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ORIGINAL PAPER

Biological nitrogen fixation in maize (*Zea mays* L.) by ^{15}N isotope-dilution and identification of associated culturable diazotrophs

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Abstract The nitrogen-fixing capacity of a range of commercial cultivars of maize *(Zea mays* L.) was evaluated by the ^{15}N isotope-dilution method. Biological nitrogen fixation (BNF) expressed as percent nitrogen derived from air (Ndfa) ranged from 12 to 33 regardless of nitrogen fertilization. BNF was not affected by mineral nitrogen fertilization except on cultivar Topacio and PAU-871 cultivars. Subsequently, culturable bacterial diazotrophs were isolated from endophytic tissue of maize: seed, root, stem, and leaf. All isolates were able to grow on N-free semisolid medium. Eleven bacteria isolates showed nitrogen-fixing capacity by the reduction of acetylene to ethylene and confirmed by PCR the presence of *nif*H gene in their genome. Identification of the 11 isolates was performed by bacteriological methods, 16S rRNA gene sequences, and phylogenetic analysis, which indicated that the bacteria isolated were closely related to *Pantoea*, *Pseudomonas*, *Rhanella*, *Herbaspirillum*, *Azospirillum*, *Rhizobium* (*Agrobacterium*), and *Brevundimonas.* This study demonstrated that maize cultivars obtain significant nitrogen from BNF, varying by maize cultivar and nitrogen fertilization level. The endophytic diazotrophic bacteria isolated from root, stem, and leaf tissues of maize cultivars may contribute to BNF in these plants.

Keywords *Zea mays* L. · ^{15}N isotope-dilution · Culturable diazotrophs · 16S rDNA sequence · *nif*H

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Introduction

Breeding programs for crop plants are designed to improve yield, fertilizer-use efficiency, and disease resistance. Usually, these programs do not consider interactions between plants and rhizospheric and plant endophytic communities, some of which have the ability to fix nitrogen. It is well known that maize (*Zea mays* L.) plants can establish rhizospheric and/or endophytic associations with various N_2-fixing bacteria such as *Azospirillum* (Christansen-Weniger and Vanderleyden 1994), *Klebsiella* (Chelius and Triplett 2000a; Dong et al. 2001), *Pantoea*, *Herbaspirillum* and *Bacillus* (Palus et al. 1996; Chelius and Triplett 2000b), *Rhizobium etli* (Gutiérrez-Zamora and Martinez Romero 2001), and *Burkholderia* (Di Cello et al. 1997; Caballero-Mellado et al. 2004; Perin et al. 2006), all or some of which may supply plants with fixed N_2. Most of these diazotrophs can inhabit intercellular plant tissue without causing disease and, for this reason, are referred

to as endophytic (facultative or obligate) diazotrophs (Baldani et al. 1997; Rosenblueth and Martínez-Romero 2006).

The potential for plant-associated diazotrophic bacteria to promote the growth of many cereals and grasses has been demonstrated in several studies (e.g. Baldani et al. 1997; Hallmann et al. 1997). Plant-associated diazotrophs may stimulate plant growth directly or indirectly through a combination of mechanisms, in addition to their potential ability to provide fixed nitrogen. Diazotrophs can affect plant growth directly by the synthesis of phytohormones and vitamins, the inhibition of plant ethylene synthesis, the stimulation of nutrient uptake (solubilization of inorganic phosphate as well as mineralization of organic phosphate), and improvement of stress resistance and control of pathogenic microorganisms (Berge et al. 1990; Triplett 1996). Therefore, plant growth/health can benefit from diazotrophic endophyte and associated bacteria if the appropriate bacterial and plant genotypes are used under optimized growth conditions (Baldani et al. 1997; Boddey et al. 2000; Chelius and Triplett 2000b). Diazotrophs associated as endophytes with maize should colonize the plant interior and establish themselves within niches protected from oxygen, which is an inhibitor of nitrogenase and thus limit nitrogen fixation. The integration of biological nitrogen fixation (BNF) into crop production strategies can improve the sustainability of agricultural systems. The screening of plant genotypes for an enhanced ability to acquire nitrogen by BNF is a key factor for the eventual reduction of N fertilizers in several important crops like sugar cane (Urquiaga et al. 1992; Fuentes-Ramírez et al. 1999; Reis et al. 2000), rice (Boddey et al. 1995), wheat, maize, and other plants (Baldani et al. 1997; Tapia-Hernandez et al. 2000).

It is known that the ^{15}N isotope dilution method has low accuracy in quantifying low levels of symbiotic nitrogen fixation (Reichardt et al. 1987; Hardarson et al. 1988); however, the method is more reliable than the acetylene reduction assay or the N balance approach (Chalk 1985; Rennie 1986; Boddey 1987; Danso 1988; Boddey et al. 1995). The BNF of maize plants inoculated with nitrogen-fixing bacteria has been monitored by ^{15}N-labeled N_2 gas (Alexander and Zuberer 1989) or by the ^{15}N isotope dilution method (Rennie 1980; García de Salamone et al. 1996), all with significant values.

