a mixture of three bacterial strains (01-36062-R2, 02-36062-H4, and 03-36062-V2, which were originally isolated from *Brachiaria* CIAT 36062) (treatment 2), 65 days after inoculation and maintained without nutrients under greenhouse conditions. Values are means of 10 plants per treatment. a and b indicate significant differences according to the *t*-test.

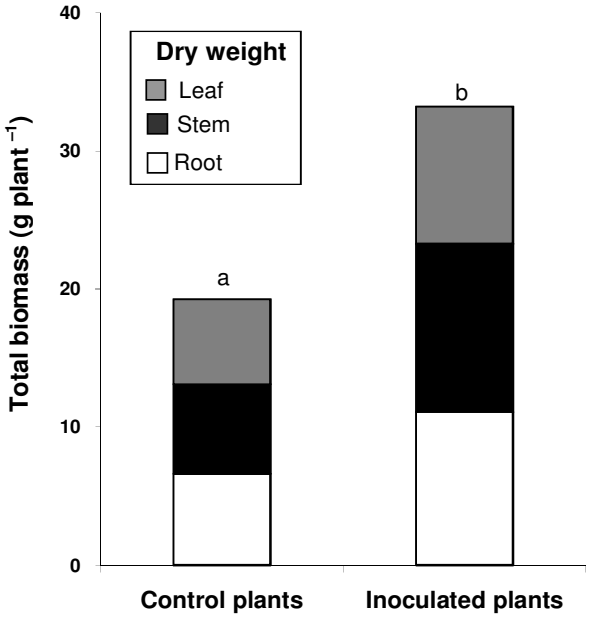

Figure 10. Total tissue biomass production in plants of *Brachiaria brizantha* CIAT 6294 that were untreated (control) and inoculated with a mixture of three bacterial strains (01-36062-R2, 02-36062-H4, and 03-36062-V2, which were originally isolated from *Brachiaria* CIAT 36062), 60 days after inoculation. Plants were maintained under greenhouse conditions with no nutrients. Values are means of six plants per treatment. a and b indicate significant differences according to the *t*-test.

number of leaves and leaf area, than those of control plants (Figure 11). ANOVA also showed that the leaf chlorophyll content (SPAD units) from control plants of *B. brizantha* CIAT 6294 (43.4) was significantly ($P < 0.05$) less than that from inoculated ones (50.3). Furthermore, the total N content in stems and N uptake values in leaves and stems were significantly higher in inoculated plants than in control ones (Table 2).

These data strongly suggest that endophytic bacteria have a direct beneficial effect on plant growth and development and possibly on associative N_2 fixation in *Brachiaria*. Although not all endophytic bacteria associated with plants are N_2 fixing (Barraquio et al., 1997 and Martinez et al., 2003), the possibility that this beneficial effect on plant growth is through associative N_2 fixation is corroborated by the endophytic bacteria sequence data described in this study (Figure 6). PCR amplification of *nifH* genes was used to identify N_2-fixing bacteria in sweet potato grown in N_2-poor soils (Reiter et al., 2003). Some of these bacteria include *Klebsiella* spp. and *Paenibacillus odorifer*.

