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ORIGINAL ARTICLE

Composition and activity of endophytic bacterial communities in field-grown maize plants inoculated with *Azospirillum brasilense*

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Abstract The application of agricultural practices in which non-leguminous plants are inoculated with growth-promoting diazotrophic bacteria is gaining importance worldwide. Nevertheless, an efficient strategy for using this inoculation technology is still lacking, and a better comprehension of the environmental factors that influence a plant's ability to support its associative bacterial community is indispensable to achieving standardized inoculation responses. To address the effects of nitrogen (N)-fertilization on the diversity of both the total and metabolically active endophytic bacterial communities of field-grown maize plants, we extracted total DNA and RNA from maize plants inoculated with *Azospirillum brasilense* strain Ab-V5 that were growing in Oxisol and treated with regular and low levels of N-fertilizers (RN and LN, respectively). Four clonal libraries were constructed and sequenced and the dominant populations analyzed. Partial description of the bacterial diversity indicated that plants receiving RN- and LN-treatments can maintain bacterial communities with similar diversity indexes for the total endophytic bacterial community, although the communities of *Novosphingobium* and *Methylobacterium* were unevenly distributed. Fertilization management had a stronger effect on the dominant populations of the metabolically active bacterial community, and 16S rRNA gene libraries from RN plants suggested a lower diversity of such populations in comparison with libraries from LN plants. The agronomic parameters obtained at the end of the crop season indicated that the inoculation treatment was efficient in promoting plant growth. However, the combination of regular treatments with N-fertilizers and plant inoculation did not have an additive effect and actually tended to decrease crop productivity.

Keywords Bacterial diversity · Plant growth-promoting bacteria · Non-legume inoculation · 16S RNA gene library

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Introduction

The search for ways to achieve high crop productivity while concurrently minimizing various environmental hazards associated with current agricultural practices has led researchers to increasingly focus on alternative practices that will enable a reduction the use of chemical fertilizers (Döbereiner 1992; Pérez-Montaño et al. 2013). The enormous biodiversity of

plant–bacteria relationships is emerging as an important source of industrially important bioproducts, such as medically relevant and insecticidal compounds. A better understanding of these relationships can also result in improvements in the practice of inoculating plants with beneficial bacteria. These bacteria are generally known as plant growth-promoting bacteria (PGPBs), and their utilization can represent an alternative low-cost and highly efficient means to protect crops and increase plant resistance against biotic and abiotic stresses, thereby improving agricultural production. Diazotrophic endophytic and associative bacteria represent a specific group of PGPBs of great interest due to their potential to (at least partially) replace nitrogen (N)-fertilizers. However, the mechanisms involved in transferring biologically fixed nitrogen within non-leguminous plant–bacterial associations are not completely understood and, in addition, these bacteria are well known for their capacity to affect plant metabolism directly by synthesizing molecules with phytohormone-like activity (Glick 2012).

Plant-associated bacteria encompass a high diversity of species and occupy a broad range of habitats. It has been noted that attention should be paid to the endophytes within this myriad of species (Döbereiner 1992; Reinhold-Hurek and Hurek 2011). The endophytic bacterial community is thought to affect host plant development and to interact with other plant-associated microbial populations through the following relations: protocooperation, mutualism, commensalism, competition, predation, parasitism and amensalism (Ryan et al. 2008). Furthermore, agricultural practices are also likely to influence the endophytic community because the plant plays an active role in selecting the bacterial species which best fits the resulting endophytic environment (Gaiero et al. 2013). In fact, the establishment of an endophytic community within any plant species represents a dynamic event that is driven by biotic and abiotic influences, with the plant actively modifying the soil microbiota to select the bacterial species most able to colonize the endophytic habitat and thereby regulating its populations (Hartmann et al. 2009). Different crop management methods influence edaphic conditions and plant growth; however, the precise mechanisms through which agricultural practices impact particular bacterial species are still not well known. Hence, the identification of practices that could stimulate (or at least not harm) beneficial associative species could lead to an improved physiological, environmental and productive status in agroecosystems (Singh et al. 2011).

Maize (*Zea mays* L.) ranks first in the global production of cereals, accounting for much of the total calories produced by agriculture. Its high productivity and relatively low price in comparison with other cereals has resulted in a growing global demand, and it is estimated that maize production will increase from 867.5 million tons in 2012 to up to 1,178 billion tons in 2050 (FAO 2012). To achieve the increase in food production needed to meet the demands of this growing global population, food productivity has to be increased in parallel with reductions in production costs and the environmental harm often caused by high agricultural inputs. N is the most limiting nutrient for maize production, but significant losses of N-fertilizer in cultivated fields can damage the environment and represent severe economic costs. For these reasons, there is a great potential in exploiting natural associations between plants and beneficial diazotrophic bacteria. Field-grown maize plants are colonized by a variety of epiphytic and endophytic microorganisms, including enshrined PGPB species such as *Azospirillum*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Herbaspirillum* and other putative species that have yet to be confirmed as PGPBs (Montañez et al. 2012).

Agricultural practices with an emphasis on mineral and organic fertilizer use have been reported to influence the endophytic community of maize roots (Seghers et al. 2004). In addition, maize genotypes also influence the diversity of culturable endophytes (Ikeda et al. 2012). However, the introduction of *Azospirillum brasilense* at high population densities into crop ecosystems seems to induce different responses in the native microbial communities, as pointed out by the authors of various studies who reported little or no effect (Herschkovitz et al. 2005; Lerner et al. 2006), a transient or limited effect (Baudoin et al. 2009; de-Bashan et al. 2010) or strong shifts in the native bacterial community (Correa et al. 2006; Baudoin et al. 2010). The response was found to vary according to plant genotype, bacterial strain, environmental conditions, agricultural practices and other factors, as well as with the introduction of PGPBs at high levels per se (Castro-Sowinski et al. 2007). Correlations between the composition of the endophytic bacterial community and plant productivity have barely been studied. Therefore, we report here a step towards increasing our understanding of the diversity of the total and metabolically active endophytic bacterial community in maize plants that have been inoculated with the PGPB *A. brasilense* Ab-V5 strain under low and regular N-fertilizer input, along with the resulting agronomic and productivity parameters.

Materials and methods

Experimental conditions

Hybrid maize plants AG 2040 (Monsanto Co., St. Louis, MO) were grown at the experimental station of the Universidade Estadual de Londrina (23°20′S, 51°12′W), Paraná State, Brazil. The climate is classified as humid subtropical (Cfa type according to the Köppen climate classification system), with an average temperature and a relative humidity of 20.2 °C and 75 %, respectively. The soil is an Oxisol with a high clay content (82.1 %) that is characterized by a pH (in H_2O) of

5.40, base saturation of 67.75 %, organic matter content of 2.06 %, phosphorus (P) content of 10.7 mg kg^{-1} and total N content of 1.47 g kg^{-1}. The cation exchange capacity of the Oxisol is (in $cmol_c/dm^3$): H + Al, 3.97; K, 0.46; Ca, 6.6; Mg, 1.30; Al, 0.01. The amount and type of fertilizer, which was applied in the plant rows at the time of sowing, was based on this chemical analysis of the soil. The low N-fertilization (LN) treatment consisted of 136 kg ha^{-1} potassium (KCl), 77 kg ha^{-1} P (super triple phosphate) and N-urea at 30 kg ha^{-1}. An additional input of N-urea was manually supplied as side-dressing to the plots in the regular N-fertilization (RN) treatment at 20 days after sowing at a rate of 130 kg ha^{-1}, resulting in approximately 1.67 g of N-urea per plant (calculated based on the distribution of rows and number of plants per row).