The aim of this study was to quantify the symbiotic biological nitrogen-fixing activity of a range of commercial maize cultivars currently grown in Uruguay and to characterize the culturable diazotrophs that were associated with maize plant tissues. The nitrogen-fixing ability of 19 maize cultivars was estimated using the ^{15}N isotope dilution methodology (McAuliffe et al. 1958; Hardarson and Danso 1990). Culturable diazotrophic bacteria isolated from seed, root, stem, and leaf of all maize cultivars were characterized by standard bacteriological methods, *nif*H gene analysis, and 16S ribosomal RNA (rRNA) gene phylotype.

Materials and methods

Maize cultivars and growth conditions

Nineteen commercial maize cultivars—DK-682, DK-758, DK-688, PAU-479, PAU-785, PAU-674, PAU-871, Quelu, Tendem, Topacio, Lumina, Dinamo, Condor, Suco, NK-940, NK-900, Maizon, Vikingo, and Cheyenne—were obtained from local private companies in Uruguay. The majority of maize cultivars were hybrids, including the NK-900 TDMAX (GMO-Bt) cultivar, and only Maizon was a Uruguayan one. Maize plants were grown in 3-kg plastic pots previously washed with a 70% hypochlorite solution and filled with low-nitrogen-content sandy soil [pH 6; organic matter, 1.9%; 0.095% total N, extractable P (BrayI), 17 mg kg^{-1}; exchangeable K, 0.27 Meq/100g; exchangeable Ca, 13.8 Meq/100g; exchangeable Mg, 5.0 Meq/100g; and exchangeable Na, 0.30 Meq/100g] and sterilized sand in a 1:2 (*v*/*v*) ratio. Surface-sterilized and pre-germinated seeds of maize cultivars were sown two per pot. Plants were always watered from the bottom plate with sterilized distilled water or one-half N-free Fähraeus's (1957) nutrient solution. The pots were randomly placed in a plant growth room, and plants were grown under controlled conditions at 30°C/20°C day/night temperature and 14/10 h day/night cycle with 40%/60% humidity.

^{15}N isotope dilution method

The ^{15}N isotope dilution method was used to quantify BNF in DK-682, DK-688, Tendem, Topacio, Condor, Suco, NK-940, NK-900, Maizon, and PAU-871, the most common cultivars used by Uruguayan farmers. This method involves the testing of a N_2-fixing crop and a suitable non-N_2-fixing control, both grown in a substrate with homogeneous ^{15}N enrichment. In addition, it is important that the ^{15}N enrichment of the soil changes slowly with time, that the fixing and reference crops have similar N uptake patterns, and that the roots of both crops explore the same volume of soil (Witty 1983; Boddey 1987; Danso 1988). Thus, with the sole supply of N by the soil and ^{15}N-labeled fertilizer, a fixing plant and a non-fixing reference plant will contain the same ratio of $^{15}N/^{14}N$, as they are taking up N of the same $^{15}N/^{14}N$ composition, though not necessarily the same total quantity of N. In both plants, the $^{15}N/^{14}N$ ratio within plant tissues is lowered by the N absorbed from the unlabelled N source. Thus, the extent to which the $^{15}N/^{14}N$ ratio in the fixing crop is decreased, relative to

the non-fixing plant, is used to calculate BNF by the equation:

$$\%\text{Ndfa} = \left(1 - \%^{15}\text{N a.e.}_{\text{F}} / \%^{15}\text{N a.e.}_{\text{NF}}\right) \times 100$$

where %Ndfa is the percentage of nitrogen derived from air and percent ^{15}N a.e. (%^{15}N atom excess) is the enrichment of the fixing (F) and non-fixing (NF) crops, respectively (Hardarson and Danso 1990; Danso et al. 1993; Boddey et al. 1995; IAEA 2001). For this reason, a solution containing 10% ^{15}N a.e. ammonium sulfate, equivalent to 5 mg N kg^{-1} soil or 25 mg N kg^{-1} soil, was used as a tracer to evaluate nitrogen fixation. Each treatment was replicated three times. The maize cultivar that had incorporated the highest levels of N from the tracers and had the highest %^{15}N a.e. values for both 5 and 25 mg N kg^{-1} solutions, respectively, was used as a control or reference for the calculation of %Ndfa, assuming that this cultivar was not fixing N or had insignificant nitrogen fixation. All plants were harvested after 90 days of seeding, and ^{15}N enrichment of plant tissue was determined by mass spectrometry (VG Prism Series II, Universidad Autónoma de Madrid). Nitrogen content of the shoot was obtained by the Kjeldahl method (Bremmer and Mulvaney 1982).

