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REGULAR ARTICLE

Field response of rice paddy crop to *Azospirillum* inoculation: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants

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Abstract The response of rice plants to the application of inoculant containing two *Azospirillum brasilense* strains was studied under field conditions. The experiment was performed as three treatments with four replicates in randomized complete blocks arranged as plots of 60 m^2 in an area on a Vertic Argiudol soil type in the province of Entre Ríos, Argentina. The bacterial rhizosphere community and also the diazotrophic isolates obtained from control and inoculated rice plants were analyzed in relation to their physiology and biological nitrogen fixation (BNF). The MPN of diazotrophs in the rhizosphere varied during the ontogenic cycle. The patterns of distribution of the microbial physiological activities obtained by principal component analysis of community-level physiological profiles (CLPP) showed differences in the utilization of carbon sources by the rhizosphere communities among treatments. Although the analyses of DGGE 16S and *nifH* profiles have not indicated that the inoculation influenced the genetic diversity of bacterial communities among treatments, they revealed that the banding profiles were altered in different parts of the rice plant by each *Azospirillum* inoculation treatment. These observations suggest that physiological responses of plant tissues to the inoculation may have occurred. According to agronomic parameters of each treatment, the *Azospirillum* inoculation increased aerial biomass at the tillering and grain-filling stages. Although the N content accumulated in rice plants increased by 16 and 50 kg ha^{-1}, the BNF contribution could not be estimated under our experimental conditions by the ^{15}N balance technique. Based on this field inoculation experiment to rice plants, it is noteworthy that our data suggest that due to *Azospirillum* inoculation the increase of total N accumulated in rice plants could be a tool to help farmers to improve production and maintain high input of plant residues, providing more organic matter to the soil and guaranteeing sustainability of the system.

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Introduction

Rice (*Oryza sativa* L.) is the staple food for a great proportion of the world's population. The northeast region of Argentina produces high yields of this cereal, but it is important to search for sustainable strategies to increase and maintain high levels of production. Current approaches involve application of agro-chemicals (fertilizers, pesticides and herbicides) that, in general, increase costs and can generate negative environmental impacts.

Nitrogen (N) is one of the most important elements in plant nutrition, particularly in Latin American countries where most soils show low N availability and the agricultural subsidies to N fertilizer are low or nonexistent. For these reasons, there is a great interest in exploring the diversity of plant growth-promoting bacteria (PGPB) as substitutes for some chemical inputs to agriculture. N_2-fixing microorganisms, or diazotrophs, can be inoculated into crops to increase the number of beneficial organisms contributing to plant growth and crop production. In general, it has been observed that cereal plants are capable of associating with diverse bacterial species including diazotrophs that can promote growth (Okon and Labandera-Gonzalez 1994; García de Salamone et al. 1996; Rodrigues et al. 2008). Most of these PGPB can colonize the exterior of plant roots and aerial tissues in several plants, and can also associate endophytically to most of them, including rice (Baldani and Baldani 2005; Döbereiner and Pedrosa 1987; Olivares et al. 1996; Rodrigues et al. 2008).

The inoculation of cereal crops with PGPB is relevant for both economic and ecological reasons. In Argentina and several other countries, *Azospirillum* strains have been used as PGPB in inoculation field trials, mostly because of their ubiquitous behavior and association with grasses and cereals, such as rice, wheat and maize. *Azospirillum* spp. have shown great versatility to adapt to diverse soil conditions and have been isolated from the rhizosphere, the root surface and the inside of several tissues of wide variety of crops and wild plants worldwide (Bashan et al. 1991, 2004; Döbereiner and Pedrosa 1987). This genus belongs to the α subclass of the proteobacteria, and very recently several new species have been described. Nonetheless, *A. brasilense* is the species that has been widely used for both experimental and commercial formulations.

Plant–bacteria associations can occur on different levels of interaction. Thus, the genetic characteristics of both bacteria and host plant can define the level of the inoculation response during association (García de Salamone and Döbereiner 1996; Olivares et al. 1996; Azevedo et al. 2005). When the same strain is applied as inoculant to different crops, significantly different levels of response can be obtained, and several mechanisms of plant growth promotion have been attributed to them (Bashan and Holguin 1997; Bashan et al. 2004).

Under controlled conditions, when *Azospirillum amazonense* strains were used as inoculants, it has been shown that contribution of the biological nitrogen fixation (BNF) for rice productivity could range from 9.2 to 27.7% of Ndfa accumulation in the plant improving the potential of BNF in the inoculated plants up to 4-fold relative to noninoculated controls (Rodrigues et al. 2008). Using selected strains of diazotrophs which can supply high levels of N through BNF could further increase the efficiency of this metabolic activity under field conditions. In this regard, previous data demonstrated that BNF could represent a significant N supply for the production of two commercial genotypes of maize that were inoculated with selected *A. brasilense* strains (García de Salamone et al. 1996).

