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Inoculation of paddy rice with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact of plant genotypes on rhizosphere microbial communities and field crop production

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ABSTRACT

Rice is the third largest global food crop. Traditional practice to achieve maximum yields of rice is associated with the availability of mineral nitrogen and fertilization. This can lead to pollution of waterways. This can be particularly important in paddy rice production in north-eastern Argentina. Biofertilization or inoculation with plant growth-promoting bacteria (PGPB) is a sustainable alternative for agro-ecosystems. Inoculation of wheat, maize, and soybean is a widespread agricultural practice that has proved to be efficient in increasing production and promoting nutrition of these crops. This work measures the response of three rice cultivars to PGPB inoculation under field conditions with a commercial formulation containing strains of *Pseudomonas fluorescens* and *Azospirillum brasilense*. The experiment was performed in a farm plot located near Villa Clara, Entre Ríos. A factorial complete block design with four replicates was applied. Samples were taken at tillering and physiological maturity. Aerial biomass, grain yield, and its components were determined. Culturable microorganisms were analyzed in rhizosphere samples. Counts of most probable number of microaerophilic, nitrogen-fixing microorganisms and community-level physiological profiles of carbon-source utilization were evaluated at physiological maturity. Also, DNA extraction, *nif*H gene amplification, and terminal restriction fragment length polymorphism (T-RFLP) analysis were performed to analyze molecular diversity of diazotrophic communities associated with rice roots. Data showed differences between rice genotypes. Inoculation with PGPB did not have significant impact on culturable microbial communities and patterns of T-RFLP. Some fragments obtained by restriction with enzymes *Hae*III and *Hha*I differentiated between inoculation treatments and rice genotypes. PGPB inoculation increased aerial biomass production, harvest index, and grain yield of the Supremo 13 cultivar by 4.7%, 16%, and 20.2%, respectively. Inoculation of the Yeruá cultivar increased aerial biomass by 1.9% and grain yield by 11%. On the other hand, control plants of the Cambá INTA cultivar produced 8.7% and 7.3% more aerial biomass and grain yield than inoculated plants, respectively. Inoculation reduced the percentage of chaffy grains of the three rice cultivars. The results indicate that the combined inoculation with *P. fluorescens* and *A. brasilense* has significant potential when applied to rice.

1. Introduction

Rice, wheat and maize provide two thirds of the energy in human diets. At current population growth rates, worldwide production will have to increase by 40% by 2020 (Vallejo et al., 2008). As a result of intensifying agricultural production, including fertilizers, pesticides, irrigation, genetic improvements, and double cropping, yields per surface unit and time has increased in the past two decades. However, future possibilities of increasing production are restricted by the limited additional suitable cropland. Thus, the increase of productivity is probably the only way to fulfill food demands. Increasing production without harming the environment is a challenge that requires improvement of soil quality and precise handling of all agricultural practices (Cassman, 1999; Naiman et al., 2009).

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Rice exhibits wide adaptability to different environments, which makes it the most widespread crop in the world. Breeding rice by selecting specific characteristics can adapt the crop to different areas with dissimilar levels of environmental stress. It can grow under drought conditions, shallow flooding, and water up to 50 cm of water (Catling, 1992). It is cultivated in a wide range of latitudes, as well as up to 3000 m elevation. In north-eastern Argentina, rice crops are grown under flood irrigation with few events of extreme temperatures (Arguissain, 2006).

Negative impacts of agriculture on the environment and high costs of production has led to initiatives to develop sustainable agriculture, production systems that provide reasonable economic returns to farmers and rural communities without affecting productive resources, preserving environmental quality, and creating safe and healthy food in sufficient quantities to meet the demands of growing populations (Benbrook, 1999).

Part of the strategy of sustainable agriculture is to find technologies that increase yields and reduce the use of chemical fertilizers (García de Salamone et al., 2010). One alternative is plant growth-promoting bacteria (PGPB) applied as inoculants (Bonilla et al., 2000). Among the PGPB, strains of *Azospirillum* are one of most studied genera. They can increase growth, development, and yield of a large number of plant species (Bashan et al., 2004; Cassán and García de Salamone, 2008). Numerous strains have been isolated from the root surface of many plants, including maize, wheat, rice, sorghum, oats, and forage grasses (Caballero-Mellado, 2007). *Azospirillum* spp. can produce and metabolize plant growth regulators as one of the main mechanisms to promote plant growth and development in inoculated plants (Okon and Labandera-Gonzalez, 1994). Biological nitrogen fixation is another mechanism in which these PGPB are involved (Baldani and Baldani, 2005; García de Salamone and Döbereiner, 1996). Importantly, grasses are capable of establishing symbiosis with diazotrophic bacteria, such as the genus *Azospirillum*. Nitrogen is the major limiting nutrient for rice production and so, bacterial contributions of nitrogen to plants could be vital at critical stages of plant development, such as reproduction and generation of tillers (Velazco, 2001). Physiological changes in rice and wheat inoculated with *Azospirillum* could favor biological nitrogen fixation and modify activity and number of microorganisms associated with the nitrogen cycle (García de Salamone et al., 2009). In further work, the bacterial strains responsible for efficiently supplying high levels of nitrogen through biological nitrogen fixation were identified (García de Salamone et al., 2010). Although numerous studies have shown the benefits of inoculation with *Azospirillum* on growth and production of different crops, the use of these PGPB is not widespread in rice production. It is known that plant-PGPB interactions can occur at different levels, influenced by genotype-environment interactions in response to inoculation (García de Salamone and Döbereiner, 1996; Olivares et al., 1996; Azevedo et al., 2005). The interaction of genotype-environment-inoculant points out the need for studies to increase the efficiency of inoculation (García de Salamone and Monzón de Asconegui, 2008).

Maintaining soil fertility is essential for sustainable production of grain and soil microorganisms play a fundamental role. The benefits of microbial inoculation on soil fertility have been documented in many studies (Jeffries, 1985; Kaushik, 1985; Roger et al., 1993; Gaur, 1990).

PGPB as biocontrol agents of diseases has also emerged as a tool in sustainable management programs. *Pseudomonas fluorescens* controls diseases, such as rice stem rot (*Sclerotium oryzae*) and rice sheath rot (*Rhizoctonia* spp.), under *in vitro* and under field conditions in north-eastern and north-western Argentina (Pedraza et al., 2009). This PGPB produces increased yields in wheat (Naiman et al., 2009) and produces high levels of cytokinins (García de Salamone et al., 2001). Initially, monoxenic inoculants have mainly been used.