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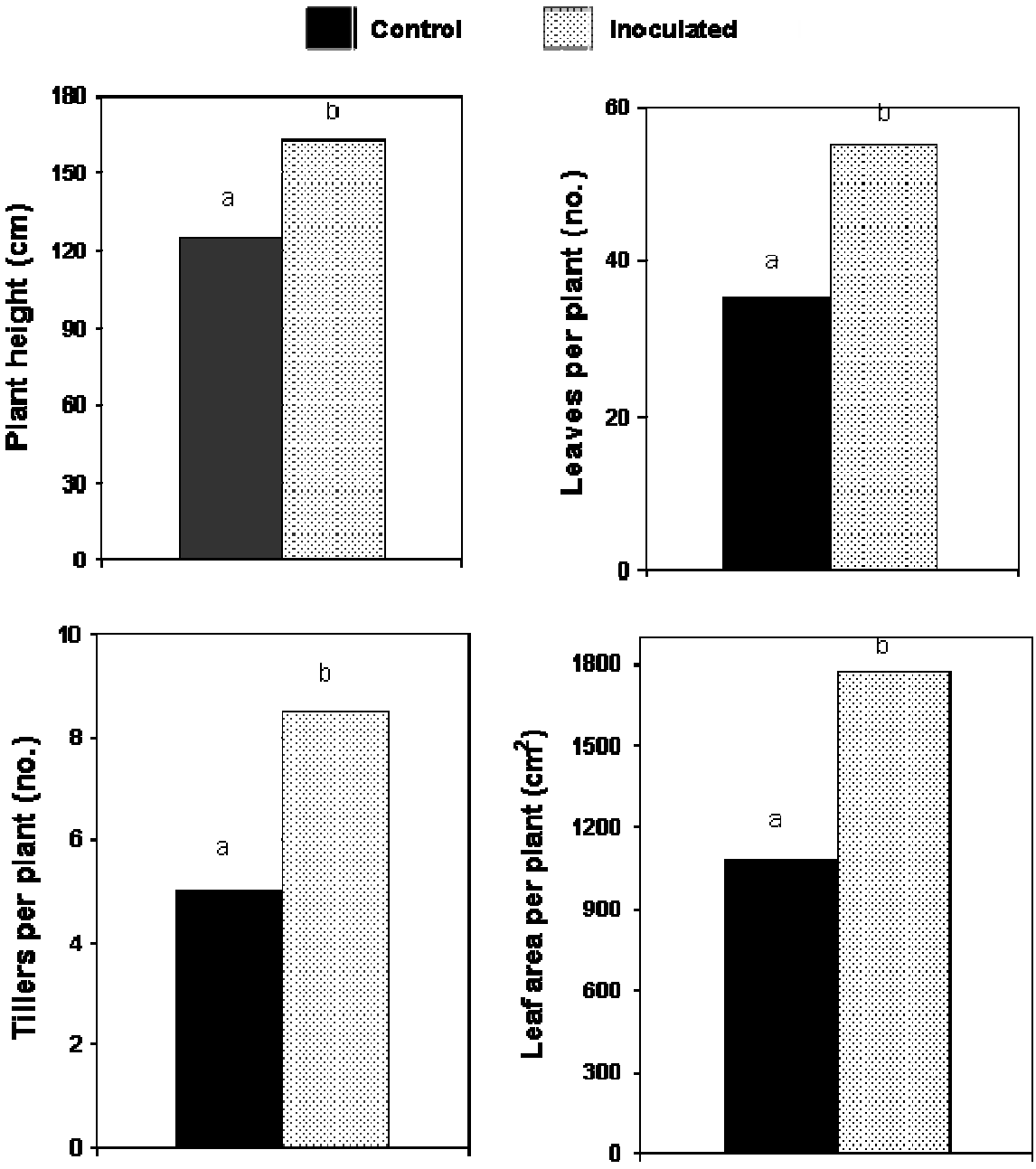

Figure 11. Effect of bacterial isolates (a mixture of three bacterial strains; 01-36062-R2, 02-36062-H4, and 03-36062-V2, which were originally isolated from *Brachiaria* CIAT 36062) on four growth parameters of *Brachiaria brizantha* CIAT 6294, 60 days after inoculation. Plants were maintained under greenhouse conditions and given no nutrients. Values are means of six plants per treatment. a and b indicate significant differences according to the *t*-test.

Table 2. Content and uptake of nitrogen in *Brachiaria brizantha* CIAT 6294 plants that were either left untreated (control) or inoculated with endophytic bacteria.

Plant part	Nitrogen content (%)		Nitrogen uptake (mg per pot)	
	Control	Inoculated	Control	Inoculated
Leaf	2.481a	2.414a	153b	240a***
Stem	0.826b	1.308a***	54b	159a***

Each value is the mean of six plants per treatment. ***, Significantly different at the 0.001 probability level. Data in each row followed by the same letter are not significantly different according to the *t*-test.

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