Diazotrophic endophyte isolation

The 19 maize cultivars were used to isolate culturable diazotrophic bacteria from endophytic plant tissue. Ninety days after seeding, maize plants were harvested and root-plants were carefully washed with tap water to eliminate soil particles and then rinsed several times with sterile distilled water. Subsequently, root, stem, and leaf tissues were surface disinfected by immersion in 70% ethanol. Maize seeds (ten per cultivar) were rinsed with tap water until the liquid was clear and then rinsed five times with sterile distilled water. Cleaned plant material including seeds, stems, roots, and leaves were surface sterilized with 4% sodium hypochlorite for 5 min and then washed five times with sterile distilled water.

Surface-sterilized seed, root, stem, and leaf tissue cut in sections of 2 mm were aseptically placed in vials (two replicates each) containing N-free semisolid media, LGI, JNfb, and JMV (Cavalcante and Döbereiner 1988; Gillis et al. 1989; Döbereiner 1992; Reinhold-Hurek et al. 1993; Döbereiner 1995), and incubated at 30°C until growth. Samples of surface-sterilized plant material were placed on plates with TY media (Josey et al. 1979) and incubated at 30°C as controls for sterility. Vials showing a fine subsurface bacterial growth were transferred to fresh semisolid JNfb medium and new growth was streaked out on TY agar plates for characterization. Colonies were purified and assayed for nitrogenase activity by the acetylene reduction activity (ARA) method (Hardy et al. 1968). Duplicates of all isolates were stored in 50% (*v*/*v*) glycerol at −20°C and −80°C, and named as EMA followed by progressive numbers.

Bacterial endophyte characterization

Identification of bacterial colonies was based on colony morphology: shape, size, color, margin, elevation, opacity, and texture.

Acetylene reduction assay

The nitrogenase activity of the isolated bacteria was determined by the reduction of acetylene to ethylene (Hardy et al. 1968; Burris 1972). To standardize ARA assays, all isolates were tested using the jNfba medium (Döbereiner 1995). The vials were sealed with rubber stoppers. Subsequently, 0.75 ml of acetylene (approximately 10% of air volume) were injected with a needle through the rubber stopper, and 1 ml was sampled from the vial after 24 h of incubation at 30°C. Ethylene concentration was analyzed by gas chromatography (Shimatzu GC 4B-PTF). *H. seropedicae* strain Z69 was used as positive control for ARA analysis.

Polymerase chain reaction amplification of nifH fragments

A rapid lysis procedure was used to generate genomic fragments of ARA-positive isolates. Isolated bacteria were grown on TY plates and a single colony was picked and suspended in 250 µl 2 mM Tris–HCl and 0.2 mM EDTA at pH 7.5. The suspension was heated to 95°C for 5 min and then centrifuged for 10 min at 11,000×*g*. The *nif*H gene fragments were amplified by a degenerate *nif*H primer pair designed to amplify the specific fragment from aerobic and micro-aerophilic diazotrophs; the forward primer was 5′ ATXGTCGGXTGXGAXCCYAARGC3, whereas the reverse primer was 5′ATGGTGTTGGCGGCRTAZAKYGC CATCAT3′ (where X=C or T; Y=G or C; R=G or A; Z=C, G, or A; and K=G or T; Olson et al 1998). The polymerase chain reaction (PCR) operating conditions were 1× PCR buffer (AmpliTaqGold^{TM}), 30 mM $MgCl_2$, 0.8 µM deoxyribonucleotide triphosphates (dNTPs), 1.6 µM forward and reverse primer, 0.5U Taq (AmpliTaqGold^{TM}), and the thermo-cycling conditions were 94°C for 4 min, five cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, 30 cycles of 94°C for 30 s, 49°C for 30 s, 72°C for 30 s, and 72°C for 10 min. The PCR products were visualized by electrophoresis on ethidium bromide-stained 1.0% agarose gels.

16S rRNA gene sequencing

Eleven *nif*H-positive isolates from root, stem, and leaf tissue of maize plants were selected for 16S rDNA

sequencing. PCR amplification was conducted using 16S rRNA gene universal primers fD1 and rD1 (Weisburg et al. 1991). The PCR operating conditions were 1× PCR buffer (AmpliTaqGoldTM), 30 mM $MgCl_2$, 0.8 μM dNTPs, 0.8 μM forward, and reverse primer, 0.5U Taq (AmpliTaqGoldTM), and the thermo-cycling conditions were 94°C for 4 min, 35 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, and 72°C for 10 min. The PCR products were visualized by electrophoresis on ethidium bromide-stained 1.0% agarose gels and purified from the gels using a Sephaglas Band Prep PCR purification kit. Sequencing was done by the Macrogen Corp SA (http://www.Macrogen.com). The sequences obtained were compared with those identified in the GenBank DNA database.

Phylogenetic analysis

Phylogenetic analysis was conducted using partial 16S rDNA sequences of EMA-15, EMA-68, EMA-77, EMA-82, EMA-83, EMA-84, EMA-109, EMA-117, EMA-134, EMA-176, and EMA-177. These sequences were compared with related bacteria sequences of the GenBank, and sequence similarities were determined using BLAST Sequence Similarity Search (Altschul et al. 1997) and aligned by using Clustal W, version 1.83 (Thompson et al. 1994). Overhanging ends were removed from both ends of the sequences to ensure multi-alignment of sequences of the same length. The tree was constructed with the MEGA software package by using the neighbor-joining method (Saito and Nei 1987) with the distance calculated by the two-parameter model proposed by Kumar et al. (2001). Gaps were treated by pairwise deletion. Bootstrap analyses were done by using 2000 pseudoreplications.