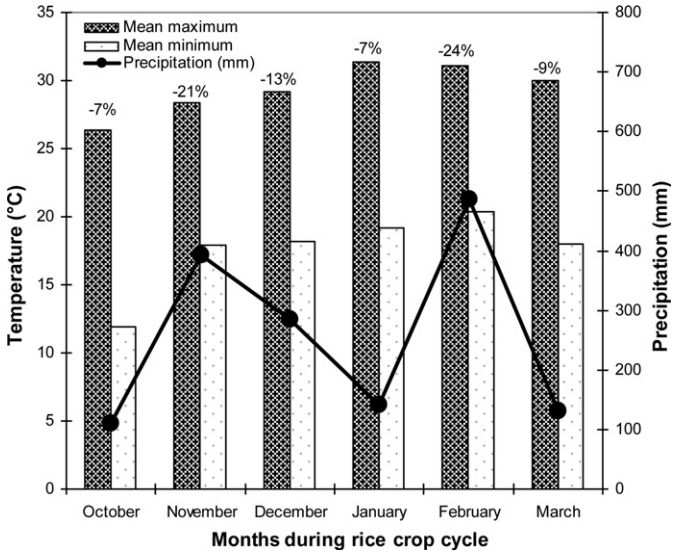

Fig. 1. Monthly precipitation, mean monthly maximum and minimum temperature, and relative differences in solar radiation during the rice crop cycle. Percentages for each month indicate relative differences in solar radiation with respect to average historical values at the site.

In recent years, there have been attempts to combine *Azospirillum brasilense* and *P. fluorescens*.

This study was planned to test the hypothesis that combined inoculation of PGPBs can modify rhizosphere microbial communities and growth and yield of rice grown under field conditions. We evaluated the response to a commercial liquid microbial inoculant containing *A. brasilense* and *P. fluorescens* to promote growth and yield of three rice cultivars under field conditions in Argentina. Additionally, we analyzed the effects of this mixed microbial inoculant and rice cultivars on the structure and physiology of microbial communities associated with rice root system.

2. Materials and methods

2.1. Field site and climate conditions

The experiments were conducted on farmland (31°44′25.4″S, 58°46′12.2″W) near Villa Clara in the Department of Villaguay in the Province of Entre Ríos. This region experiences warm and humid weather with an average temperature ranging between 17 and 20 °C and average annual rainfall of 1200 mm. Fig. 1 shows monthly maximum and minimum temperatures and rainfall during the field trials. The landscape of the experimental area mostly consisted of gently sloping alluvial plain. Fine textured clayed soils, mostly consisting of montmorillonite dominated in the experimental plots, thus hampering free movement of water through the soil profile. Chemical characteristics of the upper soil layer (30 cm) just before sowing were: pH (7.5 of 1:2.5 soil:water), electrical conductance (2.5 dS m^{-1}), total organic matter (3.1%), total N (0.16%), and available P (12 mg kg^{-1}).

2.2. Rice cultivars

We used certified seeds of the cultivars Cambá INTA, Supremo 13, and Yeruá supplied by the San Salvador Rice Coop of San Salvador, Entre Ríos, Argentina. Characteristics of the cultivars were described by Livore (2006); this is summarized in Table 1.

Table 1
Characteristics of rice cultivars (*Oryza sativa*) used in the experiment.

Cultivar[a]	Subspecies	Height	Days between emergence and anthesis[c]	Type of grain	Potential yield
Cambá INTA[b]	*indicus*	Semi-dwarf	94	Thin, long	High
Supremo 13	*indicus*	Semi-dwarf	100	Thin, long	High
Yeruá	*japonicum*	Tall	85	Wide, long	Medium

[a] Susceptible to the *Sclerotium oryzae* fungus that causes stem rot and collapse.
[b] Produced at the Instituto Nacional de Tecnología Agropecuaria (INTA), Concepción del Uruguay, Entre Ríos.
[c] On average, 35 days between anthesis and physiological maturity for the three rice cultivars.

2.3. Sowing and crop management

Before sowing, we applied to the soil a 48% concentration of Star Glyphosate™ at 2.5 L ha^{-1} (Asociación de Cooperativas Argentinas), Tordon 24K™ at 130 mL ha^{-1} (Dow AgroSciences, Argentina), Metsulfuron 50™ at 5 g ha^{-1} (Asociación de Cooperativas Argentinas), and NG Maxion™ coadjuvant at 80 mL ha^{-1} (Speed Agro Argentina). The trials were conducted in a commercial rice field under a no-tillage system, where the previous crop was rice in a rice-rice-soybean rotation sequence. Sowing occurred on 10 October 2009, based on temperatures, day length, and water and soil conditions typical for the region. The sowing density was adjusted to 180 kg ha^{-1} with row spacing of 15 cm and a working width of 3.6 m. Every 100 kg of seeds were subjected to 150 mL Terapico 30 + 30™ (Asociación de Cooperativas Argentinas) and 150 mL 75 Zn Basfoliar flo™ (Compo, Argentina) seed treatment on the day prior to sowing. The seeds were air-dried, then put in plastic bag until sowing. PGPB inoculation was performed on the day of sowing. Crop management conditions were the same as those applied in adjacent fields. At sowing, the entire experimental plot was fertilized with 50 kg ha^{-1} of a commercial grade N–P–K mixture (7–30–15). After emergence of the seedlings, the following herbicides were applied: 2 L ha^{-1} Clincher EC™ and 120 mL ha^{-1} Tordon 24K™ (Dow AgroSciences) and 750 mL ha^{-1} Natural Oil (Stoller™). Usually, flooding of the crop starts at the beginning of the tillering stage, which is approximately 30 days after emergence of the crop. Heavy rainfall during the experiment (Fig. 1), forced the crop to grow under flooded conditions for 20 days after sowing, similar to conditions of irrigation. Normally, irrigation ends when the crop reaches maturity, about 15 days before harvest.

2.4. Treatments and experimental design

The experiment consisted of two inoculation treatments (control and inoculated) and three rice cultivars. Each plot was 25.2 m^2 (14 m × 1.8 m). Single inoculations were carried out on the day of sowing, applying 300 mL per 30 kg of seeds. The inoculant was a liquid commercial formulation developed by Laboratorios CKC Argentina. It contains the PGPB *A. brasilense* and *P. fluorescens*. Counts of culturable declared microorganisms and determination of contaminants in the inoculants were performed using specific PAF™ medium (Laboratorios Britania, S.A., Argentina) and NFb medium (Döbereiner and Pedrosa, 1987). Counts of viable bacteria were 0.5×10^9 CFU/mL for *A. brasilense* and 1×10^9 CFU/mL for *P. fluorescens*. No contaminants were detected. The trial had a factorial design with four completely randomized blocks with three replicates of each treatment. Blocks were applied perpendicularly to the topographic slope (0.58%).

2.5. Sampling and determinations

Samples were taken at two ontogenetic stages of the crop, tillering and physiological maturity (PM). Tillering samples were taken at 44 days after sowing for Blocks 1 and 2, at 47 days for Block 3, and at 51 days for Block 4. These differences result from the difficulty of entering the plot during rainfall. PM sampling was carried out according to how the varieties reach this stage. Yellowing of leaves and panicles, widespread dropping of panicles in the canopy, and grains easily threshed by hand were the determinants for sampling this stage. Harvest was at 124 days after sowing for Yeruá; at 128 days for Cambá INTA, and 130 days for Supremo 13.