The objective of this work was to study, under field conditions, the eco-physiological response of rice plants to the inoculation with two *A. brasilense* strains previously characterized by their ability to fix N_2 in association with maize plants (García de Salamone et al. 1996). The rhizosphere bacterial community of inoculated rice plants and isolates of diazotrophic bacteria were analyzed in relation to BNF and their physiology. In addition, the genetic diversity of the endophytic bacterial communities, including diazotrophs, was characterized in different parts of rice plants.

Materials and methods

Bacterial strains and inoculant preparation

Azospirillum brasilense strains 40 M (GenBank accession number HM002661) and 42 M (GenBank accession number HM002662), isolated from inside the roots of field-grown maize (García de Salamone

and Döbereiner 1996) were used for inoculant formulation. Liquid formulations were prepared based on NFb medium with addition of ammonium chloride 1 g l^{-1} (Döbereiner and Pedrosa 1987). Both strains were cultured separately and the inoculant was prepared using a 1:1 ratio (v/v) of each strain. Counts of viable cells in the formulated inoculant (MI) were performed 24 h before sowing using a selective medium with Congo red dye (Rodríguez-Cáceres 1982), and indicated that the inoculant contained 10^9 cfu ml^{-1} of *A. brasilense*.

Experimental design

Paddy rice inoculation was conducted under field conditions near the city of San Salvador, located in the province of Entre Ríos, Argentina (31°26'03"S, 58°33'47"W). A total of 12 completely randomized, 60 m^2 plots provided three treatments and four replicates. The soil was a Vertic Argiudol, which had been previously used for soybean and rice crops. Chemical characteristics of soil at sowing were pH 6.7, electrical conductivity 0.88 dS m^{-1}, 18 g kg^{-1} of oxidizable carbon, 2.2 g kg^{-1} total nitrogen, 54.2 mg kg^{-1} nitrate, 10 mg kg^{-1} of ammonia and 9.3 mg kg^{-1} of P. Seeds of the rice cultivar CAMBA-INTA were pre-treated as recommended with fungicide mix (thiram + carbendazin) Biagro TC™. After drying, the seeds were subjected to the following treatments: Control: addition of 0.7 L per 100 kg of seeds of Stoller™, a commercial product containing the following micronutrients (%): Zn (5), Mn (3), Cu (0.5), B (0.5) and S (4); MI1: Stoller + inoculant; and MI2: inoculant only. The inoculation was performed immediately before sowing on October 21, 2006 and the dose per each 50 kg of seed was 300 ml of the inoculant formulation containing 10^9 ufc ml^{-1} of *A. brasilense*. Application of 70 kg ha^{-1} of NPK 5:30:20 fertilizer was performed at sowing and 1 month later 70 kg ha^{-1} of urea were applied. Irrigation began 35 days after sowing (DAS) and was maintained with a 5-cm layer up to 30 days before harvest. Weeds were controlled in pre-emergence with 3 L ha^{-1} of Glyphosate (Monsanto, Argentina).

Evaluation of agronomic parameters

Aerial biomass was determined at tillering (35 DAS) and grain-filling (117 DAS) stages. At physiological maturity, grain yield and its components were estimated. Samples were dried at 70°C and weighed after a week at constant weight.

Both N content and BNF contribution were estimated for aerial parts of plants sampled at the grain-filling stage (Boddey et al. 2001). Quantification of soil ^{15}N natural enrichment of rice plants with and without inoculation and weed plants were obtained to estimate. the percentages of BNF by ^{15}N dilution technique, calculated as suggested by Boddey et al. (2001). N content in plant tissues was calculated using the percentage values of total N obtained with a modification of the Kjeldahl method (Boddey et al. 2001).

Isolation and enumeration of bacteria

For the first two sampling dates, samples of the rhizosphere were obtained from the 0–20 cm layer. Rhizosphere soil, considered as the soil attached to roots, were obtained after hand-shaking the bulk soil and washed off the roots with tap water. Ten-fold serial dilutions were prepared and the most probable number (MPN) of N_2-fixing *Azospirillum*-like bacteria was analyzed by using N-free NFb semisolid medium (Döbereiner and Pedrosa 1987). The percentage of *Azospirillum*-like colonies was determined as % of cfu using selective Congo red medium (Rodríguez-Cáceres 1982).