The epiphytic bacterium *Azospirillum brasilense* Ab-V5 (Hungria et al. 2010) was used as the inoculant strain. Bacterial cells were grown in Dyg's liquid medium (Rodrigues Neto et al. 1986) for 48 h at 28 °C, and the culture density was determined with a Neubauer cell counting chamber. The cultures were normalized to 1×10^{10} cells mL^{-1} using sterile Dyg's medium as a diluent, and the diluted cultures were used to prepare a peat inoculant with a final concentration of 1×10^9 cells g^{-1}. The seeds were inoculated 12 h before mechanical sowing by first mixing the seeds with a sucrose solution (10 mL sucrose solution kg^{-1} seeds) to increase adhesion of the peat, followed by manually mixing of the treated seeds with 10 g of peat inoculant and air-drying until sowing. Each experimental plot in the field consisted of six rows, each 6 m long, with an inter-row spacing of 0.9 m; planting density was 7 plants m^{-1}. Each plot was separated from its neighboring plot by 1.5 m. The result was a completely randomized block design in a 2 (with and without *A. brasilense* Ab-V5 inoculation) × 2 (RN and LN) factorial scheme with three replications. The "useful" plot area comprised the four central rows of each plot (total of 168 plants). Inoculated plants growing under RN and LN conditions were randomly sampled 35 days after sowing, with three plants taken (subsamples) from the "useful" plot of each replicate (total of 9 plants per treatment); these plants were washed in tap water immediately after sampling to remove the soil particles and the stems immersed in liquid N and stored at −20 °C for molecular analysis.

The productivity parameters of the inoculated maize plants under the RN- and LN-fertilization regimens were determined at the end of the crop season. A total of ten plants from each "useful" plot were randomly selected to determine stem diameter, plant height, ear height, ear length, and the number of kernels per cob. After adjustment to 13 % moisture, the grain yield and 100 seed weight were calculated by harvesting the plants grown in the "useful" plot and by randomly sampling ten samples of 100 seeds, respectively. All data were tested for normality of the variables and homogeneity of variances before subjected to analysis of variance (ANOVA); each assumption required for the ANOVA was verified. Significant differences were followed by comparisons of the means using the LSD test at the 5 % significance level.

Nucleic acid extraction and PCR amplification

For the DNA and RNA extractions, the samples were aseptically processed in a laminar air flow workbench using sterile tweezers and scalpels to obtain the core of the stem base in order to access the endophytic communities. To avoid cross-contamination of samples, the instruments were washed after each contact with a plant sample with sterile water and 70 % ethanol and then wiped dry with sterile absorbent paper. The frozen cores of the maize stems obtained as described above were ground into a fine powder in liquid nitrogen. Total DNA was extracted from 0.5-g subsamples of the ground plant material by the standard phenol–chloroform method (Sambrook and Russel 2001); total RNA was extracted from a second 0.5-g subsample of ground plant material using TRIzol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Nucleic acid extracts were qualitatively evaluated by electrophoresis in agarose gels and quantified by spectrophotometry at 260 nm; the 260/280 ratio was also determined as a quality parameter for each sample.

PCR reactions were performed for each subsample using the universal eubacterial primers 27f and 1492r (Gurtler and Stanisich 1996). The PCR mixtures contained 50 ng of DNA extracts in 50 μL of PCR buffer (Life Technologies), 2 mM $MgCl_2$, 200 nM of each primer, 200 nM of deoxynucleoside triphosphate, and 1 U AmpliTaq DNA polymerase (Life Technologies). The reaction mixtures were incubated in a Life Express thermocycler (BIOER, Bingjiang, China) for an initial cycle of 5 min at 94 °C, followed by 35 cycles of 25 s at 94 °C (denaturation), 2.5 min at 57 °C (annealing), and 2 min at 72 °C (extension), with a final extension step of 10 min at 72 °C. Fragments of the expected size (approx. 1, 430 bp) were excised from the agarose gels and subjected to semi-nested PCR amplification using primers 27f and 518r (with fragment lengths of approx. 450 bp; Muyzer et al. 1993) with the aim of decreasing the number of amplified fragments from the mitochondria and chloroplasts in the libraries. Fragments obtained by semi-nested PCR were mixed according to each treatment to form a single 16S rRNA gene fragment pool, which was used to construct the DNA-derived 16S rRNA gene clone libraries. Reverse transcription (RT) PCR was performed for each subsample using 500 ng of RNA extracts synthesized into cDNA with M-MuLV reverse transcriptase (New England BioLabs, Ipswich, MA) using primer 778r (Rösch and Bothe 2005). Reactions without reverse transcriptase were included as controls to ensure that the amplification products originated exclusively from RNA. Control and template reactions were subjected to PCR

amplification using primers 27f (Gurtler and Stanisich 1996) and 518r (Muyzer et al. 1993). The amplification products were mixed according to the treatment to form a single 16S rRNA fragment pool and used to construct the 16S rRNA gene clone libraries that were derived from RNA.

16S libraries: cloning and sequencing

The PCR products were purified with a PureLink Quick Gel Extraction and PCR Purification Combo Kit (Life Technologies) and cloned with a TOPO TA Cloning Kit (Life Technologies) with a pCR 2.1-TOPO vector according to the manufacturer's instructions. Random inserts were sampled and verified by PCR when the correct size was identified by digestion with the *Eco*RI restriction enzyme (Life Technologies). Chemically competent *Escherichia coli* DH5α cells were transformed with the cloned fragments, resulting in two libraries from DNA-derived PCR fragments (DNA-derived libraries) and two libraries from RNA-derived fragments (RNA-derived libraries). The mean transformation efficiency was approximately 1×10^4 transformants μg^{-1} PCR product. The DNA-derived clone libraries were named EEM160N (regular N-fertilization) and EEM30N (low N-fertilization); RNA-derived clone libraries were named RTEE M160N (regular N-fertilization) and RTEEM30N (low N-fertilization). The plasmids were isolated by using a miniprep protocol from Sambrook and Russel (2001), and the isolated plasmids were evaluated by electrophoresis in agarose gels and by spectrophotometry at 260 nm to determine the quantity and quality of the extracts.

Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) with primer 518r (Muyzer et al. 1993) on an ABI 3500 automated sequencer (Applied Biosystems, Foster City, CA). Base-calling was performed with the PHRED program (Ewing and Green 1998), and the sequences were trimmed to exclude low-quality ends before being assembled using BioEdit software (Hall 1999). A chimera check of the 16S rRNA gene sequences was performed using the Bellerophon (Huber et al. 2004), Pintail, and Mallard programs (Ashelford et al. 2005).

Biodiversity and phylogenetic analysis

Rarefaction curves and Shannon–Wiener diversity indexes were obtained using the FastGroupII platform (http://fastgroup.sdsu.edu; Yu et al. 2006) and defining the operational taxonomic units (OTUs) at 97 % sequence identity (PSI). The homogeneous coverage of clone libraries (*C*) was calculated according Singleton et al. (2001). The species richness was calculated by dividing the number of OTUs by the homogeneous coverage of clone libraries *C*. Sequence identities were assigned based on the closest match to sequences available at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) by using the BLAST algorithm. All sequences were submitted to GenBank (accession no. JX899827–JX900131). The RDP Lib Compare tool was used to estimate abundance difference within a given phylogenetic taxon (Wang et al. 2007).