2.5.1. Aboveground biomass production and grain yield

Aerial plant biomass was determined for the three cultivars by cutting the plants growing in a line of 0.5 m, which was representative of the canopy and randomly selected. At both sampling times, aerial portions of the plants and their roots were sampled. Aerial biomass was determined by drying the samples in an oven at 60 °C until constant weight was reached. Number of panicles, filled and chaffy grains, and weight of 1000 grains were determined soon after PM was reached, as described by García de Salamone et al. (2010). Nitrogen and phosphorus content of the filled grains were determined according to (AOAC, 1945) adapted to the Kjeltec System (Tecator, Hillerød, Denmark) and Shaw (1959), respectively.

2.5.2. Culturable microbial communities in the rhizosphere

Root samples obtained at PM were washed with tap water and kept at 4 °C until analysis of culturable microbial communities. Counts of N_2-fixing, microaerophilic microorganisms were performed as described by García de Salamone et al. (2010). Community-level physiological profiles (CLPP) of carbon sources were studied, as described by Naiman et al. (2009).

2.5.3. Molecular characterization of rhizosphere associated diazotrophic bacterial communities

2.5.3.1. DNA extraction. Rice roots were washed with tap water, dried at 55 °C to preserve the samples and kept dry at room temperature until analysis, as previously described by García de Salamone et al. (2010). Roots were pulverized in sterilized pre-cooled mortars with liquid nitrogen. Total DNA was extracted from pooled rice roots (0.1 g) with a kit (UltraClean Soil DNA Isolation Kit #12800, Mo Bio Laboratories, Carlsbad, CA).

Pure cultures (1.5 mL) of *A. brasilense* strain AZ39 from the collection of the Instituto Nacional de Tecnología Agropecuaria (INTA, Argentina), *A. brasilense* strain Sp7 (ATCC29145), *A. amazonense* (Loaces et al., 2011), *Herbaspirillum seropedicae* (cited by Loaces et al., 2011), *Methylogaea oryzae* (Geymonat et al., 2010), and *Klebsiella* spp. (EU887702) were centrifuged for 10 min at 15,000 × *g* at 4 °C, and the pellets were extracted using a DNA purification kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI).

2.5.3.2. nifH gene amplification and screening of diversity by T-RFLP. Extracted DNA from root samples and isolates were used as templates for *nif*H gene amplification with ZMRf and ZMRr primer pairs (Zehr and McReynolds, 1989). ZMRf primer was 5′ labeled with 6-carboxyfluorescein. Three aliquots per sample of the extracted DNA were used to perform PCR amplification. All PCRs were carried out in 40 μL (total volume) solutions containing 100 ng of DNA solution, 0.1 mmol L^{-1} of each primer, 25 mmol L^{-1} $MgCl_2$, BSA (0.2 g L^{-1}), *Taq* 10× buffer, 0.25 mmol L^{-1} of each dNTP, and

Table 2
Effect of the interaction between rice genotype and inoculation on the most probable number (MPN) of microaerophilic microorganisms in the rhizosphere of rice plants at physiological maturity.

Cultivar	Log MPN MN_2FM[a]	
	Control	Inoculated
Cambá INTA	8.86 a	8.81 a
Supremo 13	8.83 a	8.98 a
Yeruá	9.15 a	8.83 a
CV (4.29)		

[a] Log MPN of microaerophilic nitrogen-fixing microorganisms. Means with the same letters are not different, as determined by Tukey's test at $P < 0.05$.

Table 3
Growth of rhizosphere microbial communities on four carbon sources of three rice cultivars at physiological maturity.

Cultivar	Microbial growth (Absorbance at 590 nm)			
	Rhamnose	Cellobiose	Phenylalanine	Xylose
Cambá INTA	0.14 b	0.24 b	0.19 b	0.10 b
Supremo 13	0.07 a	0.22 ab	0.05 a	0.03 a
Yeruá	0.06 a	0.20 a	0.09 ab	0.04 a
P values	0.006	0.045	0.011	0.003

Means with the same letter are not significantly different as determined by Tukey's test at $P < 0.05$values.

1.2 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The reactions were performed in a thermocycler (GeneAmpPCR System 2400, PerkinElmer, Waltham, MA) using the following steps: initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 1 min each, 57 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 7 min. The three aliquots of each sample were pooled after PCR amplification and the labeled PCR products were concentrated and desalted with centrifugal filter columns (Microcon 100, Amicon, EMD Millipore, Billerica, MA). Approximately 300 ng of labeled PCR products were digested with *Hha*I and *Hae*III (Fermentas, Thermo Fisher Scientific, Waltham, MA).

After enzyme inactivation by heating at 65 °C, for 15 min, 4 μL aliquots of the digest were mixed with a master mix (16 μL) containing deionized formamide loading buffer (Applied Biosystems, Carlsbad, CA), and 0.5 μL of a DNA fragment length standard (ROX 500, Applied Biosystems). After DNA denaturing at 94 °C for 5 min and immediate chilling on ice, samples were loaded into a capillary automated DNA sequencer (ABI 3100 genetic analyzer, Applied Biosystems).

After electrophoresis, lengths of fluorescently labeled terminal restriction fragments were analyzed by GeneScan 3.1 software (Applied Biosystems). Terminal restriction fragment (T-RF) sizes between 35 and 350 bp, with peak heights larger than 100 fluorescence units, were considered for the analysis to obtain reproducible T-RF profiles. Total fluorescence intensity present in each electropherogram was compared within each data set; T-RFLP data was standardized to the lowest quantity, as described by Dunbar et al. (2001). All profiles were aligned and considered identical if the T-RFs differed by <2 bp. Results were contrasted with *in silico* digestion made with 36 sequences of the *nif*H gene available in the database of the National Center for Biotechnology Information (NCBI; Bethesda, MD).

2.6. Statistical analysis

Absorbance records of CLPP were analyzed using principal component analysis. Data of relative abundance of each fragment of T-RFLP, were used to perform both UPGMA clustering analysis with the Morisita similarity index programmed in the Past 1.42 software (Hammer et al., 2001) and principal component analysis. These analyses included the two data sets (accounting for 75 different T-RFs) of amplicons obtained by *Hha*I and *Hae*III. Microbial counts and plant data were analyzed by ANOVA and comparison of means using Tukey's test at $P < 0.05$ with the software INFOSTAT/Professional 1.1 (Universidad de Córdoba, Argentina).

3. Results

3.1. Microbial rhizosphere communities

3.1.1. Culturable microbial communities

Microaerophilic nitrogen-fixing microorganisms in the rhizosphere of rice plants at the PM stage showed no significant differences between inoculated and control treatments for either cultivar (Table 4). Also, there were no differences between cultivars. The average of microaerophilic nitrogen-fixing microorganisms was 8.9 expressed as log MPN per gram of root. The CLPP of carbon-source utilization of microbial rhizosphere communities in samples obtained at the PM stage is shown in Fig. 2. On average, PC1 and PC2 explained 30% and 15%, respectively, of the total variation. The PCA only showed differences between the cultivars. Thus, the application of the inoculant did not change the physiological profiles of any of the cultivars. Culturable microbial communities associated with the rhizosphere of the Cambá INTA cultivar were significantly different from those associated with the other two cultivars. The analysis of variance of the absorbance values recorded at every carbon source showed significant differences between cultivars for some of them. These were rhamnose, cellobiose, phenylalanine, and xylose (Table 3). Microbial rhizosphere communities associated with the Supremo 13 and Yeruá cultivars can similarly use rhamnose and xylose and they were significantly different from those associated with the Cambá INTA cultivar. Cellobiose can distinguish the physiology of the microbial rhizosphere communities associated with the Cambá INTA cultivar, with respect to the Yeruá cultivar and phenylalanine, with respect to the Supremo 13 cultivar (Table 3). The average values of absorbance for arginine showed significant differences between the rhizosphere microbial communities associated with inoculated (0.12) and control (0.15) plants. No interactions between cultivars and inoculation treatments were observed.