Rhizosphere community-level physiological profiles (CLPP) analysis

At grain-filling stage, the physiological activity of the rhizosphere samples were assessed using community-level physiological profiles (CLPP) of carbon-source utilization. A modified approach of the technique described by Mills and Garland (2002) was used as follows. The 10^{-3} dilution of each rhizosphere sample was exposed simultaneously to 23 carbon sources (CSs), and four absorbance values were recorded in successive days at 590 nm using an automated microplate reader Multiskan EX™ (Thermo, Vantaa, Finland). Average well-color development (AWCD) was calculated to adjust data and thus to minimize artefacts caused by possible initial differences in microbial community members among treatments. After the last reading, 50 µl of the content of the seven wells with the highest absorbance records were

used to inoculate flasks with N-free NFb semisolid medium to estimate the proportion of N_2-fixing *Azospirillum*-like bacteria per treatment in each selected well. After 3 days of incubation at 30°C, the number of flasks showing typical pellicle formation was recorded. A loop from this typical pellicle was streaked onto plates with solid Congo red medium to estimate percentages of *Azospirillum*-like colonies after 9 days of incubation at 30°C.

Statistical analysis

The multivariate absorbance readings obtained from CLPP were analyzed using principal component analysis (PCA). Microbiological and plant production data were subjected to analysis of variance and comparison of means using the Tukey test. The statistical package INFOSTAT/Professional™ (version 1.1; University of Córdoba) was used.

Molecular microbial community analysis of total and diazotrophic bacteria

DNA extraction

Roots, aerial parts and grains of rice plants were collected and macerated prior to incubation at 50°C for 48 h. Macerated and dried samples of different tissues of rice plants were weighed and 0.3 g were used for DNA extraction in triplicate. The protocol used for DNA extraction includes CTAB (cetyl trimethylammonium bromide) and PVP (polyvinylpirrolidone) according to Khan et al. (2007).

PCR-nifH

Presence of DNA of diazotrophs among the total DNA extracted from several parts of rice plants was evaluated by PCR amplification of the *nifH* gene. Primers 19F and GC278R (Ueda et al. 1995; Direito and Teixeira 2002) were used to amplify DNA fragments from samples of roots, aerial part and grain collected from all treatments. The reaction was performed in 50 µl volume and contained the following reagents per reaction: 1 µl (0.5–20.0 ng) of total DNA, 1× PCR Buffer, 0.3 µM of each primer (19F and GC278R), 200 µM dNTPs, 2 mM $MgCl_2$ and 1 U of Taq polymerase (Invitrogen). The amplification was performed by touchdown PCR according to Direito and Teixeira (2002). The thermocycler program (Eppendorf Mastercycler gradient; Netheler–Hinz, Hamburg) was as follows: denaturation step at 95°C for 2 min, 20 cycles of decreasing annealing temperature (93°C for 1 min, 55 to 48°C for 1 min, 72°C for 2 min) followed by 15 cycles at constant annealing temperature (93°C for 1 min, 48°C for 1 min, 72°C for 2 min), and final extension at 72°C for 5 min.

PCR-16S rDNA

16S rDNA from the bacteria community associated with plant samples were amplified directly or subjected to pre-amplification with a primer set proposed to avoid amplification of chloroplast sequences and interference during the fingerprinting analysis (Chelius and Triplett 2001). The PCR mixture, made to a final volume of 50 µl, contained per reaction: 1 µl (0.5–20.0 ng) of total DNA, 0.3 µM of each primers (799f and 1492r), 200 µM of dNTP, 2 mM $MgCl_2$, 0.5 mg ml^{-1} BSA, 1x PCR Buffer and 1 U of Taq DNA polymerase (Invitrogen) in 20 mM pH 8.4 Tris-HCl and 50 mM KCl. The PCR program was as follows: first denaturation step at 95°C for 3 min, cycles of 94°C for 20 s, 53°C for 40 s, 72°C for 40 s (35×), and a final extension step at 72°C for 7 min. To generate fragments of small size and include the GC clamp for DGGE analysis, products of the first PCR were used as substrate for amplification of V6–V8 region of bacterial 16S rRNA using primers 968GCf and 1401r (Nübel et al. 1996) in the second amplification. The PCR mixture was made in a final volume of 50 µl containing: 1 µl of total DNA extract (0.5–20.0 ng), or from a 20-fold dilution of PCR products, 0.3 µM of the primers 1401r and 968GCf, 200 µM of dNTP, 3.75 mM $MgCl_2$, 0.5 mg/mL BSA and 0.1 U of Taq DNA polymerase (Invitrogen) in 20 mM pH 8.4 Tris-HCl and 50 mM KCl. A negative control (PCR mixture without DNA) and positive control (*G. diazotrophicus* PAL5 DNA) was included in all PCR experiments. The fragments were amplified using the following PCR program: 95°C for 2 min first denaturation step, cycles of 93°C for 1 min, 55°C for 1 min, 72°C for 2 min (35×), final extension step at 72°C for 5 min. Five µl of the PCR product were analyzed by electrophoresis in a 1% (w/v) agarose gel with 1 x TAE buffer (Sambrook et al. 1989) and stored at −20°C for DGGE analysis.