Representative sequences from each OTU as determined by the FastGroupII platform output were aligned using the ClustalX2 program (Larkin et al. 2007), and the resulting multiple sequence alignment was corrected manually using BioEdit (Hall 1999). Phylogenetic inference was obtained using MEGA6 software (Tamura et al. 2013), the evolutionary history was inferred by using the UPGMA method, and the evolutionary distances were computed with the Maximum Composite Likelihood method. The resulting tree output was obtained by bootstrap test (1,000 replicates), and the tree was compressed at 1 % base substitution per site to improve visualization of the results. Statistical comparisons of the individual clone libraries were performed with the RDP II Library Compare tool (http://rdp.cme.msu.edu/comparison/comp.jsp; (Cole et al. 2009).

Results

The DNA and RNA extracted from the stem core sections of field-grown maize plants was used to amplify and perform RT-PCR of the 16S rRNA gene fragments from the endophytic bacterial community. From among all of the clones in each library, we randomly chose 114, 111, 38, and 42 clones from the EEM160N, EEM30N, RTEEM160N, and RTEEM30N libraries, respectively, for sequencing. From the DNA-derived libraries, 60 sequences (26.7 %) were identified as plastid 16S rRNA genes (chloroplast DNA) and discarded from further analysis. No plastid-related 16S rRNA gene sequences were found in the RNA-derived libraries. The clone sequences remaining in the four libraries reflected the dominant bacterial groups associated to maize, although it must be noted they provide only a partial overview of the total bacterial diversity.

The rarefaction curves of the 16S rRNA clone libraries (Fig. 1) indicate that the number of screened clones was insufficient to access the complete diversity of the endophytic bacterial community within all clone libraries, with the exception of the RTEEM160N library (Fig. 1f). The library coverage ranged from 71.7 to 97.% and supports the finding that more clones should be sequenced to characterize the total diversity of endophytic species associated with maize plants grown under the conditions studied here (Table 1). The Shannon–Weaver indices and species richness estimates indicate a similar diversity among DNA-derived libraries, whereas no clear N-fertilization effect was noted. Data from

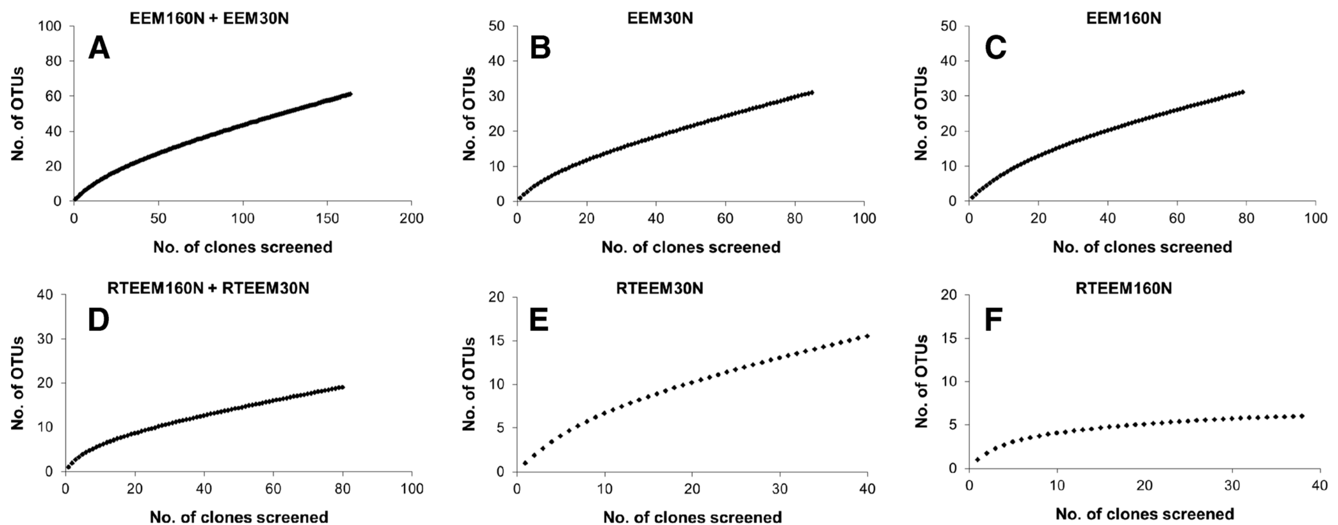


Fig. 1 Rarefaction analysis of 16S rRNA clone libraries [DNA-derived clone libraries: *EEM160N* regular nitrogen (N)-fertilization treatment (*RN*), *EEM30N* low N-fertilization treatment (*LN*); RNA-derived clone libraries: *RTEEM160N* RN, *RTEEM30N* LN] recovered from field-grown maize (AG 2040 hybrid) inoculated with *Azospirillum brasilense* Ab-V5 under different N-fertilization conditions. **a** Clones screened from DNA-derived libraries of plants grown under 160 kg N ha^{-1} (RN plants) and 30 kg N ha^{-1} (LN plants), **b** DNA-derived clones from LN plants, **c** DNA-derived clones from RN plants, **d** clones screened from RNA-derived libraries of plants grown under 160 kg N ha^{-1} and 30 kg N ha^{-1}, **e** RNA-derived clones from LN plants, **f** RNA-derived clones from RN plants. *OTU* Operational taxonomic units

combinations of the genomic libraries showed higher diversity estimates and higher numbers of OTUs for DNA-derived libraries relative to the RNA-derived libraries, indicating that the active endophytes made up a portion of the total endophytic bacterial community associated with maize. Comparison of the diversity estimates and numbers of OTUs among the RNA-derived libraries revealed that both values were higher among LN plants than among RN plants. In addition, the numbers of OTUs observed when libraries were jointly analyzed were lower than the sum of OTUs identified in each single library, suggesting that few OTUs were shared by both libraries, except for the combined analysis of the EEM160 and RTEEM160 libraries (Table 1).

The distribution of representative OTUs with respect to the phylogenetic composition of the 16S rRNA gene libraries as determined by the RDP Classifier and its respective closest relatives in GenBank are presented in Electronic Supplementary Material (ESM) 1, 2, 3, and 4. *Proteobacteria* was the dominant taxon in all libraries (90.5–100 %), with the most abundant class being the

Table 1 A statistical analysis and description of the 16S rRNA gene clone libraries from the stem core samples of field-grown maize (AG 2040 hybrid) inoculated with *Azospirillum brasilense* Ab-V5

Library[a]	Nucleic acid[b]	Fertilization condition	Sequences	OTUs[c]	Homogeneous coverage (%)[d]	Species richness	Shannon–Weaver index
EEM30N	DNA	LN	85	33	71.7	46.0	2.89
EEM160N	DNA	RN	80	31	73.8	42.0	2.89
RTEEM30N	RNA	LN	42	16	73.8	21.7	2.27
RTEEM160N	RNA	RN	38	6	97.4	6.2	1.48
EEM30N+EEM160N	DNA	LN+RN	165	61	63.0	96.8	3.59
RTEEM30N+RTEEM160N	RNA	LN+RN	80	18	77.5	23.2	2.17
EEM30N+RTEEM30N	DNA+RNA	LN	127	46	75.6	60.8	3.27
EEM160N+RTEEM160N	DNA+RNA	RN	118	37	82.2	45.0	3.13

RN, Regular nitrogen (N)-fertilization treatment; LN, low N-fertilization treatment; OTU, operational taxonomic unit

[a] See text (section 16S libraries: cloning and sequencing) and caption to Fig. 1 for a definition of the libraries