3.1.2. Molecular characterization of root-associated diazotrophic bacterial communities

The enzymatic restriction of amplicons of the *nif*H genes with *Hae*III yielded a fragment distribution pattern in the T-RFLP analysis (Fig. 3). Four fragments whose sizes were 44–46 bp, 66–68 bp, 100–102 bp, and 155–157 bp dominated all samples. Each of them represented at least 7% of the signal. In any sample, the sum of the four fragments was always over 60% of the signal. The sum of the signals of the fragments that represented less than 10% of the total signal and occurred only in a few samples were grouped and named "others" in Fig. 3. None of these fragments was unique for any treatment or cultivar. The enzymatic restriction of amplicons with *Hha*I yielded a fragment distribution pattern in the T-RFLP analysis that showed the same trend as the pattern obtained with *Hae*III (data not shown).

Principal component analysis of all restriction fragments obtained with both enzymes (Fig. 4) provided significant mean values for each variety ($P = 0.0246$), which was different for PC1. Fragment sizes that had the highest Pearson correlation coefficients with PC1 were 59–61 bp (*Hha*I, 0.21), 58–60 bp (*Hae*III, 0.19), 91–93 bp (*Hae*III, 0.19), 249–251 bp (*Hae*III, 0.19), and 252–254 bp (*Hae*III, 0.19). Analysis of variance of all fragment sizes showed significant differences between cultivars for some of them (Table 4). No significant differences were obtained with PC2 (Fig. 4), and neither PC1 nor PC2 revealed differences related to inoculation

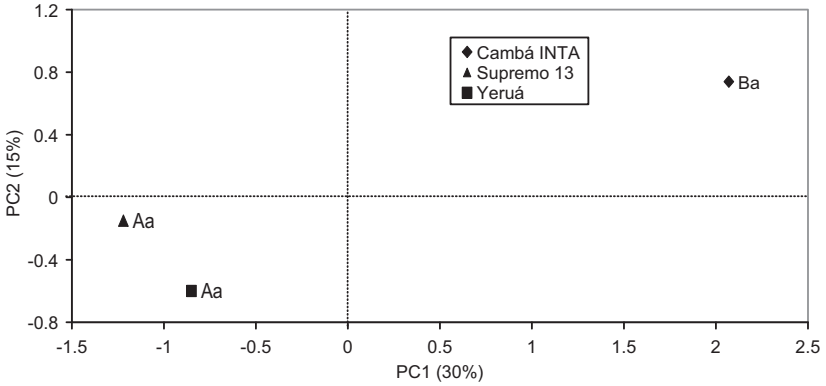

Fig. 2. Principal component analysis of the microbial communities in the rhizosphere of three rice cultivars at physiological maturity. Similar uppercase letters indicate no significant differences between means for 24 h absorbance values of each cultivar for PC1. Similar lowercase letters indicate no significant differences between means for absorbance values of each cultivar for PC2. Means were compared by Tukey's test at $P < 0.05$.

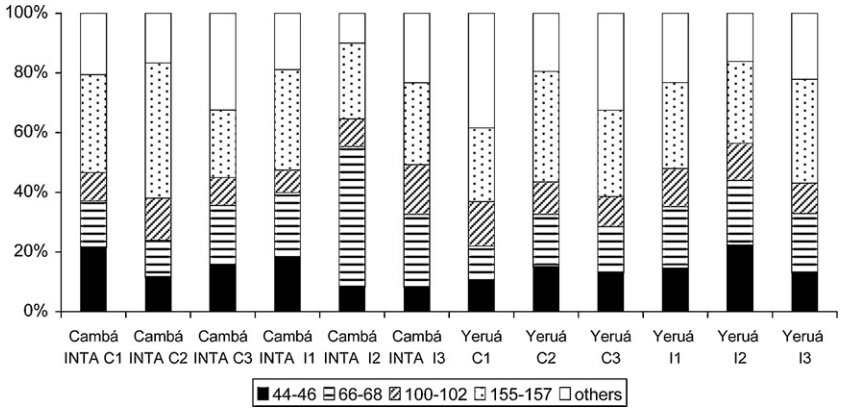

Fig. 3. Distribution of DNA fragment sizes obtained by amplification of the *nif*H gene and digestion with *Hae*III of root samples from two rice cultivars, with and without PGPB inoculation. C1–C3 are samples from uninoculated plants (controls) and I1–I3 are samples from inoculated plants for the two cultivars. The pattern inside every bar indicates different fragment sizes (number of base pairs). "Others" indicate the sum of the signal of fragments that represent <10% of the total signal.

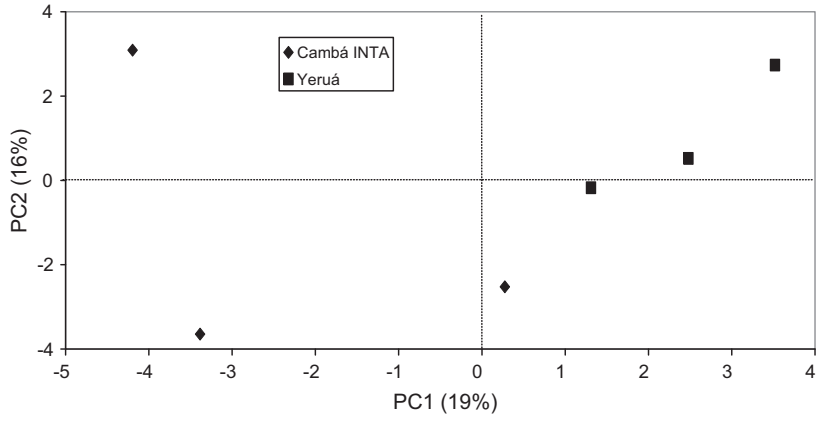

Fig. 4. Principal component analysis of restriction fragments obtained with enzymes *Hha*I and *Hae*III from roots samples of two rice cultivars at physiological maturity.

Table 4
Comparisons of means of six DNA fragment sizes for two rice cultivars obtained with restriction enzymes *Hha*I or *Hae*III for two inoculation treatments.

Cultivar	Fragment size (bp) and restriction enzyme					
	59–61 (*Hha*I)	40–42 (*Hae*III)	58–60 (*Hae*III)	69 (*Hae*III)	74–76 (*Hae*III)	183–185 (*Hae*III)
Cambá INTA	1045.8 a	315.6 a	298.1 a	169.5 b	432.7 a	309.3 a
Yeruá	2283.3 b	707.5 b	492.8 b	0.0 a	902.9 b	1,339.4 b
P	0.013	0.046	0.026	0.024	0.001	0.001

Means with the same letter are not significantly different as determined by Tukey's test at $P < 0.05$.