Phenotypic characterization

EMA-15, EMA-68, EMA-77, EMA-82, EMA-83, EMA-84, EMA-109, EMA-117, EMA-134, EMA-176, and EMA-177 were characterized using Gram reactions (Gregersen 1978) and cell morphology, evaluated by phase contrast microscopy. Carbon source assimilation tests were performed using liquid minimal medium (MM) containing $NH_4H_2PO_4$ 1.25 g l^{-1}, KCL 0.25 g l^{-1}, $MgSO_4.7H_2O$ 0.25 g l^{-1}, and K_2HPO_4 0.50 g l^{-1} (pH 7; modified from medium CM, Kennedy 1994). The carbon sources utilized were sucrose, glucose, lactose, and D-raffinose. All carbon sources were added to MM at final concentration of 0.25% (*w*/*v*). Bacterial isolates were grown in 5 ml TY for 48 h and incubated at 30°C. Cultures were centrifuged three times and re-suspended each time in NaCl 0.85% and then adjusted to an OD_{610} of 0.6. An aliquot of 95 μl of each cell suspension was used to inoculate 3 ml of MM supplemented with each carbon source. Three tubes per treatment were incubated at 30°C for 4 days, after which carbon source utilization was evaluated.

Statistical analysis

Data were analyzed for significant differences between maize cultivars using analysis of variance (ANOVA). Differences between cultivar means were analyzed by the least significant differences test (SPSS for Windows 6.0). Correlation among variables was assessed by the Pearson correlation.

Results

Quantification of BNF using ^{15}N isotope dilution methodology

Ten maize cultivars were evaluated in pot experiments at two nitrogen fertilizer rates, 5 mg (T5) or 25 mg N kg^{-1} soil (T25). Significant ($P<0.05$) differences were observed in $\%^{15}N$ a.e. and %Ndfa among maize cultivars (Table 1). The one with the highest $\%^{15}N$ a.e. at T5 was DK682 and at T25 were Topacio and PAU871. Table 1 shows that $\%^{15}N$ a.e. was affected by the N fertilization rate because the average for the T5 treatment was 0.45, whereas that of T25 treatment was 2.31 ($P<0.05$). The %Ndfa values of cultivars ranged from 12.3 (Tendem) to 33.1 (Topacio) regardless of the levels of available N. In general, %Ndfa was not significantly affected by the N fertilization rate, except for Topacio and PAU871 that benefit from BNF under low N inputs (T5). The % N varied with the N fertilization rate in most maize cultivars, with Topacio being the cultivar with the highest % N, and DK-688 and DK-682 cultivars with the lowest values (Table 1). There was a negative correlation between $\%^{15}N$ a.e. and shoot N accumulation at T5 ($r=0.76$, $P<0.05$, $n=30$).

Isolation of diazotrophic endophytes from maize cultivars

One hundred and seventy-eight endophytic bacteria were isolated from 19 maize cultivars. The lowest number of isolates was obtained from DK-758 (3) and the highest from Tendem (21). The number of isolates in different culture media was 85 with jNfb, 62 with LGI, and 31 with JMV. Additionally, differences were seen in the relative number of isolates obtained from different plant tissues: 83 (47%) were from stem tissue, 54 (30%) from roots, 30 (17%) from seeds, and 20 (11%) from leaves. Endophytic bacteria were obtained from the stems of all cultivars, while isolates from seeds, roots, and leaves were from only some of the cultivars. PAU-785 was the only cultivar in which bacteria could be isolated from all plant tissues examined.

Table 1 Evaluation of the nitrogen-fixing capacity of maize cultivars in a pot experiment using ^{15}N isotope dilution method

Maize cultivar	T5			T25		
	Shoot N content (%)	$\%^{15}N$ a.e.	%Ndfa	Shoot N content (%)	$\%^{15}N$ a.e.	%Ndfa
DK-682	0.68 c	0.55 a	–	0.72 c	2.37 ab	12.4 b
DK-688	0.68 c	0.48 ab	13.6 b	0.78 c	2.24 b	17.3 ab
Tenden	1.02 bc	0.48 ab	12.3 b	1.17 a	2.26 b	16.6 ab
Topacio	1.62 a	0.37 c	33.1 a	1.18 a	2.71 a	–
Condor	0.86 bc	0.45 bc	18.5 ab	1.04 ab	2.28 ab	15.7 ab
Suko	1.11 b	0.41 bc	26.2 ab	0.91 bc	2.26 b	24.2 a
NK-940	1.04 bc	0.40 bc	27.2 ab	0.99 ab	2.05 b	24.4 a
NK-900	0.91 bc	0.46 abc	16.6 ab	0.86 bc	2.31 b	14.6 ab
Maizon	0.82 bc	0.43 bc	20.8 ab	0.89 bc	2.10 a	22.3 ab
PAU-871	0.92 bc	0.43 bc	21.9 ab	0.86 bc	2.52 b	12.4 b