[b] The nucleic acid extract used to construct the respective clone library

[c] OTUs were defined at 97 % sequence identity

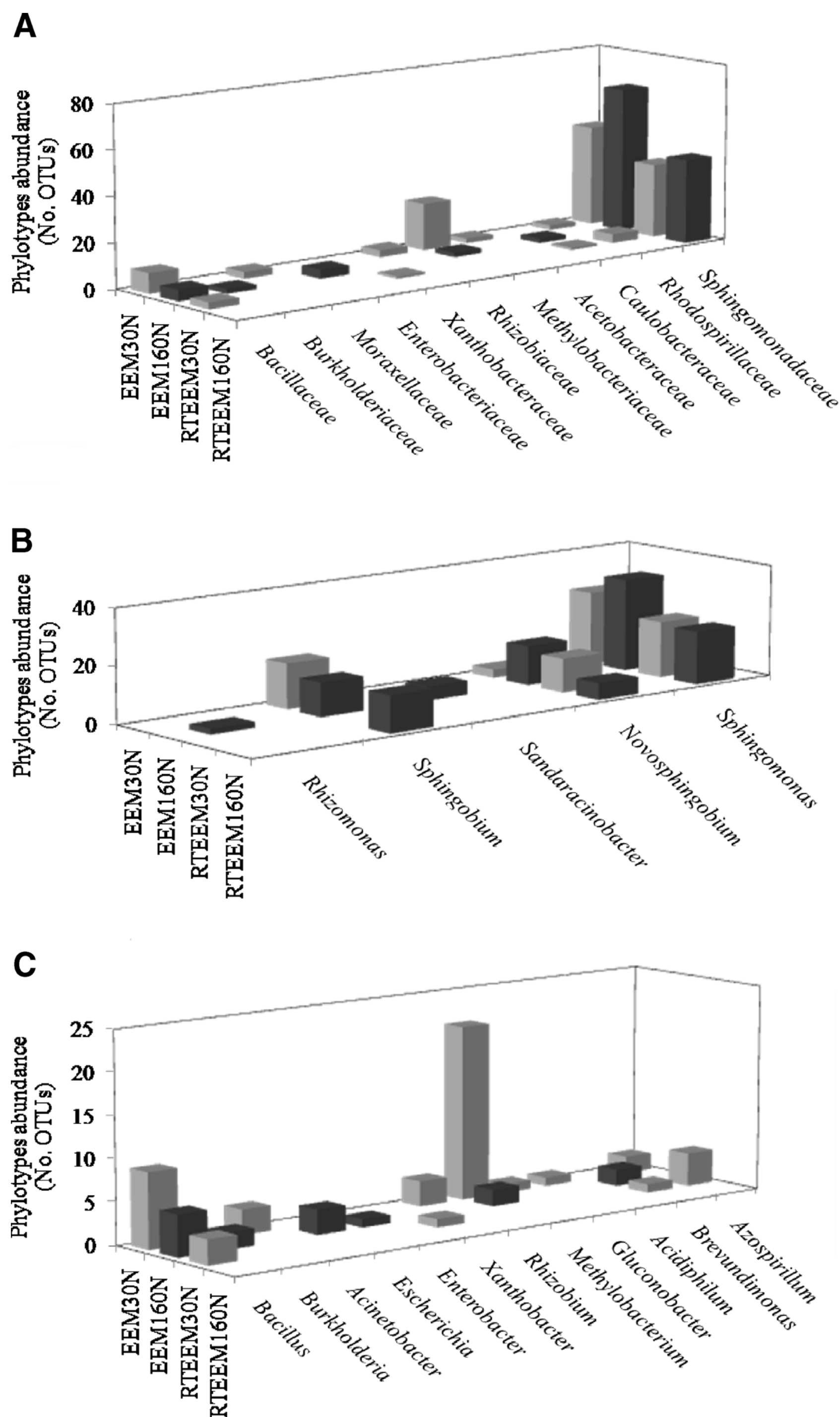
[d] $C_x=1-(n_x/N)$, where n_x is the number of OTUs represented by a single clone sequence in a library and N is the total number of clones

Alphaproteobacteria (88.1–100 %). Representative sequences of *Gammaproteobacteria* and *Betaproteobacteria* were not detected in the RNA-derived libraries, despite N-fertilization. Representative *Firmicutes* sequences were also identified in three of the libraries, ranging from 4.7 to 14.1 %, with the except being the library constructed from the RNA extracts of maize plants receiving the RN-fertilization treatment. At the family level, the *Sphingomonadaceae* encompassed the majority of the OTUs in both RN and LN plants (73.8 % of all sequenced clones), reaching up to 100 % of OTUs in the RNA-derived library from RN plants. Following the *Sphingomonadaceae*, only the OTUs related to the families *Bacillaceae* and *Methylobacteriaceae* were present at relative abundances of >5 %, reaching up to 6.9 and 6.5 % of the total sequenced clones in these two families, respectively. While most of the non-sphingomonad-related taxa occurred at a low relative abundance (<5 % of cloned sequences) in each particular library, these taxa were responsible for shifts in the endophytic bacterial communities of plants grown under contrasting N-fertilization levels (Fig. 2).

The results of a pairwise comparison between DNA-derived libraries that was performed with RDP's LibCompare showed significant differences among the composition of total endophytic bacterial communities in plants grown under different N-fertilization conditions (Fig. 2). Although the *Sphingomonadaceae* were predominant and a major component of the endophytic community under both LN- and RN-fertilization conditions, members of the genus *Novosphingobium* were identified in higher abundance in RN plants (*P* value>9.6E-4), which is at a lower taxonomic level (Fig. 2b). The non-sphingomonads were also unevenly distributed among LN and RN plants, with the relative abundance of members of genus *Methylobacterium* decreasing in RN plants (*P* value>3.5E-3; Fig. 2c). When only the RNA-derived clone libraries were analyzed, pairwise comparisons of the metabolically active endophytic bacteria provided an unclear picture of the fertilization effect on OTU abundance (Fig. 2b, c). Nevertheless, maize plants receiving the regular amount of N-fertilization (e.g., 160 kg N ha^{-1}) showed a narrower diversity of physiologically active endophytes than those under the low N-fertilization regimen (30 kg N ha^{-1}). Surprisingly, the sequencing results of the RNA-derived 16S rRNA gene library from RN plants, which presented a relatively higher homogeneous coverage (97.4 %) and a rarefaction curve reaching a plateau, was formed by as few as six OTUs in terms of the active endophytic bacterial community, all of which are related to the *Sphingomonadaceae* family (Fig. 2b, c; ESM 3). However, the active bacteria associated with LN plants (ESM 4) showed a broader diversity, with up to 16 OTUs, of which seven belonged to non-sphingomonads (*Bacillaceae*, 3 OTUs; *Rhodospirillaceae*, 2; *Caulobacteraceae*, 1; *Xanthobacteraceae*, 1). While these results should be confirmed in future studies, differences in the sphingomonad-related 16S rRNA gene sequences from the LN and RN plant libraries suggest the influence of N-fertilization on the colonization of the endophytic maize habitat by these taxa.