Table 5
Comparison of inoculation treatments of the average for two DNA fragment sizes obtained with the restriction enzyme *Hae*III for cultivars Cambá INTA and Yerúa.

Inoculation treatments	Fragment size (bp)	
	66–68 (*Hae*III)	183–185 (*Hae*III)
Control	5453.5 a	1158.7 b
Inoculated	10,927.6 b	490.0 a
P	0.029	0.008

Means with the same letter are not significantly different as determined by Tukey's test at $P < 0.05$.

Table 6
Number of emerged plants and aerial biomass at the beginning of tillering for three rice cultivars.

Cultivar	Emerged plants (number m^{-2})	Aerial biomass (kg ha^{-1})
Cambá INTA	510 ab	1137.8 ab
Supremo 13	642 b	1463.9 b
Yeruá	425 a	1022.2 a

Means with the same letter are not significantly different as determined by Tukey's test at $P < 0.05$.

treatments. Analysis of variance of the 66–68 bp and 183–185 pb fragments obtained with digestion using the *Hae*III enzyme had significant differences among inoculation treatments for an average of two cultivars (Table 5).

3.2. Plant stand and biomass production

At tillering, there were significant differences in the number of emerged plants and in aerial biomass among rice cultivars (Table 6). At this stage, there were no demonstrated effects of inoculation treatments. The Supremo 13 cultivar had the highest values for the number of plants and aboveground biomass, average increases with respect to the other cultivars of 27.1% in plant stand and 26.2% in biomass. Analysis of aerial biomass showed no differences between cultivars and inoculation treatments (Fig. 5); however, it is possible to indentify performance differences between cultivars. The Cambá INTA cultivar produced the highest plant biomass, an estimated average of 12,822 kg ha^{-1}. Comparatively, the Supremo 13 cultivar was lower by 3.6% and the Yeruá cultivar was lower by 14.1%. Inoculation increased by 2.4% the straw biomass of the Yeruá cultivar; inoculated Cambá INTA and Supremo 13 cultivars yielded a reduced straw biomass, compared to the controls.

3.3. Grain yield and components

The Cambá INTA cultivar had the highest grain yield, which averaged 6048 kg ha^{-1}. The Supremo 13 cultivar yield was 4645 kg ha^{-1} (23% less) and the Yeruá produced 36% less grain yield than the Cambá INTA cultivar (Fig. 5). None of the cultivars showed significant differences between inoculation treatments; however, the largest difference resulting from inoculation occurred in the Supremo 13 cultivar, with a 20.2% increase in yield. Inoculated Yeruá cultivar also exceeded grain yield over control plants by 11%. For the Cambá INTA cultivar, inoculation reduced grain yield by 7.3%.

The analysis of grain yield components after physiological maturity is shown in Table 7. No significant differences between inoculation treatments for number of panicles, filled grains, and chaffy grains of the three genotypes were observed; however, PGPB inoculation increased the number of panicles of the Supremo 13 and Yeruá cultivars by 0.4% and 7.4%, respectively. Average nitrogen and phosphorus content of rice grains were 1.24% and 0.35%, respectively. These values represent 62.7 kg ha^{-1} of nitrogen and 17.3 kg ha^{-1} of phosphorus. No significant differences were observed among the three cultivars.

For aerial biomass and grain production, inoculation of the Cambá INTA cultivar reduced panicles by 12.9%, compared to the controls, with significant differences among the inoculated cultivars: Supremo 13 had the highest numbers of panicles (average of 621 panicles m^{-2}), which was 28.6% and 43.2% higher than the results for the Cambá INTA and Yeruá cultivars, respectively. The Yeruá cultivar produced significantly less filled grains, compared to the other two cultivars. The inoculation treatment resulted in fewer chaffy grains among the three rice cultivars compared to the control plants. Decrease in chaffy grains was 9.7%, 14%, and 18.3% for Cambá INTA, Supremo 13, and Yeruá cultivars, respectively. The mean weight of 1000 grains was significantly different among the cultivars. Yeruá had the highest value; PGPB inoculation significantly increased this yield (4.5%). Finally, the Cambá INTA and Supremo 13 cultivars had the highest and lowest harvest index, respectively. Inoculation increased the index of the Supremo 13 cultivar by 14%.

4. Discussion

Although *A. brasilense* and *P. fluorescens* are diazotrophic bacteria that efficiently colonize roots and the inoculation with PGPB was at a high dose, competition between the native microorganisms and the added strains was probably severe. This is suggested because the rhizospheres of inoculated and control plants had similar numbers of culturable rhizobacteria (Table 2). Besides, rice has a particularly adapted microflora (Doi et al., 2011; Loaces et al., 2011). Although differences were not significant, the microaerophilic nitrogen-fixing microorganisms were very high in the non-inoculated rhizosphere. Counts of culturable bacteria were made on a selective medium, which allows only an approximation because commercial inoculant did not contain strains that could selectively be identified. Because the inoculation procedure was applied as suggested by the manufacturer, it is clear that will be necessary to achieve better colonization to ensure a substantial inoculation response. *Pantoea ananatis* and *Pseudomonas* spp. are widely distributed and persistent bacteria in rice plant rhizospheres. They can produce siderophores and some of them have strong antagonistic activity against *Azospirillum* spp. or *Herbaspirillum* spp. strains (Loaces et al., 2011). Some reports indicate that application of microbial inoculants can influence, at least temporarily, the microbial community of the resident associated bacteria (Conn and Franco, 2004; Naiman et al., 2009). Accordingly, inoculation with an experimental inoculant containing different *A. brasilense* strains changed the CLPP of the culturable microbial communities associated with the Cambá INTA cultivar at the grain-filling stage in the same geographical region in a previous experiment (García de Salamone et al., 2010). CLPP gives an estimate of growth and catabolic potential of culturable microorganisms in the original community (Mills and Garland, 2002). Also at the grain-filling stage, it was possible to distinguish the different microbial physiological profiles between the rhizosphere of plants inoculated with the same *A. brasilense* strains used here and another strain of *P. fluorescens* (Naiman et al., 2009). Although the analysis of absorbance values showed that the microbial communities associated with inoculated and control plants had different capabilities to use arginine, in our study, most of the variables showed significant differences of CLPP among rice cultivars (Fig. 2, Table 3). Other authors state that inoculation of *A. brasilense* did not influence the dominant members of the endophytic microbial communities in the phyllosphere, but improved nitrogen content and production of rainfed rice when applied cultivation-based techniques and cultivation-independent methods involving PCR-16S

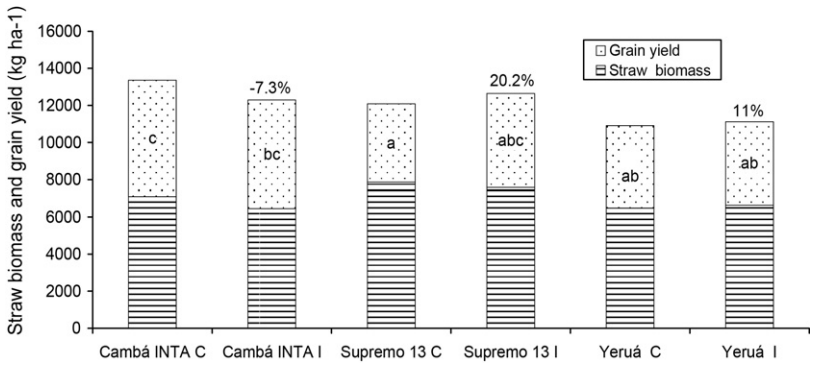

Fig. 5. Impact of PGPB inoculation on aerial (total and straw) biomass and grain yield at physiological maturity of three rice cultivars. Numbers above the bars indicate percentage of change in grain related to inoculation with respect to the control (C). Similar letters indicate no significant differences in grain yield with Tukey's test at $P < 0.05$.

rRNA and denaturing gradient gel electrophoresis (Pedraza et al., 2008).