DGGE and statistical analysis

DGGE analysis, using the DGGE-Phor U 2×2 apparatus (Ingeny, Goes, The Netherlands), was performed as described previously (Muyzer et al. 1993). PCR samples were loaded onto 6% (wt/vol) polyacrylamide gels in 0.5 × TAE buffer. The polyacrylamide gels were prepared with denaturing gradients ranging from 55 to 65% for 16S rDNA fragments or from 60% to 80% for *nifH* gene fragments (where the 100% denaturant contained 7 M urea and 80% formamide). The gels were run for 16 h at 100 V and 60°C, after which they were silver stained according to Sanguinetti et al. (1994). The markers used for 16S rDNA and *nifH* fragments analysis by DGGE were prepared from PCR amplification of different diazotrophs (*Herbaspirillum eropedicae*, *H. rubrisubalbicans*, *Azospirillum amazonense*, *A. brasilense*, *Burkholderia tropica*, *Gluconacetobacter diazotrophicus*) from the Embrapa Agrobiologia Diazotrophic Bacteria Culture Collection, including the strains used to prepare the experimental inoculant.

DGGE analysis of partial 16S PCR products was performed in triplicate for two different parts of rice plants (roots and aerial parts), but the triplicates of the PCR products from grains were combined prior to DGGE analysis. The triplicates of partial *nifH* gene PCR products from each different part of the rice plants were combined prior to DGGE analysis to increase the amount of DNA target and banding profile of this specific group of bacteria. All gels were scanned at 400 dpi. The DGGE bands were identified and calculated from the densimetric curves of the scanned DGGE profiles with the software GelCompar II (version 4.2; Applied Maths, Kortrijk, Belgium). Similarity indices were calculated for DGGE profiles and cluster analysis using UPGMA and similarity Jaccard index were calculated with GelCompar II.

Elution of DNA from DGGE bands, reamplification and sequencing

Selected bands were cut from DGGE gels and transferred to 1.5 ml microtubes containing 50 µl of sterile DNase-free water. After overnight incubation at 4–8°C, the gel were crushed and centrifuged at 14,000*g* in an Eppendorf microtube centrifuge. The supernatant containing DNA in solution were transferred to another microtube, then 1–5 µl of each DNA eluted solution were used in PCR reactions as previously described for partial amplification of each gene. Sequencing was performed using the DYEnamic ET Dye Terminator Cycle Sequencing Kit and MegaBace 1000 automatic sequencer (GE Healthcare, Life Sciences).

Results

Culturable microbial communities in the rhizosphere

The MPN of *Azospirillum*-like diazotrophs in the rhizosphere of rice plants varied during the ontogenic cycle (Table 1). MPN were significantly higher at tillering compared to the grain-filling stage, 8.9 and 6.4 at $P=0.05$, respectively. At tillering, the percentage of *Azospirillum*-like diazotrophs occurrence in the rhizosphere of inoculated plants varied between 61 and 78% without significant differences between treatments. At the grain-filling stage, the percentage of microaerophilic diazotrophs were significantly different among treatments and the control.

PCA revealed distinctive CLPP among samples at the grain-filling stage (Fig. 1). The rhizosphere of the control plants were clearly separated from those of the inoculation treatments along PC1 and PC2, which explained 25 and 23%, respectively, of the total variance in the data. Certain CSs, including malic acid, oxalate, mannitol and maltose, showed high coefficients of correlation in the Pearson matrix

Table 1 MPN of *Azospirillum*-like diazotrophs in the rhizosphere of inoculated paddy rice plants at two phenological stages

Treatments	*Azospirillum*-like diazotrophs (Log MPN g of root^{-1})	
	Tillering[a]	Grain-filling[b]
Control	8.5	6.2 a
MI 1	9.3	6.4 ab
MI 2	8.9	6.7 b
P	ns	0.05

Means with the same letter are not significantly different as determined by Tukey test at the rejection level of $P=0.05$

ns Not significant

[a] Samples representative of plants collected 35 days after sowing (DAS)

[b] Samples representative of plants collected 117 DAS

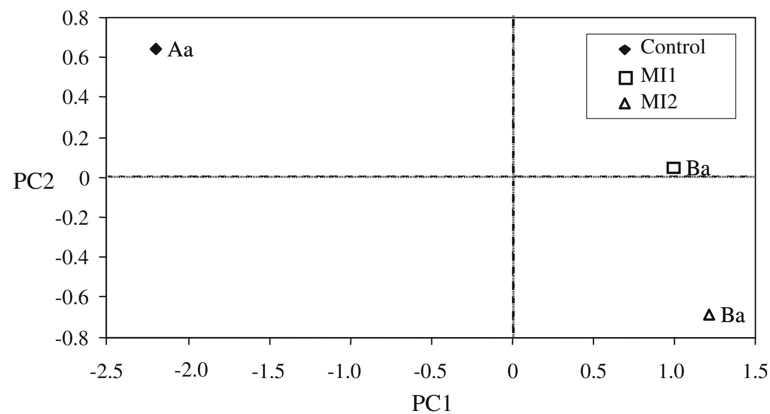

Fig. 1 Principal component (PC) analysis from CLPP of carbon source utilization communities associated with the rhizosphere of rice plants at the grain-filling stage. Values are the means of each inoculation treatment. Data of absorbance of 72 h reading were adjusted for average well-color development. *PC1* and *PC2* scores with the same *uppercase and lowercase letters*, respectively, indicate that they were not significantly different as determined by Tukey test at the rejection level of P=0.05

