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Original article

Azospirillum inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop

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ABSTRACT

We assessed the *Azospirillum* inoculation and N-fertilization effect on grain yield and on the phyllosphere endophytic diversity of nitrogen-fixing bacteria in a rice rainfed crop. We used cultivation-based techniques and cultivation-independent methods involving PCR-16S rRNA and denaturing gradient gel electrophoresis (DGGE). In general, we observed that grain yield was improved when inoculated with *Azospirillum* (depending on the genotype) and/or fertilized with urea. A similar behavior was observed in total N-content in grain and the MPN determination, as the highest values occurred when seeds were inoculated with *A. brasilense* REC3 (S1) than with *A brasilense* 13-2C (S2). A positive nitrogenase activity and PCR-*nifH* amplification suggests that the bacteria associated to inner tissues of rice phyllosphere could have contributed to the different N-contents detected. The bacterial diversity, observed in the number and intensity of DGGE profiles, showed a higher number of bands when total DNA was obtained using only CTAB than with CTAB + PVP. The DGGE profiles revealed great stability in the dominating bands, which presumably represent numerically dominant species. Application of *A. brasilense* strains as inoculants did not influence the dominant members of the endophytic microbial communities in the phyllosphere, but improved N-content and production of rainfed rice crop.

1. Introduction

Plants may be considered complex ecosystems where different niches are inhabited by a broad diversity of bacteria. Such niches include not only the external surfaces of plants, but also the internal tissues where endophytic bacteria live without apparent harm to the host or external structure [25]. In most plant species, endophytic bacteria are ubiquitous, colonizing locally as well as systemically, and influencing plant health by suppression of disease, degradation of contaminants, and promotion of plant growth [34,42]. Furthermore, the plant-associated habitat is a dynamic environment in which many factors, such as plant tissues, soil type, and interaction with other microorganisms, may affect

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the structure and species composition of the bacterial communities that colonize plant tissues [1,2,15,25,26,30].

Considerable advancement has been made in understanding the interaction between rice plants, soil biogeochemistry, and microorganisms [10,11,14,21,24,28], and thus rice fields are one of the best-studied model systems in soil microbial ecology [31]. Nitrogen-fixing endophytes in rice were reported to be higher in stems than in roots, indicating that rice stems probably provide a suitable niche for them [7]. However, little information exist on the diazotrophs and other bacteria associated to leaf inner tissues of rice, and most work published so far has ignored their diversity in the phyllosphere. A study of ascending migration of endophytic gfp-tagged rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology was considered as a model of beneficial plant–bacterium interaction [12]. According to the observation that this kind of endophytic plant–bacterium association is far more inclusive, invasive and dynamic than previously thought, these authors stress its potential value for future exploitation as a inoculant strategy in sustainable agriculture to produce the world's most important cereal crop [12]. At present, it is known that microbial phyllosphere populations are more complex than previously realized [46], but no study was applied to assess the diversity of cultivable and non-cultivable bacteria associated to rice endophyllosphere.

Therefore, the aim of this work was to assess the effect of nitrogen fertilization and inoculation of *Azospirillum brasilense*, a plant growth-promoting bacterium, on grain yield and on the diversity of phyllosphere endophytic bacteria in rice rainfed crop. For that, analysis of agronomic parameters and methods to assess microbial diversity, as cultivation-based techniques and cultivation-independent methods involving 16S rDNA-PCR fragments and denaturing gradient gel electrophoresis (DGGE), were used to evaluate the response of rice rainfed crop and the bacterial genetic diversity in the endophyllosphere of rice plants, under different treatments.

2. Materials and methods

2.1. Bacterial strains and inoculant preparation

Two local strains of *A. brasilense* were used to inoculate the rice seeds:

- *A. brasilense* REC3 (GenBank accession number FJ012319 for the 16S rRNA gene sequence), isolated from strawberry roots [36], named Strain 1 (S1) in this study. It produces 1.6 ± 0.01 μg total indoles per mg protein, when using tryptophan as precursor in the culture medium, and 0.5 ± 0.01 μg total indoles mg protein^{-1}, without addition of Trp as precursor.
- *A. brasilense* 13-2C (GenBank accession number FJ012320 for the 16S rRNA gene sequence), isolated from sugarcane roots [35], named Strain 2 (S2) in this study. It produces 0.84 ± 0.01 μg total indoles per mg protein, when using Trp as precursor in the culture medium, and 0.2 ± 0.01 μg total indoles mg protein^{-1}, without addition of Trp as precursor.