Before analyzing the response from inoculation, several ecophysiological implications need to be considered. The 2009–2010 crop season showed lower productivity in the Province of Entre Ríos, compared to the 2006–2009 crop seasons, when average yields were >8000 kg ha^{-1} (García de Salamone et al., 2010). This is probably the result to lower available solar radiation during the crop cycle and difficulties of properly applying herbicides and fertilizers on schedule because of the many intense rain storms during the early period of cultivation, mainly November and December (Fig. 1). These delays impacted crop performance (Arguissain et al., 2010), although sowing dates and plant density were in accordance with recommendations (Quintero, 2009). The range of sowing density in this agroecological region is very wide (110–210 kg ha^{-1}); generally, most of the rice cultivars can adjust their performance through a higher or lower production of tillers. The number of emerged plants (Table 6) indicates that the stand of plants for each cultivar was formed with 0–3 tillers per plant with an initial density of ~300 plants m^{-2}. Usually, no grain yield limitations are observed in a range of 100–500 plants m^{-2}. Certain cultivars reduce the number of plants after emergence because of zinc deficiency in response to excess calcium and/or soil pH >6.5 (Arévalo et al., 2000; Quintero et al., 2006). Zinc was applied to avoid this problem. The ratio between the number of plants (Table 6) and the number of panicles (Table 7) indicate that the cultivars in this experiment had different sensibility to this situation. Thus, the Yeruá cultivar had fewer plants at the beginning of tillering (Table 6), and the number of panicles (Table 7) was even lower (ratio of 0.81 panicles/plant), while the other two cultivars have an average ratio of 1.1 panicles/plant. Rice cultivars responded differentially to PGPB inoculation. Biofertilization increased the number of panicles of the Supremo 13 and Yeruá cultivars by 15.5% and 11%, respectively (Table 7). The opposite occurred with the Cambá INTA cultivar, which decreased 16.7%. Although no significant differences were found between inoculation treatments, increases of grain production for the Supremo 13 and Yeruá cultivars were economically relevant for the farmers (Fig. 5). The different responses with the Cambá INTA cultivar with respect to data obtained by García de Salamone et al. (2010) in a previous experiment was most likely related to the interaction of the plant genotype, the bacterial strains, and the environment. These interactions were previously observed in other crops, such as maize (García de Salamone and Döbereiner, 1996).

Molecular analysis of the root samples accords with the ecophysiological response of the cultivars in this experiment. The restriction of amplicons with *Hae*III and *Hha*I supports the conclusion that all samples were highly similar to each other. The results of cluster analysis made from the same data showed that all analyzed diazotrophic communities were similar, >74% (data not shown). All analyses yielded the same four predominant fragment sizes (Fig. 3). Although, this similarity cannot be explained by a single organism, T-RFLP analysis of the *nif*H gene with known strains indicated that the 44–46 bp fragments obtained with the *Hae*III enzyme could correspond to *H. seropedicae* because it showed a fragment of 43 pb with that enzyme (Fig. 3). However, *in silico* digestion of 36 *nif*H gene sequences obtained from the database of NCBI showed that 19 of them also had a cutting site for this enzyme at 46 bp.

The analysis of strains isolated from similar ecosystems shows that the 66 pb fragment could correspond to a methanotrophic bacteria associated with rice, which was isolated in this region, which is *M. oryzae* (Geymonat et al., 2010). The fragments of 100–102 bp and 156–157 bp could also be assigned to two diazotrophic microorganisms: *A. amazonense* and *Klebsiella* spp., respectively. The former is a known endophyte of various crops (Pedraza and Díaz Ricci, 2000; Reis et al., 2004; Azevedo et al., 2005) and the latter was isolated as the predominant diazotrophic endophyte in rice (James et al., 2000; Fig. 3). However, *in silico* digestion performed on 36 selected sequences mentioned above indicates that the larger fragment could also be assigned to *nif*H genes of the genera *Gluconacetobacter*, *Sphingomonas*, *Paenibacillus*, and *Burkholderia*.

The restrictions *in silico* of the 36 sequences of *nif*H gene obtained from the NCBI database and performed with the

Table 7
Effects of PGPB inoculation on the yield of three rice cultivars after physiological maturity.

Treatment	Panicles	Filled grains	Chaffy grains	Weight of 1000 grains (g)	Harvest index (%)
	(number m^{-2})				
Cambá INTA (C)	564 b	26,357 c	4283 a	23.7 b	0.47 b
Cambá INTA (I)	500 b	24,622 bc	3869 a	23.8 b	0.48 b
Supremo 13 (C)	620 b	20,093 b	8542 c	20.9 a	0.35 a
Supremo 13 (I)	622 b	24,061 bc	7350 bc	21.1 a	0.40 ab
Yeruá (C)	332 a	13,086 a	5652 ab	33.6 c	0.40 ab
Yeruá (I)	357 a	12,724 a	4619 a	35.1 d	0.40 ab

Same letters in each column indicate no significant differences in grain yield with Tukey's test at $P < 0.05$.
C and I indicate control and inoculated treatments, respectively.

*Hae*III and *Hha*I show that fragment sizes of predominant microorganisms that may be present are: *Zoogloea oryzae* A-4 A-7 (AB201045.1, AB201046.1), *Azospirillum* spp. (AB542346.1, AB542349.1, FN813561; FJ799358.1, AB185395), and *Sphingomonas* spp. (FJ455053; FJ455038).

Most of the microorganisms mentioned above were isolated from crop roots, and in most cases, authors report that these strains are capable PGPB. *Zooglea oryzae* A-4 and A-7 strains were isolated from rice paddy soil associated with *Oryza sativa* roots (Xie and Yokota, 2006). *Azospirillum zeae* strain gr31 (FN813561.1) was isolated from the rhizoplane of wheat plants and has the ability to fix nitrogen and produce IAA (Venieraki et al., 2011). *Azospirillum oryzae* strain COC8 (AB185395.1) was isolated from rice paddy soil associated with *Oryza sativa* roots (Xie and Yokota, 2006). *Azospirillum doebereinerae* strain DSM 13131 (FJ799358.1) was isolated from roots of Chinese silvergrass (*Miscanthus sinensis*) and was tested for its ability to fix nitrogen (Eckert et al., 2001). *Sphingomonas* spp. BR12261 strain (FJ455053.2) and *Sphingomonas* spp. BR12249 strain (FJ455038.2) were obtained from the rhizosphere of rice plants grown in hydromorphic soils of Brazil (Videira et al., 2009).