for PC1.The analysis of variance of the absorbance recorded at the respective CS wells showed significant differences between inoculation treatments with respect to the control (Table 2). Samples from seven wells, selected for their highest absorbance records at the last CLPP analysis reading, were transferred to N-free semisolid NFb medium, and the percentages of *Azospirillum*-like diazotrophs were determined by their ability to grow and typical pellicle formation (Table 3). These results revealed that the numbers of diazotrophs in the rhizosphere of paddy rice plants were very high. Also, it can be observed that the *Azospirillum* inoculation favors the presence of this PGPB in the rhizosphere of rice representing between 8 and 13%, respectively, in respect to the native culturable population of bacteria associated with control plants (Tables 1 and 3).

Table 2 Absorbance values for four carbon sources with the highest Pearson correlation coefficients for PC1

Treatments	Absorbance at 590 nm			
	Malic acid	Mannitol	Oxalate	Maltose
Control	0.265 b	0.203 b	0.018 b	0.251 b
MI 1	0.184 a	0.174 a	0.010 a	0.181 a
MI 2	0.177 a	0.160 a	0.010 a	0.166 a
P	0.05			

Means with the same letter are not significantly different as determined by Tukey test at the rejection level of P=0.05

Non-culturable microbial communities

The banding profile of 16S rDNA DGGE analysis, mostly to Gram-negative bacteria because of DNA extraction procedure, revealed that the endophytic populations of this group of bacteria varied among all treatments and different rice plant tissues (Fig. 2). During 16S rDNA DGGE analysis, we observed that, when the same sample from roots or aerial parts of rice plants was independently amplified, variability of banding profile was generated, but it was mainly related to the intensity of the bands, as can be seen in the Electronic supplementary material (ESM) Fig. S1. Since we were just considering the technique to describe the genetic diversity associated with the effect of *Azospirillum* inoculation to rice cropped under field conditions, we decided to use the most representative banding profile to construct our cluster analysis as shown in Fig. 2. The cluster analysis revealed that the highest levels of similarity were

Table 3 Percentages of isolates showing *Azospirillum*-like pellicle and *Azospirillum*-type colonies inside seven wells of each sample of rhizosphere at the grain-filling stage

Treatments	*Azospirillum*-like pellicle (%)	*Azospirillum*-type colonies (%)
Control	52 (±36)	25 (±16)
MI1	38 (±22)	33 (±16)
MI2	43 (±14)	38 (±16)

Standard deviation (SD) shown in parentheses

observed between Gram-negative bacteria communities associated with aerial part of the control and roots of MI2 treatment (80%) but no clear influence of treatments could be assigned from this data (Fig. 2). The endophytic community of Gram-negative bacteria associated with aerial parts and grains from treatment MI2 showed very low similarity values between each other and also to those present in all tissues from control and MI1 treatments. This suggests that the inoculation of *Azospirillum brasilense* in rice altered the Gram-negative bacteria communities associated within these tissues in relation to the control and could be evidence that a physiological response of plant tissues to inoculation occurred. We eluted, amplified and sequenced the DNA from several bands corresponding to 16S rRNA and *nifH* genes (dinitrogenase reductase) partial sequences separated during DGGE analysis (ESM Fig. S1 and Table S1; Fig. 3). Not all DNA eluted from bands of 16S rRNA and *nifH* could be amplified. DGGE 16S rDNA analysis indicated bands corresponding to *Azospirillum* sp. in the same position on the bands shown for the 40 M and 42 M strains used as inoculants (Fig. S1). Sequencing data from bands of *A. brasilense* strains used as inoculant and of all treatment samples revealed that at least one of the sequenced bands corresponds to *Azospirillum* sp. Interestingly, this band corresponds to position 44 of RMI2 showing similarity of partial sequences of 16S rRNA genes to *Azospirillum brasilense* strain CW301 (AY518780.1), *Azospirillum* sp. YM 195 (GU396257.1) and the sequences from the genome of *Azospirillum* sp. B510 (AP010951.1 and AP010949.1) at 89% and e-value of 4^{e-102}, including to the first sequence of *Azospirillum oryzae* (Xie and Yokota 2005) and another strain JCM 21588 (GU256443) deposited in GenBank in January 2010. However, the bands corresponding to those at positions 35 and 39 could not be amplified and sequenced. In addition, sequencing data from bands eluted from the DGGE gel revealed predominance of *Pantoea* spp. and other uncultured bacteria in endophytic association with these parts of rice plants (ESM Fig. S1 and Table S1).