Values for each column not followed by the same letter differ significantly at $P<0.05$.
T5 ^{15}N equivalent to 5 mg N kg^{-1} soil, *T25* ^{15}N equivalent to 25 mg N kg^{-1} soil, *%^{15}N a.e.* percent atom excess ^{15}N, *%Ndfa* percent N derived from atmosphere, e*n dash* value used as baseline for %Ndfa calculation

In addition, the highest number of isolates from seeds was obtained from the PAU-785 and Tendem cultivars.

The isolates showed different colony morphologies when grown on various media. The predominant colony type seen on TY were white translucent, round, and flat with entire margins and diameter ranging from ≤0.5 to 1.0 mm. All 178 bacteria isolated showed subsurface growth in N-free semisolid medium. Many isolates formed fine or thick and diffuse pellicles at a depth of 2 to 4 mm below the surface and some had an alkaline reaction.

Acetylene reduction assays

All 178 isolates were tested for their ability to reduce acetylene to ethylene in jNfb N-free semisolid medium. Nitrogenase activity was detected in 11 isolates from DK-688, Tendem, Topacio, Suco, NK-940, NK-900, and PAU-871 cultivars. Most ARA-positive isolates were recovered from the stem tissue (Table 2) under the assayed conditions used.

PCR amplification of *nifH*

Isolates showing ARA activity were analyzed for the presence of *nif*H genes. Primers used amplified a fragment of DNA of 300 to 400 bp. All tested isolates had the *nif*H fragment (Table 2).

Sequence and phylogenetic analysis of 16S rRNA genes

DNA of 11 isolates with *nif*H gene fragments was amplified by PCR amplification of 16S rRNA that resulted in an amplification fragment of about 1,450 bp. Partial 16S rRNA gene sequencing was used initially to identify the bacteria isolated at the genus level and to determine whether there were clusters of similar organisms. The results of the partial 16S rDNA sequencing of isolates indicated that EMA-15 (805 bp) had 16S rDNA sequences closely related to *Pantoea* spp. (99% similarity); EMA-68 (348 bp), *Pseudomona* spp. (99% similarity); EMA-82

Table 2 Diazotrophic bacteria isolated from different plant tissue of maize cultivars

Strain[a]	Maize cultivars	Plant tissue	Close relatives based on 16S rDNA	Percent homology[b]
EMA-15	DK688	Root	*Pantoea* spp.	99
EMA-68	Tendem	Stem	*Pseudomona* spp.	99
EMA-82	Topacio	Stem	*Pantoea* spp.	98
EMA-83	Topacio	Stem	*Rhanella* spp.	99
EMA-84	Topacio	Stem	*Rhanella* spp.	99
EMA-77	Topacio	Stem	*Rhanella* spp.	99
EMA-109	Suco	Leaf	*Herbaspirillum* spp.	99
EMA-117	NK940	Stem	*Herbaspirillum* spp.	99
EMA-134	NK900	Leaf	*Azospirillum* spp.	95
EMA-176	PAU871	Root	*Rhizobium* spp. (*Agrobacterium* spp.)	99
EMA-177	PAU871	Root	*Brevundimonas* spp.	99

[a] Acetylene reduction assay and *nifH* positive
[b] Percentage of homology between 16S rDNA sequences of each strain to that of close relatives

(750 bp), *Pantoea* spp. (98% similarity); EMA-83 (431 bp), *Rhanella* spp. (99% similarity); EMA-84 (411 bp), *Rhanella* spp. (99% similarity); EMA-77 (757 bp), *Rhanella* spp. (99% similarity); EMA-117 (575 bp), *Herbaspirillum* spp. (99% similarity); EMA-109 (826 bp), *Herbaspirillum* spp. (99% similarity); EMA-134 (198 bp), *Azospirillum* spp. (95% similarity); EMA-176 (533 bp), *Rhizobium* spp. (99% similarity); and EMA-177 (489 bp), *Brevundimonas* spp. (99% similarity; Table 2).

Figure 1 shows the phylogenetic tree of nitrogen-fixing bacteria isolated from different maize varieties and their relatives in the Proteobacteria by neighbor-joining grouping of the aligned partial sequences of the 16S rRNA.

16S rDNA sequence accession numbers

The sequences from 16S rDNA were submitted to the GenBank and received the following accession numbers: EMA-177 (EU723214), EMA-15 (EU723215), EMA-68 (EU723216), EMA-83 (EU723217), EMA-176 (EU723218), EMA-117 (EU723219), EMA-134 (EU723220), EMA-109 (EU723221), EMA-84 (EU723222), EMA-82 (EU723223), and EMA-77 (EU723224).