Fig. 2 Phylogenetic distribution of 16S rRNA gene clone libraries from field-grown maize (AG 2040 hybrid) inoculated with *A. brasilense* Ab-V5 under different N-fertilization conditions. *Light-gray bars* Libraries constructed from LN plants, *dark-gray bars* libraries constructed from RN plants. **a** Phylogenetic distributions at the family level, **b** relative abundance of *Sphingomonadaceae* genera, **c** relative abundance of non-*Sphingomonadaceae* genera. Clone libraries as as described in Fig. 1. Classification of family within phylum (for clarity, only the family within each combination is in italics): *Bacillaceae*, Firmicutes; *Burkholderiaceae*, Proteobacteria; *Moraxellaceae*, Proteobacteria; *Enterobacteriaceae*, Proteobacteria; *Xanthobacteraceae*, Proteobacteria; *Rhizobiaceae*, Proteobacteria; *Methylobacteriaceae*, Proteobacteria; *Acetobacteraceae*, Proteobacteria; *Caulobacteraceae*, Proteobacteria; *Rhodospirillaceae*, Proteobacteria; *Sphingomonadaceae*, Proteobacteria. Classification of genera according to class, order, and family (in this sequential order; for clarity, only the genus is given in italics): *Rhizomonas*, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae; *Sphingobium*, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae; *Sandaracinobacter*, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae; *Novosphingobium*, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae; *Sphingomonas*, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae; *Bacillus*, Bacilli, Bacillales, Bacillaceae; *Burkholderia*, Betaproteobacteria, Burkholderiales, Burkholderiaceae; *Acinetobacter*, Gammaproteobacteria, Pseudomonadales, Moraxellaceae; *Escherichia*, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae; *Enterobacter*, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae; *Xanthobacter*, Alphaproteobacteria, Rhizobiales, Xanthobacteraceae; *Rhizobium*, Alphaproteobacteria, Rhizobiales, Rhizobiaceae; *Methylobacterium*, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae; *Gluconobacter*, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae; *Acidiphilum*, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae; *Azospirillum*, Alphaproteobacteria, Rhodospirillales, Rhodospirillaceae; *Brevundimonas*, Alphaproteobacteria, Caulobacterales, Caulobacteraceae

To better evaluate the cross-distribution of OTUs among the four libraries, we performed a clustering analysis of 16S rRNA sequences using the representative OTUs from the full set of clone sequences as determined by the FastGroupII program (Figs. 3 and 4). The phylogenetic distribution of the *Sphingomonadaceae*-related OTUs is presented in Fig. 3; despite the identification of 45 OTUs representing the *Sphingomonadaceae* from all libraries, the phylogenetic tree compressed at 1 % base substitution per site resulted in 37 clusters. From these, six OTUs, namely, RTEEM30N 086, EEM30N 403, RTEEM160N 056, EEM160N 485, RTEE M30N 045, and EEM160N 600, were shared by two or more clone libraries and corresponded to the vast majority of sphingomonad-related sequences (110 sequences, 60.8 % of total sphingomonads). The representative clones for these six OTUs were closely related to *Sphingobium* (two OTUs, 46 clone sequences), *Sphingomonas* (two OTUs, 35 sequences),



and *Novosphingobium* (two OTUs, 29 sequences). Of these individuals, two OTUs (RTEEM160N 056 and RTEEM30N 045) were restricted to the libraries constructed from RNA extracts of both LN and RN maize plants (41 sequences, 22.7 % of total) and were closely related to *Sphingomonas* (25 clone sequences) and *Novosphingobium* (16 sequences). A total of 14 sphingomonad-related OTUs were found to be exclusive to RN maize plants (32 sequences, 17.7 % of total), from which one OTU was restricted to the RNA-derived library (RTEEM160 082). These clones were related to *Sphingomonas* spp. (11 OTUs, 29 clone sequences) and *Novosphingobium* spp. (three OTUs, three sequences). In addition, endophytic sphingomonads from maize plants grown under LN-fertilization conditions formed a total of 17 exclusive OTUs (39 sequences, 21.5 % of the total), with none of these found in the RNA-derived library. These OTUs were

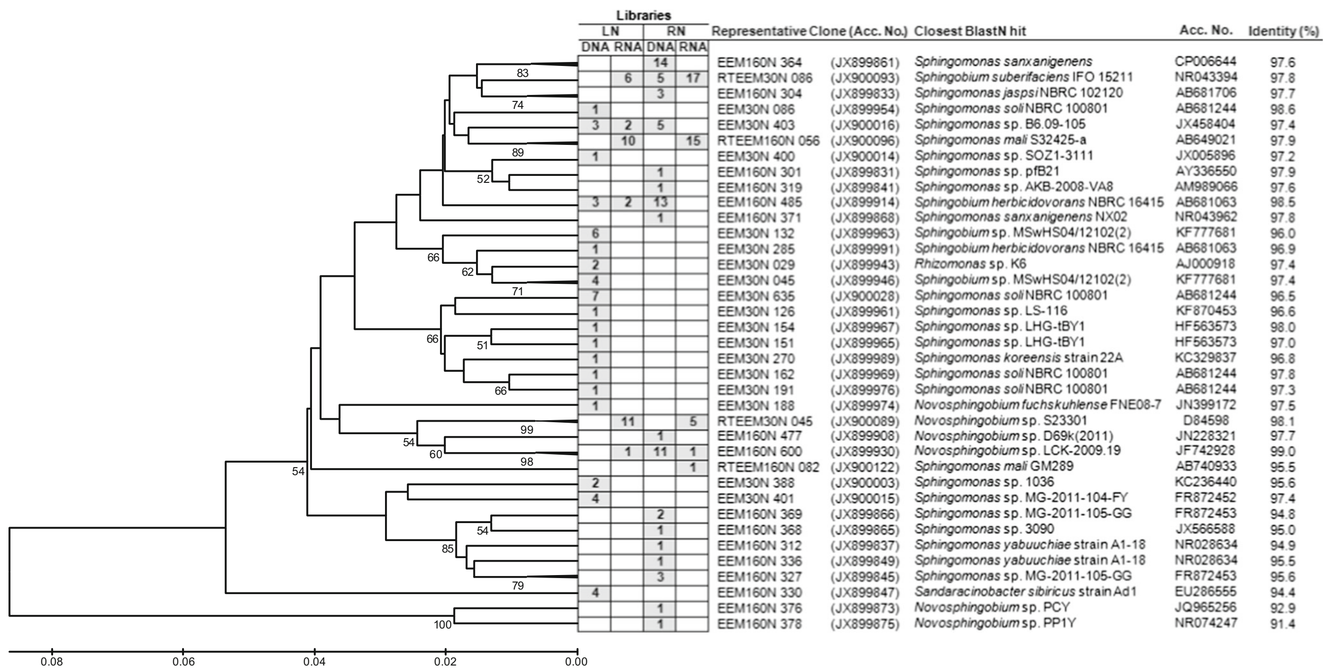


Fig. 3 Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences from clone libraries of field-grown maize (AG 2040 hybrid) inoculated with *A. brasilense* Ab-V5 under different N-fertilization conditions. Representative sequences of each OTU assigned to the *Sphingomonadaceae* were used to infer the evolutionary history, with distances computed by Maximum Composite Likelihood method and presented as the number of base substitutions per site. *Numbers at branch points* Percentages of 1,000 bootstrap values above a 50 % threshold. The tree was compressed at 1 % base substitution per site. *Colored squares* Clone library to which each OTU belongs, *number in squares* number of sequences of each OTU in its respective library

closely related to *Sphingomonas* (11 OTUs, 21 sequences), *Sphingobium* (3 OTUs, 11 sequences), *Novosphingobium* (one OTU, 1 sequence), *Sandaracinobacter* (1 OTU, 4 sequences), and *Rhizomonas* (1 OTU, 2 sequences). Seven sphingomonad-related OTUs were assumed to indicate the active groups of the maize endophytic habitat (71 sequences, or 39.2 % of the total) due to the clustering of clone sequences from the RNA-derived libraries with these representative sequences. Analysis of this phylogenetic distribution revealed a shift in the structure of the endophytic community in maize in response to N-fertilization, with a decrease in the relative abundance of *Novosphingobium* in the DNA clone library of LN plants and an increase in their relative abundance in the RNA clone library under the same growth condition.