The presence of diazotrophic Gram-negative bacteria associated within different rice plant tissues was also detected. The partial amplification of *nifH* gene from DNA extracted from all treatments, including the control, showed DGGE banding profile of *nifH* gene that represents mostly the genetic diversity of Gram-negative diazotrophs present in rice plant tissues. Bands located at the same position of those observed in *A. brasilense* strains used as inoculant are clearly seen amongst the banding profile from diazotrophic bacteria from all evaluated treatments.

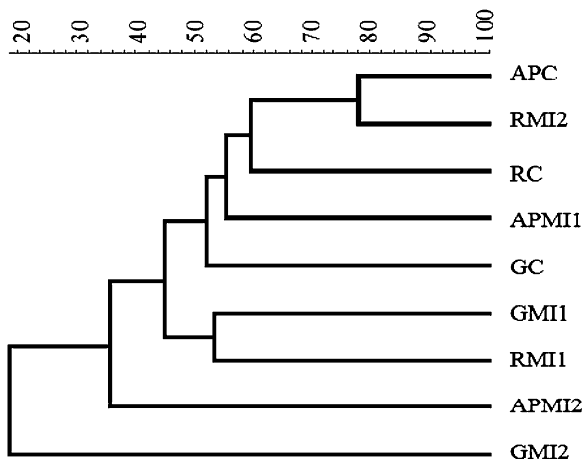

Fig. 2 16S rDNA-based cluster analysis of Gram-negative bacteria community associated with different parts of rice plant. The UPGMA dendrogram was constructed based on Jaccard similarity coefficient (1.6% of position tolerance) at 2.65% of band optimization (calculated by Pearson correlation). The *C* (control), *MI1* and *MI2* represent the treatments. Parts of the plants analyzed were roots (*R*), aerial parts (*AP*) and grain (*G*)

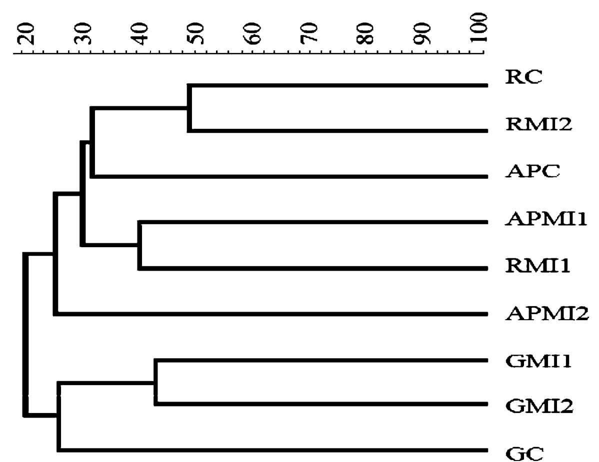

Fig. 3 Dendrogram based on DGGE profile of *nifH* fragments indicating the diversity index of diazotrophic Gram negative bacteria community analysis associated with different parts of rice plants. The UPGMA dendrogram was constructed based on Jaccard similarity coefficient (1.6% of position tolerance) at 2.65% of band optimisation (calculated by Pearson correlation). The *C* (control), *MI1* and *MI2* represent the treatments. Parts of the plants analyzed were roots (*R*), aerial parts (*AP*) and grain (*G*)

However, the DNA elution and sequencing of one of these bands (corresponding to band 35) did not confirm their identity, showing highest similarity with those of cyanobacteria.. The sequences of bands 24 (HM134905) and 25 (HM134906) isolated from DGGE gel were 96 and 98% similar to deduced sequences of uncultured bacteria *nifH* gene for dinitrogenase with accession numbers AB184928.1 and AB208261.1, respectively. These bands also showed a lower level of similarity (88 and 91%) to the deduced amino acid of nitrogenase iron protein nitrogenase component II sequence of *Desulfovibrio magneticus* RS-1 (AP010904.1). The sequence of band 35 (HM134907) showed similarity varying from 56 to 58% with deduced amino acid sequences of *nifH* sequence from representatives of cyanobacteria group, *Cyanobacterium* endosymbiont of *Rhopalodia gibba* (AY728387.1), *Cyanothece* sp. ATCC 51142 (CP000806.1) and *Gloeothece* sp. KO68DGA (AB293988.1).

Presence of PCR artefacts were detected after amplification of *nifH* DGGE-eluted bands resulting in PCR products smaller than 250 bp. Sequencing data of these amplified DNAs showed that most of them have similarities with rice (*Oryza sativa*) sequences or no significant similarity was found.