In principal component analysis (Fig. 4), the fragment sizes showing high Pearson correlation coefficient with PC1 did not match any of the dominant fragment sizes between samples, and did not correspond to the fragment sizes predicted by *in silico* digestion of the selected strains. This shows that fragment sizes that explain the variability of the samples are diazotrophic microorganisms, which are a minority of the population. Some of these diazotrophic bacteria were not in the set considered for *in silico* digestion, either because they were not selected from the database or because their *nif*H sequence have not been deposited in the database. Some of the fragment sizes obtained by restriction with *Hae*III that differentiate both inoculation treatments and the cultivars (Tables 4 and 5, respectively), are the same size as those obtained from the selected analyzed strains. Thus, digestion of *H. seropedicae* generated a predominant fragment size of 43 bp, which could correspond to the 40–42 fragment size of *Hae*III that showed significant differences among rice genotypes. Besides, digestion of *M. oryzae* generated a predominant fragment of 66 bp that could correspond to the 66–68 bp fragment of *Hae*III that showed significant differences between inoculation treatments. Finally, digestion of *A. brasilense* strain AZ39, included in the inoculant, generated a predominant 75 bp fragment. This could correspond to the 74–76 bp fragment of *Hae*III that showed significant differences among the cultivars. Some of the fragments obtained by restriction with *Hae*III permitted differentiation of inoculation treatments or rice genotypes (Tables 4 and 5, respectively) and are the same fragment sizes obtained by *in silico* digestion. The 69 bp fragment of *Hae*III, which allowed us to differentiate cultivars, could correspond to the 70 bp fragment obtained in three sequences in *Pseudomonas stutzeri* (AF117977; CP002622.1; FN813565.1) and one in *Paenibacillus fujiensis* AB489070). The 185 bp fragment obtained by *in silico* digestion of four sequences of *A. brasilense* accord with the 183–185 bp fragment that distinguished between cultivars and inoculation treatments.

In summary, we demonstrated that PGPB inoculation had a moderate effect on the physiological and genetic characteristics of microbial communities associated with certain rice cultivars under field conditions. These moderate effects were also observed in the agronomic responses. Our results demonstrate that CLPP, T-RFLP, and analysis of plant attributes indicate a strong effect of plant genotypes on the associated microbial community. Further studies would clarify how different environmental and management conditions change the response to inoculation of specific bacteria–plant associations. This could help to obtain a better understanding of rice inoculation responses with PGPB in order to help farmers improve production and soil health.

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References

AOAC, 1945. Official Methods of Analysis, 6th ed. AOAC International, Gaithersburg, MD.

Arévalo, E., Quintero, C.E., Boschetti, N., Bracony, D., Valenti, R., Martinez, R., Spinelli, N., Bucari, E., 2000. Fertilización foliar de arroz en suelos con tosquilla. Resultados experimentales 1999–2000, vol. 9. INTA-Fundación Proarroz, pp. 60–75.

Arguissain, G.G., 2006. Ecofisiología del cultivo de arroz. In: Director de obra: René A. Benavidez, El arroz su cultivo y sustentabilidad en Entre Ríos. 1st ed. Universidad Nacional de Entre Ríos: Universidad Nacional del Litoral. Concepción del Uruguay, Entre Ríos, Argentina. p. 75.

Arguissain, G.G., Gregori, L., Pirchi, H.J., 2010. Informe climático campaña de arroz 2009-2010. Resultados experimentales 2009–2010, vol. XIX. Jornada Técnica Nacional de Arroz, Concordia, Entre Ríos, Argentina, pp. 17–21.

Azevedo, M.S., Teixeira, K.R.S., Kirchhof, G., Hartmann, A., Baldani, J.I., 2005. Influence of soil and host plant crop on the genetic diversity of *Azospirillum amazonense* isolates. Pedobiologia 49, 565–576.

Baldani, J.I., Baldani, V.L.D., 2005. History on the biological nitrogen fixation research, in: graminaceous plants: Special emphasis on the Brazilian experience. An. Acad. Bras. Cienc. 77, 549–579.

Bashan, Y., Holguin, G., de-Bashan, L.E., 2004. Azospirillum-plant relationships: physiological, molecular, agricultural and enviromental advances (1997–2003). Can. J. Microbiol. 50, 521–577.

Benbrook, C.M., 1999. World food system challenges and opportunities: GMOs, biodiversity, and lessons from America's heartland. In: University of Illinois World Food and Sustainable Agriculture Program <http://pmac.net/IWFS.pdf>.

Bonilla, R., Novo, R., Venegas, N., Galvis, A., Martinez, M., Parra, D., Vanegas, O., 2000. Generación de tecnologías para la utilización de fijación no simbiótica de nitrógeno como alternativa de fertilización. Corpoica-Regional 3, 40.

Caballero-Mellado, 2007. El género Azospirillum. Programa de Ecología Molecular y Microbiana. Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Morelia, México.

Cassán, F.D., García de Salamone, I.E., 2008. *Azospirillum* spp.: Cell Physiology, Plant Interactions and Agronomic Research in Argentina. Asociación Argentina de Microbiología, Buenos Aires, Argentina.

Cassman, K.G., 1999. Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. Proc. Natl. Acad. Sci. U.S.A. 96, 5952–5959.

Catling, D., 1992. Rice in Deep Water. Macmillan Press, London.

Conn, V., Franco, C., 2004. Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA clones. Appl. Environ. Microbiol. 70, 1787–1794.

Doi, T., Abe, J., Shiotsu, F., Morita, S., 2011. Study on rhizosphere bacterial community in lowland rice grown with organic fertilizers by using PCR-denaturing gradient gel electrophoresis. Plant Root 5, 5–16.

Döbereiner, J., Pedrosa, F.O., 1987. Nitrogen-fixing Bacteria in Non-leguminous Crop Plants. Springer, Madison, WI.

Dunbar, J., Ticknor, L.O., Kuske, C.R., 2001. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl. Environ. Microbiol. 67, 190–197.

Eckert, B., Baller-Weber, O., Kirchhof, G., Halbritter, A., Stoffels, M., Hartmann, A., 2001. *Azospirillum doebereinerae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass *Miscanthus*. Int. J. Syst. Evol. Microbiol. 51, 17–26.

García de Salamone, I.E., Döbereiner, J., 1996. Maize genotype effects on the response to *Azospirillum* inoculation. Biol. Fertil. Soils 21, 193–196.

García de Salamone, I.E., Hynes, R.K., Nelson, L.M., 2001. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Can. J. Microbiol. 47, 404–411.

García de Salamone, I.E., Monzón de Asconegui, M.A., 2008. Ecofisiología de la respuesta a la inoculación con Azospirillum en cultivos de cereales. *Azospirillum* sp.: Cell Physiology, Plant Interactions and Agronomic Research in Argentina, vol. 14. Asociación Argentina de Microbiología, Buenos Aires, Argentina, pp. 209–226.