The distribution of representative non-sphingomonad-related OTUs revealed that N-fertilization had a relatively pronounced effect on the phylogenetic composition and diversity of endophytes in maize plants (Fig. 4) and that the diversity of endophyte bacterial populations of maize under the LN-fertilization condition were enriched. The non-sphingomonad taxa were represented by 33 representative sequences that clustered into 26 groups in the compressed phylogenetic tree at 1 % base substitution. These clusters were unevenly distributed among LN and RN 16S rRNA clone libraries, with a higher number of representative OTUs in LN plants. Only three clusters formed by ten non-sphingomonad sequences were identified in both the LN and RN libraries, namely, RTEEM30N 049, EEM160N 466, and EEM160N 358; the first cluster was related to *Brevundimonas* (three clone sequences) and the other two clusters were related to *Bacillus* (7 clone sequences in total). Taking both the DNA and RNA 16S rRNA libraries into consideration, LN plants showed 17 exclusive clusters (46 clone sequences, or 71.9 % of the non-sphingomonads) in contrast to the six exclusive OTUs found for the RN libraries (8 sequences, or 12.5 % of the non-sphingomonads). The OTUs which were exclusive to the RN libraries were related to *Methylobacterium* (2 sequences), *Burkholderia* (2 sequences), *Enterobacter* (1 sequence), and *Escherichia* (1 sequence). For the LN-fertilization condition, the exclusive non-sphingomonads formed three OTUs related to *Methylobacterium* (21 sequences), three OTUs related to *Azospirillum* (6 sequences), a *Xanthobacter* OTU (1 sequence), three OTUs related to *Rhizobium* (3 sequences), a *Gluconobacter* OTU (1 sequence), an *Acidiphilium* OTU (1 sequence), an OTU related to *Acinetobacter* (3 sequences), and four OTUs that were closely related to *Bacillus* (10 sequences). Metabolically active non-sphingomonad endophytes could not be detected in extracts from RN plants. An analysis of sequenced clones from 16S rRNA gene libraries in plants grown under the LN condition identified representative OTUs related to *Azospirillum*, *Xanthobacter*, *Brevundimonas* and *Bacillus*. Although a possible bias in the phylogenetic composition of maize endophytes due to the low coverage of the libraries

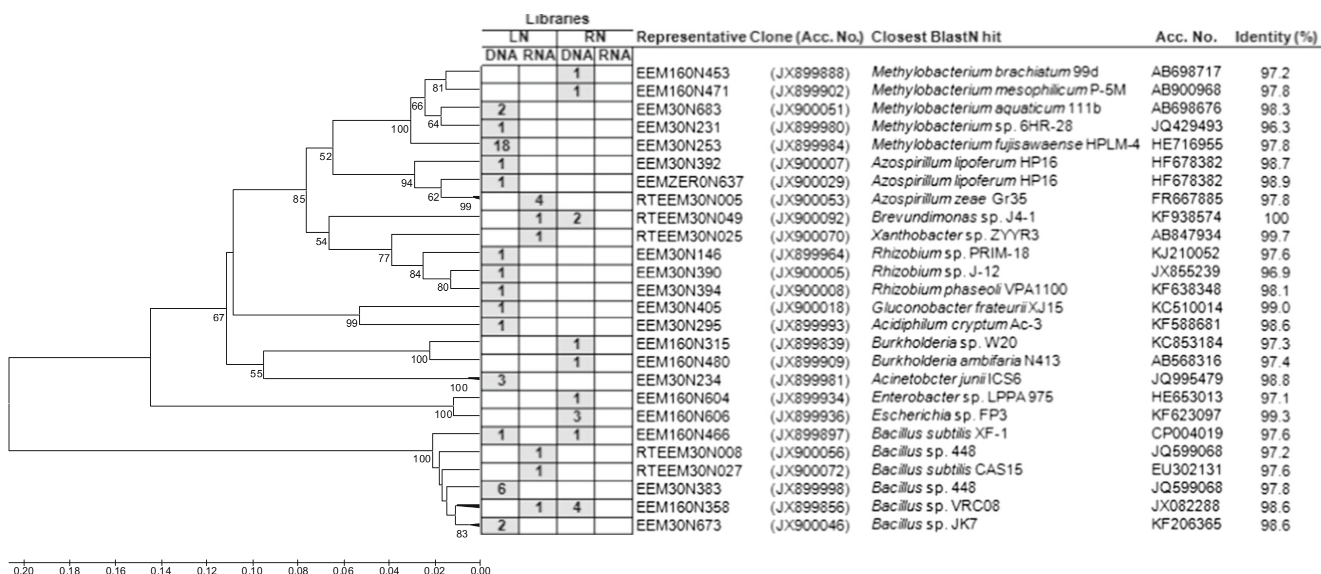


Fig. 4 Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences from clone libraries of field-grown maize (AG 2040 hybrid) inoculated with *A. brasilense* Ab-V5 under different N-fertilization conditions. Representative sequences of each OTU not assigned to the *Sphingomonadaceae* were used to infer the evolutionary history with distances computed by Maximum Composite Likelihood method and presented as the number of base substitutions per site. *Numbers at branch points* Percentages of 1,000 bootstrap values above a 50 % threshold. The tree was compressed at 1 % base substitution per site. *Colored squares* Clone library to which each OTU belongs, *number in squares* number of sequences of each OTU in its respective library

cannot be ignored, the fact that only nine clusters of 26 non-sphingomonads were identified in RN libraries, in addition to the failure to identify non-sphingomonad-related sequences in the library constructed from maize RNA extracts under the RN condition, reinforces the putative effect of N-fertilization in decreasing the metabolically active endophytic bacterial populations in field-grown maize plants.

The agronomic evaluations performed at the end of the crop season demonstrated that *A. brasilense* Ab-V5 inoculation was successful and that inoculated bacteria promoted plant growth (Table 2). Although some parameters were not significantly different between treatments, such as the stem diameter, plant height, ear height, and ear length, significant differences were observed in the other parameters. For example, the number of kernels per cob, grain weight, and estimated productivity were increased in maize plants inoculated with *Azospirillum brasilense* Ab-V5, and even those plants grown under the condition of a low N-fertilization input reached the same productivity as plants grown under the RN condition, although the latter apparently gained no benefit from inoculation with *A. brasilense* Ab-V5. These results indicate the ability of the maize plants inoculated with *A. brasilense* Ab-V5 to support a high yield when inoculation substituted for up to 80 % of the total applied N-fertilizer (Table 2).

Discussion

Maize plants are naturally colonized by a high diversity of bacterial species (Chelius and Triplett 2001; Montañez et al. 2012; Arruda et al. 2013), which are currently believed to play important roles in plant development and the expression of plant genes (Hartmann et al. 2009; Friesen et al. 2011). Bacterial communities associated with maize are likely to originate from the soil microbiome, from which the plant is able to enrich a few taxa selectively in response to root metabolism, a phenomenon known as the rhizosphere effect (Berendsen et al. 2012). The selective enrichment of bacterial taxa by plant roots results in a decreasing biodiversity gradient from the bulk soil towards the endophytic habitat (Roesch et al. 2008). This effect can be observed as soon as the plant starts its development (20 days after sowing), and it is influenced by a myriad of biotic and abiotic factors, such as the plant genome, plant age, soil nutrient availability, and soil pH, among others (Bouffaud et al. 2014).