The dendrogram shows that treatments were distributed in two main clusters (Fig. 3). One comprises the banding profile of diazotrophic bacteria obtained exclusively from grains, and reveals that grains derived from both treatments of inoculation formed a group approximately 40% similar to the control. The other group derived from the *nifH* DGGE profile of diazotrophic bacteria from roots and aerial parts of all treatments. Interestingly, the cluster of banding profile from roots of inoculation treatment MI2 with those from the control supports the evidence previously observed by 16S rDNA DGGE analysis (Fig. 2). In this case, when micronutrients are not present in the treatment of inoculation, *Azospirillum* strains have a slight influence over the diazotrophic bacteria associated with roots of rice plants.

Agronomic responses

The *Azospirillum* inoculation increased aerial biomass at both the tillering and grain-filling stages. Grain numbers could explain the grain yield differences among treatments (Table 4). Significant increases were observed for inoculation treatments in relation to the control, and the percentage increases for treatments MI1 and MI2 were, respectively, 15 and 35% at tillering, 28 and 50% at the grain-filling stage, and 8 and 4% for grain yield. Both inoculation treatments increased N content by an average of 35% (Table 5). Values of Delta $^{15}N/^{14}N$ in rice plants are also shown in Table 5. The ^{15}N natural abundance of the soil was estimated as an average of the values of Delta $^{15}N/^{14}N$ determined for three weed plants, *E. polystachia, Cyperus* sp. and *Physalis viscosa* which are non-biological N fixers growing in the same area of the experiment. The inoculation of *Azospirillum* mixtures did not alter the Delta $^{15}N/^{14}N$ compared to that observed in the weed plants, but enhanced N uptake by the rice plant in the range of 16 and 50 kg ha^{-1} for MI1 and MI2, respectively.

Discussion

The data presented are pointing out that the competence of *Azospirillum* to establish in the paddy soil with rice should have been very high, because the rhizosphere of this plant has a particularly adapted microflora. It could be seen that, although differences were not significant, *Azospirillum* inoculation established high numbers of this PGPB in the rhizosphere of the inoculated plants at tillering (Table 1). Differences between treatments could only be observed at the grain-filling stage when translocation of plant reserves to the grains and the roots are dramatically changing their physiology. Indeed, inoculation with these selected strains can modify, at least temporarily, the density and composition of the rhizosphere communities of the rice plants under field conditions (Figs. 1, 2 and 3). The specific meaning of the differences in CS utilization are related to the number of microorganisms which are able to use the CSs within the well as a sole carbon supply and are concomitantly stained with the tetrazolium violet. The importance of growth indicates that the color responses produced in this assay are a reflection of the catabolic potential of the original community. For this, it was possible to obtain a fingerprint of their growth abilities (Mills and Garland 2002). The separation of the samples in PC space is related to differences in CS utilization. Because of that, it is relevant to perform analysis of variance for CSs

Table 4 Influence of *Azospirillum* inoculation on biomass accumulation and grain productivity of rice plants grown under field conditions

Treatments	Aerial biomass (kg ha^{-1})		Grain yield (kg ha^{-1})	Grain no. (N^{o} m^{-2})
	Tillering[a]	Grain-filling[b]		
Control	7,256 a	15,183 a	8,370 a	34,993 a
MI1	8,308 b	19,367 b	9,018 b	37,455 b
MI2	9,799 b	22,800 b	8,724 ab	37,170 ab
CV%	12.1	10.8	11.5	10.9
P	0.05			

Means with the same letter are not significantly different as determined by Tukey test at the rejection level of $P=0.05$

[a] Samples representative of plants collected 35 days after sowing (DAS)

[b] Samples representative of plants collected 117 DAS

which had the highest Pearson coefficients, since they have at least half of their variance explained by the PC1 or PC2. The absorbance values for control samples of rice rhizosphere at the grain-filling stage were higher than those with *Azospirillum* inoculation (Table 2). This could indicate that the microbial communities present in the rhizosphere of inoculated plants have a reduced ability or number of micro-organisms with potential to use those CSs, which are usually found in root exudates. From these data, it could be concluded that *Azospirillum* inoculation modified the functionality of the rhizosphere. Thus, it is necessary to study whether this mechanism is transitory or continues after the plant is harvested.