Phenotypic characterization

All eleven nitrogen-fixing bacteria isolated, with *nif*H gene in their genome, were Gram-negative and motile. Cell

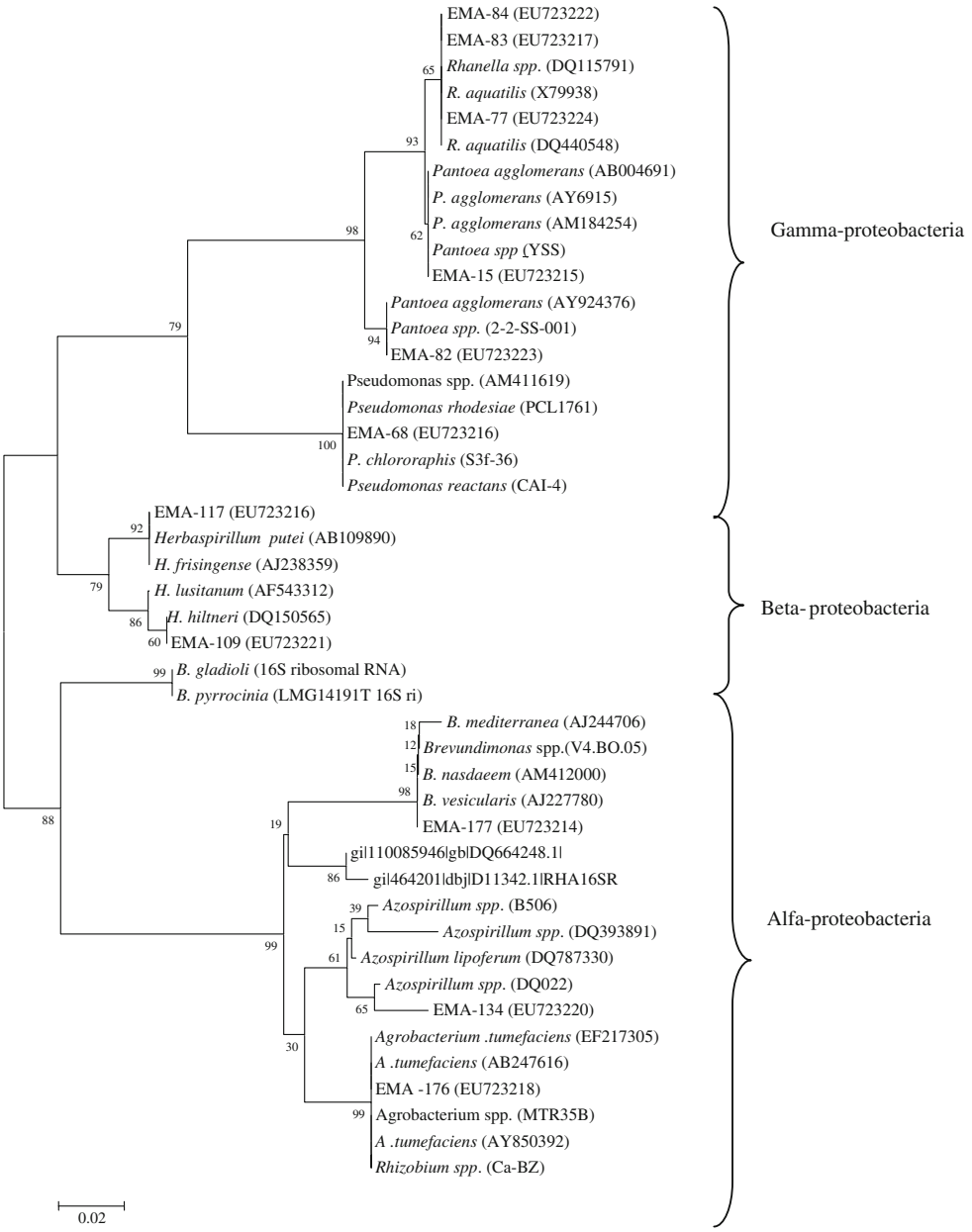

Fig. 1 Phylogenetic tree based on partial 16S rDNA sequences of EMA-84 (EU723222), EMA-83 (EU723217), EMA-77 (EU723224), EMA-15 (EU723215), EMA-82 (EU723223), EMA-68 (EU723216), EMA-117 (EU723216), EMA-109 (EU723221), EMA-177 (EU723214), EMA-134 (EU723220), and EMA-176 (EU723218). These sequences were compared with related bacteria sequences of the GenBank, and sequence similarities were determined using BLAST Sequence Similarity Search and were aligned by using Clustal W, version 1.83. The tree was constructed with the MEGA software package by using the neighbor-joining method from distance calculated by the method of Kimura (1980) two-parameter model. Gaps were treated by pairwise deletion. Bootstrap analysis were done by using 2,000 pseudoreplications

morphology observation of EMA-77, 83, 82, 15, and 84 showed short rods, occurring as single or in pair, EMA-176 and 177 showed long rods, while EMA-117, 109, and 134 were vibroid. All isolates were able to grow on sucrose, glucose, lactose, and D-raffinose as the only carbon source, except EMA-82 that could not use lactose and D-raffinose.