The variability in the response of plants in inoculation trials with PGPB has been widely reported, as has the observation that maize plants inoculated with diazotrophic PGPB rarely present a combined effect with high N-fertilization input. The main goal of this study was to address the influence of N-fertilization on the composition and activity of the bacterial community associated with maize plants inoculated with *Azospirillum brasilense* Ab-V5 (used in Brazil for the production of commercial inoculants) and improve the performance of inoculants of diazotrophic bacteria, rather than to determine the full composition of the microbial community associated with maize. Consequently, we did not sample uninoculated plants for analysis of its bacterial community, rather we focused on achieving a better understanding of a specific crop management program which aims to implement crop

Table 2 Mean square for agronomic traits of maize (hybrid AG 2040) evaluated under different N-fertilization and *Azospirillum brasilense* (Ab-V5) inoculation treatments and its effects on stem diameter, plant height, ear height, ear length, number of kernels per cob, 100 kernel weight, and grain yield

ANOVA (Mean Square)

Source of variation	*df*	Stem diameter	Plant height	Ear height	Ear length	K/C	100 Kernel weight	Grain yield
Nitrogen (N)	1	3.09*	42.79	170.52	171.17	545.61	9.00	1.38
Inoculation (I)	1	0.26	89.46	13.02	220.03	5142.09*	25.00*	8.40*
N x I	1	0.13	253.06	37.12	318.04	2653.13	25.00*	5.97*
Blocs	3	0.82	755.54*	162.60	163.10	1207.13	0.33	0.78
Error	9	0.46	140.08	43.58	95.30	766.40	4.78	1.06
Coefficient of variance (%)		4.76	4.38	5.12	6.94	6.59	6.57	11.26

Agronomic evaluations

Treatments		Stem diameter (mm)[a]	Plant height (cm)[a]	Ear height (cm)[a]	Ear length (mm)[a]	K/C[a]	100 Kernel weight (g)[a,b]	Grain yield (MG ha^{-1})[b,c]
T1. Uninoculated + 30 kg N ha^{-1}		13.58	274.83	126.28	129.25	383.75	30.00 b	7.493 b
T2. Uninoculated + 160 kg N ha^{-1}		14.63	270.15	129.75	144.63	421.10	34.00 a	9.302 a
T3. Inoculated + 30 kg N ha^{-1}		14.15	268.64	126.30	143.63	436.99	35.75 a	10.197 a
T4. Inoculated + 160 kg N ha^{-1}		14.70	272.23	132.20	142.50	428.71	34.50 a	9.603 a
Uninoculated plants	(T1 + T2)	14.10	272.49	128.01	136.90	402.38 B	32.00 B	8.40 B
Inoculated plants	(T3 + T4)	14.35	267.76	129.81	144.31	438.24 A	34.50 A	9.85 A
30 kg N ha^{-1}	(T1 + T3)	13.78 A	268.49	125.65	137.33	414.47	32.50	8.83
160 kg N ha^{-1}	(T2 + T4)	14.66 B	271.76	132.18	143.88	426.15	34.00	9.42

[a]Data represent the means of 10 replicates. Means followed by the same letter within each column are not significantly different by Scott-Knott test ($P < 0.05$). Lower case letters are used for comparisons among treatments and uppercase letters are used for comparisons among factors.

[b]Corrected for 13% humidity.

[c]The grain yield was obtained from the plants grown in the "useful area" of the plots with four replicates.

K/C, Number of kernels per cob

[a] Data represent the means of 10 replicates. Means followed by the same letter within each column are not significantly different by the Scott–Knott test ($P<0.05$). Lower case letters are used for comparisons among treatments and uppercase letters are used for comparisons among factors

[b] Corrected for 13 % humidity

[c] The grain yield was obtained from the plants grown in the "useful area" of the plots with four replicates

inoculation with PGPB as an alternative to the use of high amounts of mineral fertilizers. Maize inoculation studies have demonstrated that inoculation with PGPB positively affects plant development, but to variable extents (Hungria et al. 2010). Physiological and anatomical changes can be observed following the inoculation of maize plants with PGPB (Masciarelli et al. 2013), and these changes can be assumed to induce modifications in the plant gene expression pattern and to influence plant nutrient acquisition and productivity. However, further studies are needed to determine whether the inoculation impacts on the structure of the maize-associated bacterial community are a result of a direct effect of the introduced bacteria on plant environment or an indirect effect of the inoculated bacteria on the physiology and metabolism of the plant (Castro-Sowinski et al. 2007), including the factors that influence and control endophytic bacterial activity in plant tissues.

The sequencing results of three of our four libraries (EEM160N, EEM30N and RTEEM30N) indicate that the sampling effort did not reflect the complete taxonomic diversity within these libraries, restricting the analysis to the dominant taxa. While more clones will be needed for sampling and sequencing to access the rare taxonomic groups, the coverage of our libraries yielded similar estimates for the EEM160N, EEM30N, and RTEEM30N libraries (73.8, 71.7, and 73.8 %, respectively), allowing us to perform reliable comparisons among communities and detect statistically significant differences. Nevertheless, the number of OTUs retrieved from the RTEEM160N clone library is thought to reflect a robust view of the endophytic bacterial diversity within this library, based on the high coverage of the clone library and the shape of the rarefaction curve. In addition, the predominance of *Proteobacteria* in the four clone libraries presented in this study is in agreement with previous studies (Chelius and Triplett 2001; Seghers et al. 2004; Roesch et al. 2008; Liu et al. 2013). At the lower taxonomic level, we found that most of the OTUs present in each of the four clone libraries were related to the family *Sphingomonadaceae*, with *Sphingomonas* appearing as the dominant genus and accounting for up to 43.2 % of the total sequenced clones, followed by *Novosphingobium*, with 15.9 % of the total sequenced clones.

The significant differences observed in the distribution of dominant endophytic bacteria among DNA-derived libraries was restricted to the genera *Novosphingobium* and *Methylobacterium*, with the former identified in a larger proportion of plants under the RN-fertilization condition (17.5 % of clones) relative to the LN-fertilization condition (3.5 % of clones) and the latter predominating in LN plants (24.7 % of clones) relative to RN plants (2.5 % of clones). Both *Novosphingobium* and *Methylobacterium* genera belong to phylum *Proteobacteria*, even though they correspond to different phylogenetic lineages of the alpha subgroup. Representative species from both genera are commonly found in soil (Roesch et al. 2008) and have also reported as endophytes in maize (Liu et al. 2013). Major differences in carbon metabolism between *Novosphingobium* and *Methylobacterium* are based on the xenobiotic-degrading ability of several *Novosphingobium* strains; *Methylobacterium* strains are known to assimilate C1 compounds as carbon sources, such as methanol and formic acid. It is interesting to note that the *Methylobacterium* is phylogenetically close to the endosymbionts that nodulates legumes, and this genus includes species that are able to nodulate and fix atmospheric nitrogen in symbiosis with leguminous plants (Sy et al. 2001; Jourand et al. 2004). In legumes, high levels of N-fertilizer inhibit the development and function of root nodules (Salvagiotti et al. 2008). We report here that *Methylobacterium* abundance diminishes in the clone libraries derived from RN plants although no *Methylobacterium* sequence was found in the RNA-derived 16S clone libraries, suggesting that this taxon had a low metabolic activity at sampling time. The role of *Methylobacterium* as a plant endophyte encompasses interactions with plant pathogens (Araújo et al. 2002), reductions in toxicity from contaminated soils (Madhaiyan et al. 2007), inductions of the defense response against pathogens (Indiragandhi et al. 2008), and other plant growth-promoting effects. The depletion of certain endophytic bacterial populations in maize, but not species richness, was reported by Seghers et al. (2004) in their comparison of the use of mineral versus organic fertilizers.