Sequencing data (ESM Table S1) indicated that 3 of the 4 bands shown in *A. brasilense* strains 16S rDNA DGGE profile correspond to gene copies previously described in genome of this species (Martin-Didonet et al. 2000). Interestingly, the DNA sequencing data indicated that the band corresponding to position 44 is similar to several 16S rDNA sequences of *Azospirillum brasilense*, and *Azospirillum* spp. available at NCBI database including *Azospirillum oryzae*, a species described associated with rice crop (Xie and Yokota 2005). Our data indicated that, considering the regions amplified by the pair of primers used in this study (V6–V8), these 16S rDNA copies from the *A. brasilense* genome are different in sequence, as evidenced by the blastn analysis and alignment, but we cannot exclude effects of PCR bias and/or silver staining on sequencing data. We assume that it reflects two main features of these strains: (1) copy number and (2) variability in this region that can contribute to the lower value of similarity observed against the sequences from Gen-Bank database. This fact suggests that the sequences submitted as representative of these strains, and also most of the other sequences previously deposited, are error prone since they are generated by a mixture of 16S copies amplification products. Despite the fact that four bands were observed to DGGE profile of cultures from *A. brasilense* strains, only one band corresponding to *A. brasilense* strain 40 M or 42 M position was detected in the RMI2 treatment. This could be related to the limited resolution of the DGGE technique or that under environmental conditions not all copies could be efficiently amplified due to variation in the matching percentages for primers to 16S rDNA sequences of bacterial groups (Sánchez et al. 2007). Regarding the *nifH* DGGE

Table 5 Nitrogen content and BNF estimates values observed in rice plants at the grain-filling stage

Treatments	Delta $^{15}N/^{14}N$	Total N content (kg ha^{-1})	N accumulation increase[b] (%)
Control	16.3	95	–
MI1	14.4	111	16.8
MI2	15.4	145	52.6
Weed plants[a]	15.6	–	–
CV%	28	19	

[a] Plants of *E. polystachia, Cyperus* sp. and *Physalis viscosa* which are non-biological N_2 fixers growing in the same area of the experiment

[b] Percentage of total N increase in the rice plant

sequences, our data are in accordance with those previously shown by studies measuring the genetic diversity of environmental samples (Chowdhury et al. 2009; Coelho et al. 2009). Sequences related to cyanobacteria were not identified by Wartiainen et al. (2008) who evaluated the active community associated with rice paddy rhizosphere. These authors suggested that it might be due to template discrimination in the PCR reactions, or low abundance of cyanobacteria compared to heterotrophic nitrogen-fixing bacteria. It is noteworthy that *nifH* sequence showing similarity with deduced aminoacid sequences of dinitrogenase reductase from representatives of cyanobacteria group were detected in this study. Based on clustering and sequencing identity data from this study and others, the numbers of gene copies and the high number of bands related to unculturable microorganism sequences should be considered as bias during evaluation of genetic diversity. These biases are due to the high number of environmental sequences deposited in the database. Usually, no efforts are applied to isolate the specimen. In addition, the copy number and variation of the matching percentage of several primers used for fingerprinting analysis are not considered.

In the rhizosphere, inoculation had a clear effect on community structure (Fig. 1). On the other hand, the cluster analysis of the endophytic community of Gram-negative bacteria based on 16S rDNA and *nifH* gene showed no clear influence of treatments (Figs. 2 and 3).

The inoculation of a mixture of *Azospirillum* strains increased N accumulation by the plant, representing 16.8 and 52.6% of the total N demanded by this crop production. This effect is of great importance especially for small farmers who generally apply N fertilizers at doses similar to those applied in this study (approx. 35 kg N ha^{-1}). Effects like these could only be expected if phyto-hormonal effects mediated by the bacteria occurred, promoting root development or other mechanisms that allow better absorption and assimilation of nitrogen from the N available in the soil, which represented 16 and 50 kg ha^{-1} of the N-total accumulated by the plants. Similar values of ^{15}N natural abundance were observed in all treatments, indicating that bacteria inoculation did not contribute to N derived from BNF to this crop under our experimental conditions. Otherwise, it indirectly contributed with an increase in N accumulation by rice plants from chemical fertilizer applied in the form of urea, as previously seen for paddy rice crops in Vietnam (Cong et al. 2009). However, it is important to consider that significant differences for N-fixation contributions can be found among rice genotypes (Boddey et al. 1995). Further studies to elucidate the mechanism basis for increased N uptake should be performed.

In summary, *Azospirillum* inoculation increased biomass and N accumulation by the plants. These data suggest that a hormonal effect exerted by *Azospirillum,* rather than enhanced BNF, improved the efficiency of N absorption leading to superior yields of biomass. Inoculation improved plant growth at the grain filling stage by 28 and 50% in relation to the control; however, no significant increase in grain production could be observed. Based on these results, we can hypothesize that another factor influenced rice plant development at the grain-filling stage, interfering with the contribution of the accumulated N content to improvement of grain production under our experimental conditions. The excess N available in soil influenced the overall diversity, mainly of specific functional groups, associated with plants and soils as previously described (Freitag et al. 2005; Tilak et al. 2005; Roesch et al. 2006). However, the specific mechanisms causing these changes are not clear from the present study. While further studies are needed to evaluate this bacteria–plant association, the increased biomass due to *Azospirillum* inoculation could be a tool to help farmers to increase input of plant residues, providing more organic matter to the soil and improving the sustainability of the system.

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