Discussion

Maize is one of the most widely cultivated cereals in the world, and its production is highly dependent on chemically produced nitrogen fertilization, whereby the production of 1 ton of plant biomass requires 9 to 11 kg of N (Anuar et al. 1995). As the human population grows, the increase of maize consumed will present an economic challenge due to the impact of rising fossil energy costs in synthetic N fertilizer production, as well as an environmental challenge to maintain soil and water quality. For these reasons, the benefits from BNF in maize cultivation are very important from both an environmental and economic perspective and for sustainability (Döbereiner 1995; Triplett 1996; Chelius and Triplett 2000b; Riggs et al. 2001; Kennedy et al. 2004).

The results obtained in this study with the ^{15}N isotope dilution method confirm that there is a significant BNF contribution to maize and that there are differences among maize cultivars in their capacity to support N_2 fixation. Our finding demonstrate that, with the T5 treatment, those plants that accumulated the most nitrogen had the lowest ^{15}N enrichment, which is to be expected for plants obtaining any inputs from BNF (Boddey 1987; Chalk 1991). On the other hand, Condor, Suco, NK-940, NK-900, and Maizon cultivars benefit from BNF with both nitrogen fertilization treatments (T5 and T25). Therefore, the capacity of maize to fix nitrogen is affected by fertilization treatment, being some cultivars more sensitive than others to N inputs. It is important to consider that, in this study, both nitrogen fertilization treatments (T5 and T25) were low considering that normal N application reported in agricultural fields is twice the level of T25. This may explain why most maize cultivars were relatively unresponsive to N application and showed no variation in BNF (Urquiaga et al. 1992; Yoneyama et al. 1997). The application of ^{15}N isotope dilution method is a useful tool to screen and select maize cultivars with any potential BNF. In this study, the screening of a large number of maize cultivars revealed a large variation in $\%^{15}N$ a.e. and facilitated the choice of a reliable and suitable reference crop for BNF calculation (Urquiaga et al. 1992). Any BNF of the reference cultivar would in this case underestimate the actual level of BNF associated with a given plant.

The results presented hereby add further evidence to those of the existing literature on the contribution of BNF to maize (Von Bulow and Döbereiner 1975; Rennie 1980; Alexander and Zuberer 1989; García de Salamone et al. 1996). Cultivars may differ in BNF capacity, and such information is useful to enhance maize nitrogen fixation through genetic improvement and more efficient management. Future programs may benefit from integration of BNF into mainstream maize breeding programs that are focused on a broad range of traits, and in which all material is evaluated in low N soils to provide the most appropriate selection pressure for high BNF. However, the agronomic significance of these nitrogen inputs requires further studies. Future work in this area should focus on field studies with low N inputs, as has been done with sugar cane in Brazil (Urquiaga et al. 1992; Boddey et al. 1995; Yoneyama et al. 1997; Boddey et al. 2003).

The culturable diazotrophic endophytes associated with 19 different maize cultivars were isolated and described. Bacterial endophytes were recovered from all plant tissues, but differed in the percent of colonization. Evidences of different colonization patterns of plant tissue by endophytes have been found by several authors. For example, *Klebsiella pneumoniae* was typically found in the zone of root hair formation but only occasionally formed colonies on or in the root hair (Vande Broek et al. 1993; Chelius and Triplett 2000a). *A. diazotrophicus*, *H. rubrisubalbicans*, and *H. seropedicae* colonized xylem vessels of sugarcane and sorghum bicolor (James et al. 1994; James et al. 1997; James and Olivares 1998). Cells of *A. brasilense* demonstrate a characteristic pattern of wheat colonization that involves invasion of root hair cells (Assmus et al. 1995) and colonization of the root tip (Schloter and Hartmann 1998). *Azoarcus* spp. colonize rice and kallar grass in the root cortex, root cap, epidermis, exodermis, and xylem (Hurek et al. 1994; Reinhold-Hurek and Hurek 1998). *Pantoea agglomerans* has been found in the intercellular spaces of wheat roots (Ruppel et al. 1992). Elbeltagy et al. (2001) showed that *Herbaspirillum* spp. was never found to enter vascular tissues of wild rice species, preferring the apoplast of shoot tissues, colonizing intercellular space and root surfaces. Palus et al. (1996) reported the presence of cultivated endophytes in the apoplastic fluid of maize plants. If endophytes prefer the apoplast of shoot tissues, this could explain the higher percent of isolates obtained from stem tissue (47%) compared to root tissue (30%) in this study, as well as the spread of motile endophytes in the aerial parts of plants such as leaves via apoplastic spaces. In addition, maize cultivars showed differences in their capacity to support endophytic colonization, with Topacio being associated with up to 21 isolates and four ARA positive, while DK-758 with only three isolates, all with similar colony morphology and non-ARA positive. It is assumed that maize cultivars could be selective in the reproduction of certain endophytic bacteria, as some

endophytes are more likely to be associated with certain maize cultivars. On the other hand, due to lack of close "specificity" between diazotrophic endophytes species associated with maize, several species may simultaneously occupy the same plant. For example, one plant of PAU-871 had nine isolates, two of them having positive ARA and belonging to two different genera *Rhizobium* (*Agrobacterium*) and *Brevundimonas*. However, Topacio had 21 isolates, four of which were ARA positive and belonged to two different genera, *Pantoea* and *Rhanella* (Table 1).