Differences in the composition of the dominant taxa of the endophytic maize community observed in the DNA-derived LN and RN libraries were not reflected in their respective RNA-derived 16S gene libraries, which we assumed estimated the dominant taxa of the metabolically active endophytic populations, even though the profiles of active bacteria were quite distinct among the RN and LN libraries. Maize plants receiving the RN-fertilization treatment exhibited the lowest species richness in terms of active endophytes, with 84.2 % of the sequenced clones related to *Sphingomonas* and 15.8 % of sequences related to *Novosphingobium*. The relative abundance of these genera shifted in the RNA-derived library from the LN-fertilization condition, in which a decrease in the *Sphingomonas* and an increase in the *Novosphingobium* relative abundance were followed by the identification of several non-sphingomonad taxa (ESM 3, 4). The genus *Sphingomonas* has been reported to be a maize endophyte (Chelius and Triplett 2001; Liu et al. 2013), but to the best of our knowledge, our study is the first to identify *Novosphingobium* as a maize endophyte. Indeed, both genera are widespread in nature and have been identified as endophytes in different plant species, including their potential as PGPB and nitrogen-fixing species (Hrynkiewicz et al. 2009; Videira et al. 2009). In addition, the lower biodiversity of the active endophytic community compared with that of the total endophytic community suggests that bacterial activity is not

directly related to bacterial abundance and that plant nutritional status plays an important role in determining which bacterial groups will be able to grow and be active under particular nutritional conditions. As observed earlier in the endophytic communities associated with rice (Knauth et al. 2005) and elephant grass (Videira et al. 2013), metabolically active endophytes may comprise a portion of the total endophytic community at a specific time, a condition which may also be true for maize.

Relative shifts in the abundance of dominant phylogenetic groups in the DNA- and RNA-derived libraries indicate either an enrichment or depletion of OTUs related to these taxa in response to the amount of N-fertilization applied at maize sowing. The modification of plant metabolism in response to the amount of nitrogen available in the soil could lead to qualitative–quantitative changes in the root deposition and consequently modify the rhizosphere effect, thereby influencing the recruitment of specific bacterial taxa from the soil microbiota, which may eventually lead to changes in the endophyte populations. Both nitrogen and carbon metabolism in plants are tightly regulated, and differences in the nitrogen status have been shown to influence both the composition of the root exudates of maize by depleting the amounts of amino acids released by the roots (Carvalhais et al. 2010) and the metabolite profile of leaves by increasing the amounts of starch, carbohydrates, and secondary metabolites (Schlüter et al. 2012). Within the endophytic habitat, the nutritional status of the plant could induce changes in nutrient availability that would in turn affect the colonization, development, and activity of the endophytic microbiota, as has observed earlier for maize and other grasses (Prakamhang et al. 2009; Pariona-Llanos et al. 2010). In this context, the endophytic community associated with maize can also be regulated in a manner similar to that observed for legumes—that is, through the plant–bacteria signaling system together with the nitrogen signaling pathway, in which the infection, nodulation, and composition of endophytes are affected by the amount of N-fertilizer applied (Wahab et al. 1996; Ikeda et al. 2010).

Soil is the repository of microbial diversity which is able to interact with virtually any plant species; the intensive use of mineral fertilizer modifies the composition of such communities. Indeed, plant metabolism induces changes in the associated microbiota and is believed to exert a selective effect on microbes (Hartmann et al. 2009; Ikeda et al. 2010). This readily leads to the assumption that different agricultural practices affect plant development and metabolism differently and lead to changes in the associated bacterial communities, including endophytes (Seghers et al. 2004). Based on the comparison of our results from the 16S rRNA gene libraries under LN- and RN-fertilization conditions, we suggest that the use of low N-fertilization input (e.g., 30 kg N ha^{-1}) induces maize plants to make available a higher quantity of carbon substrates to the endosphere in a way that sustains the endophytic community. This N-fertilizer effect (and most likely other nutrients) takes place within the framework of a cost–benefit mechanism, resulting in a richer and metabolically more active endophytic bacterial community in LN plants in comparison with those grown under regular N-fertilization conditions in which the presence of endophytes with plant growth-promoting abilities are less likely to benefit the host plant (Partida-Martínez and Heil 2011). As such, bacteria with the ability to contribute to the nutritional requirements of maize under field conditions are enriched in the soil and are preferred by plants under nutritional constraints. However, plants growing under full nutritional and optimal conditions reduce the amount of carbon substrates available in the endosphere, resulting in poor colonization and activity of the endophytic community.

The results of our study in terms of species richness, the diversity index, and the composition of the dominant active endophytic bacterial communities of RN and LN plants are in agreement with the cost–benefit hypothesis. The non-sphingomonad OTUs identified in the RN-fertilization condition libraries were primarily associated with ubiquitous soil bacteria, such as *Enterobacter*, *Burkholderia*, and *Bacillus*, but they also include two OTUs related to *Methylobacterium* and a *Brevundimonas*-related OTU. Within the non-*Sphingomonadaceae* OTUs identified in the LN-fertilization condition libraries, sequences that are closely related to *Gluconobacter*, *Acidiphilum*, *Acinetobacter*, *Rhizobium*, *Sinorhizobium*, and *Azospirillum*, in addition to *Methylobacterium* and *Bacillus*, were detected. Such genera include well-established growth-promoting bacteria with the potential to provide biologically fixed nitrogen to the host plant, such as *Rhizobium*, *Sinorhizobium*, and *Azospirillum*.

The practice of inoculating non-leguminous crops with PGPBs is increasing in Brazil for a number of commercially grown crop species, such as maize. Nevertheless, the inoculation of seeds with PGPB is mostly posed as an additional agricultural recommendation because no reduction in the use of mineral fertilizer has been effectively adopted, and the effectiveness of the biological N-fixation contribution in non-legumes is still controversial. The variability in plant responses to PGPB inoculation is the primary factor that addresses this recommendation. Our agronomic evaluation of maize inoculated with *A. brasilense* Ab-V5 indicates that high productivity can be obtained with lower N-fertilizer inputs; moreover, no additive effect was observed in terms of productivity in inoculated plants receiving the regular N-fertilization treatment. The cost–benefit relation in plant–microbe interactions is an emerging field of study (Partida-Martínez and Heil 2011). Any gene expressed by a plant comes at a metabolic cost and, therefore, the aim of expressing this gene is to provide a benefit; also, it is to be expected that gene expression is under the control of endogenous and exogenous signals (Saleem et al. 2010). We argue that the application of N-fertilizers leads maize plants to decrease the activity of

endophytic bacteria and that this effect can also be seen for the rhizosphere bacterial communities. In this cost–benefit framework, no additive effect can be achieved when diazotrophic PGPB inoculation is used together with regular N-fertilization. On the other hand, we found that plants inoculated with diazotrophic PGPB and grown under low amounts of N-fertilizer were able to efficiently improve their productivity. These findings must be considered when methodologies and strategies are being developed to inoculate plants with diazotrophic PGPB technology.

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Conflict of interest None.

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