García de Salamone, I.E., Escobar Ortega, J.S., Gatica, M., Di Salvo, L.P., Vilches, M.V., Zubillaga, M.M., Urquiaga, S., 2009. Effect of *Azospirillum* inoculation on N-cycling microorganisms associated with rice and wheat crops. 16th Nitrogen Workshop, http://www.nitrogenworkshop2009.org/. Turin, Italy.

García de Salamone, I.E., Di Salvo, L.P., Escobar Ortega, J.S., Boa Sorte, M.P., Urquiaga, S., Dos Santos Teixeira, K.R., 2010. Field response of rice paddy crop to inoculation with *Azospirillum*: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants. Plant Soil 336, 351–362.

Gaur, A.C., 1990. Phosphate Solubilising Microorganisms as Biofertilizers. Omega Scientific Publishers, New Delhi.

Geymonat, E., Ferrando, L., Tarlera, S.E., 2010. *Methylogaea oryzae* gen. nov., sp. nov., a novel mesophilic methanotroph from a rice paddy field in Uruguay. Int. J. Syst. Evol. Microbiol., doi:10.1099/ijs.0.028274-0.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Paleontología Electrónica 4, 1–9.

James, E.K., Gyaneshwar, P., Barraquio, W.L., Mathan, N., Ladha, J.K., 2000. Endophytic diazotrophs associated with rice. In: Ladha, J.K., Reddy, P.M. (Eds.), The Quest for Nitrogen Fixation in Rice. International Rice Research Institute, Los Baños, Philippines.

Jeffries, P., 1985. Use of mycorrhizae in agriculture. Crit. Rev. Biotechnol. 5, 319–357.

Kaushik, B.D., 1985. Effect of native algal flora on nutritional and physicochemical properties of sodic soils. Acta Bot. Indica 13, 143–147.

Livore, A.B., 2006. Los cultivares de arroz. In: René A., Benavidez (Ed.), El arroz su cultivo y sustentabilidad en Entre Ríos. , 1st ed. Universidad Nacional de Entre Ríos: Universidad Nacional del Litoral, Concepción del Uruguay, Entre Ríos, Argentina, pp. 95–110.

Loaces, I., Ferrando, L., Fernández Scavino, A., 2011. Dynamics, diversity and function of endophytic siderophores-producing bacteria in rice. Microb. Ecol. 61, 606–618.

Mills, A.L., Garland, J.L., 2002. Application of physiological profiles to assess community properties. In: Hurst, C.J., Crawford, R.I., Knudsen, G.R., McInerney, M.J., Stenzenback, L.D. (Eds.), Manual of Environmental Microbiology. , 2nd ed. ASM Press, Washington, DC, pp. 135–146.

Naiman, A.D., Latrónico, A., García de Salamone, I.E., 2009. Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: impact on the production and culturable rhizosphere microflora. Eur. J. Soil Biol. 45, 44–51.

Okon, Y., Labandera-Gonzalez, C.A., 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem. 26, 1591–1601.

Olivares, F.L., Baldani, V.L.D., Reis, V.M., Baldani, J.I., Döbereiner, J., 1996. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems and leaves, predominantly of Gramineae. Biol. Fertil. Soils 21, 197–200.

Pedraza, R.O., Díaz Ricci, J.C., 2000. Azospirillum amazonense: its presence in the sugar cane area of the province of Tucumán. Rev. Argent. Microbiol. 32, 199–201.

Pedraza, R.O., Bellone, C.H., Carrizo de Bellone, S., Boa Sorte, P.M.F., Teixeira, K.R.S., 2008. *Azospirillum* inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop. Eur. J. Soil Biol. 45, 36–43.

Pedraza, M.V., Asselborn, M.N., Liberman, C.A., Restelli, Y., Clemente, G.E., 2009. Avance en el estudio del control biológico de enfermedades de tallo y de vaina en arroz, con *Pseudomonas fluorescens*. Resultados experimentales 2008–2009, vol. 18. Jornada Técnica Nacional de Arroz, Concordia, Entre Ríos, Argentina, pp. 83–88.

Quintero, C.E., Arévalo, E., Boschetti, N., Spinelli, N., 2006. Clorosis en suelos con calcáreo, experiencias en cultivo de arroz en Entre Ríos. In: Vázquez, M. (Ed.), Micronutrientes en la agricultura. , 1st ed. Asocación Argentina de la Ciencia del Suelo, pp. 113–125.

Quintero, C.E., 2009. Factores limitantes para el crecimiento y productividad del arroz en Entre Ríos, Argentina: Doctoral Thesis. Universidad de la Coruña. Ediciones de la Fundación Para el Desarrollo Agropecuario, 1th ed. Paraná, Argentina.

Reis Jr., F.B., Silva, M.F., Teixeira, K.R.S., Urquiaga, S., Reis, V.M., 2004. Intra-specific diversity study of the nitrogen fixing bacterium *Azospirillum amazonense* isolated from different *Brachiaria* species. Symbiosis 36, 41–56.

Roger, P.A., Zimmerman, W.J., Lumpkin, T.A., 1993. Microbiological management of wetland rice fields. In: Metting, B. (Ed.), Soil Microbial Ecology: Applications in Agricultural and Environmental Management. Marcel Dekker, New York, pp. 417–455.

Shaw, W.M., 1959. Nitric-perchloric acid oxidation of sulfur in plant and animal tissues. J. Agric. Food Chem. 7, 843–847.

Vallejo, M.M., Bonilla, C.R., Castilla, L.A., 2008. Evaluación de la asociación bacterias fijadoras de nitrógeno-líneas interespecíficas de arroz-nitrógeno, en typic haplustalf. Universidad Nacional de Colombia. Acta Agronómica 57, 43–49, http://redalyc.uaemex.mx/src/inicio/ArtPdfRed.jsp?iCve=169913318006.

Velazco, A.C., 2001. *Azospirillum* sp. como diazótrofo predominante en los cultivos de caña de azúcar y arroz. Master's Thesis. Universidad de la Habana, Havana, Cuba.

Venieraki, A., Dimou, M., Pergalis, P., Kefalogianni, I., Chatzipavlidis, I., Katinakis, P., 2011. The genetic diversity of culturable nitrogen-fixing bacteria in the rhizosphere of wheat. Microb. Ecol. 61, 277–285.

Videira, S.S., de Araujo, J.L., Rodrigues Lda, S., Baldani, V.L., Baldani, J.I., 2009. Occurrence and diversity of nitrogen-fixing *Sphingomonas* bacteria associated with rice plants grown in Brazil. FEMS Microbiol. Lett. 293, 11–19.

Xie, C.H., Yokota, A., 2006. Zoogloea oryzae sp. nov., a nitrogen-fixing bacterium isolated from rice paddy soil, and reclassification of the strain ATCC 19623 as *Crabtreella saccharophila* gen. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 56, 619–624.

Zehr, J.P., McReynolds, L.A., 1989. Use of degenerate oligonucleotides for amplification of the *nif*H gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. 55, 2522–2526.