These results suggest that multiple infections and bacterial coexistence is possible and that competition could be mediated through spatial segregation, with different diazotrophs occupying different plant tissues (root, stem, or leaf). Results also suggest that bacteria may have a "preference" for multiplying within roots and stems, while other bacteria may prefer leaves, where different conditions for propagation may be found. However, such "preferences" might change with maize cultivar, plant ontogeny, sampling time, and/or different environmental conditions (e.g. stress, low pH, high temperature, etc.; Roesch et al. 2006). Nevertheless, the mechanisms by which multiple endophyte species coexist within plant cultivars should be investigated further. If we can determine what factors enable some cultivars, receiving the same treatment, to support high rates of endophytic N_2 fixation, then perhaps we can make more accurate assessments of the potential for endophytic bacteria to benefit plant growth.

The diazotrophic nature of 11 isolates was shown by their ability to grow well in an N-free semisolid medium, the reduction of acetylene, and the presence of *nif*H gene in their genome. Among the 178 isolates capable of growing in N-free semisolid medium, only 11 displayed ARA activity. This result may be explained by the fact that the ARA assay was performed under only one experimental condition (carbon source, incubation temperature and time), which may not be the more suitable condition for nitrogenase activity for all isolates. Estrada-de los Santos et al. (2001) demonstrated that the ability to fix N_2 varied among the different diazotrophs isolated from maize and coffee plants and depended upon selective carbon sources and microaerophilic conditions. Therefore, it is possible that low BNF activity may have not been detected under the experimental conditions used in this study and more diazotrophic bacteria could be present in harvested tissues. The recovery of endophytic nitrogen-fixing bacteria within roots, stems, and leaves in maize, and the evidence of BNF in maize plants suggest which culturable bacteria genus may be contributing to the observed BNF. The different N-free media and the large number of maize cultivars used in this study allowed for the recovery of a relatively high level of genetic diversity of diazotrophs belonging to seven different genera and three subdivisions of the Proteobacteria: (a) γ-proteobacteria. *Rhanella* spp. (EMA-84, 83, and 77), *Pantoea* spp. (EMA-82 and 15), and *Pseudomonas* spp. (EMA-68); (b) β-proteobacteria: *Herbaspirillum* spp. (EMA-117 and 109); and (c) α-proteobacteria: *Azospirillum* spp. (EMA-134), *Rhizobium* spp. (*Agrobacterium* spp.; (EMA-176), and *Brevundimonas* spp. (EMA-177). The information on the homology of 16S rDNA sequence and their relationship to the different genera and phenotypic characteristics are sufficiently consistent to support these preliminary taxonomic designations.

The culturable fraction of the endophytic bacterial community within maize has previously been described (Fisher et al. 1992; McInroy and Kloepper 1995). In one study, the most frequently isolated species were *Enterobacter* spp., member of the γ-proteobacteria, followed by β-proteobacterial *Burkolderia* spp. (McInroy and Kloepper 1995). In recent years, molecular methods have greatly facilitated the study of bacterial communities and revealed a complexity of diversity not detected by conventional culture-dependent methods. For example, Roesch et al. (2008) found that the taxonomic affiliation of diazotrophic bacteria associated with maize plants and their rhizospheres revealed a high dominance of proteobacteria, mainly α-proteobacteria and β-proteobacteria. Studying the diversity of Archea and bacteria in association with the roots of maize (Chelius and Triplett 2001) found that most of the sequences analyzed were related to proteobacteria with the α-subdivision predominating and β-subdivision in second place. The results obtained in this study also showed a considerable number of members of γ-proteobacteria but also a representative number of bacteria within α-proteobacteria, with β-proteobacterial being less represented.

Conclusions

This study demonstrated that maize cultivars obtain significant nitrogen from BNF, the level of which varied by maize cultivar and nitrogen fertilization level, some cultivars being more sensitive than others to N inputs. The application of ^{15}N isotope dilution method is a useful tool to screen and select maize cultivars with any potential BNF. The endophytic diazotrophic bacteria closely related to *Pantoea*, *Pseudomonas*, *Rhanella*, *Herbaspirillum*, *Azospirillum*, *Rhizobium* (*Agrobacterium*), and *Brevundimonas*, isolated from the root, stem, and leaf tissues of maize cultivars, may contribute to the biological nitrogen fixation in these plants.

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