They were grown in NFb liquid medium [6], supplemented with 1% NH_4Cl (w/v) at 30 °C, 120 rpm for 72 h. Then, they were washed once with sterile distilled water and suspended in sterile distilled water. The optical densities of bacterial cultures were spectrophotometrically measured and cell numbers of each suspension were adjusted to 10^7 cfu/ml.

2.2. Experimental field design

The rice (*Oryza sativa*) cultivar Taranga was cultivated in an Argiudol type soil (pH 6.4 and 3% organic matter) in the experimental field of the Faculty of Agronomy and Zootechnics of the National University of Tucumán, located in the province of Tucumán, Argentina (26° 47′S–65° 16′W). The mean annual rainfall is 1188 mm and 80% occurs during rice cropping period (November to April), and the mean annual evapotranspiration is 1005 mm. Thus, the rice production is depending on the rainfall only. During winter, the cropping area was planted with lentil (*lens culinaris*).

The experimental design was a completely randomised block with four replications; the plots consisted in 12 lines of 15 m long, separated by 30 cm. The density of sowing was 100 seeds/linear meter and the treatments were: (i) seeds without bacterial inoculation or N-fertilization; (ii) seeds inoculated with S1; (iii) seeds inoculated with S1 and fertilized with urea (50 kg N ha^{-1}); (iv) seeds inoculated with S2; (v) seeds inoculated with S2 and fertilized with urea (50 kg N ha^{-1}); (vi) seeds inoculated with S1 and S2; (vii) seeds inoculated with S1 and S2, and fertilized with urea (50 kg N ha^{-1}); and (viii) fertilization with urea (50 kg N ha^{-1}). During the trial, performed in the season of 2006–2007, weeds were controlled manually and neither pesticide nor herbicide was applied. During the experiment, irrigation was dependent on the rainfall regime. Panicles were collected, 120 days after sowing, from all the plants of each plot and treatment. The grains produced from all panicles collected from each treatment were weighed and used as a composite sample for determination of total nitrogen content by the micro-Kjeldahl method. Results were subjected to ANOVA and LSD ($P = 0.05$) analysis with Statistix Analytical Software 1996 for Windows.

2.3. MPN determination of cultivable endophytic bacteria

When green panicles were present, about 10 g of leaves were collected at random from 20 plants of each treatment located in different plots of the plantation and pooled before assay. They were placed in a plastic bag and immediately transported to the laboratory where they were washed in running tap water to remove dust.

Total endophytic bacteria were isolated after removing epiphytes by surface disinfection using serial washing in 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl) for 3 min, 70% ethanol for 30 s and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto 10% trypticase soy agar (TSA) and incubating the plates at 30 °C for 5 days. After surface disinfection, 10 g of a compost sample of leaves from each treatment were cut and triturated in a 50-ml flask containing 90 ml of sterile

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phosphate-buffered saline (PBS: 0.88% (w/v) NaCl, 2.9 mM KH_2PO_4, 7.1 mM K_2HPO_4, pH 7.2). Then, serial dilutions were made in the same solution. The MPN per gram fresh leaf was determined using the McCrady table for three replicates. The following semi selective culture media were used: NFb [36], JNFb [5], LGI [36], LGI-P [45] and JMV [38]. In all cases, they were semisolid and N-free.

Presence or absence of a pellicle in the growing medium was determined. Bacterial purification was made from the highest dilution with positive growth in the different culture media, plating on Petri dishes containing the same culture media. Single colonies were picked up with a sterile stick and inoculated into semisolid N-free media, irrespective of the original media used for isolation, for further acetylene reduction assay.

2.4. *Acetylene reduction assay (ARA)*

The nitrogenase activity measurement by acetylene reduction assay (ARA) was carried out in 50-ml vials containing 25 ml of semisolid media (NFb, JNFb, LGI, LGI-P and JMV) in triplicate. Isolates obtained after purification in the MPN determination were grown in these media for 48–72 h at 30 °C. Each vial was sealed with rubber stopper and the headspace (25 ml) was injected with 10% (v/v) acetylene. Gas samples (0.2 ml) were removed after 1 h and assayed for ethylene production with a gas chromatograph (Carle Analytical Gas Chromatograph model 311), using a Porapak-N column (5 m × 0.5 mm, 35 °C) and a hydrogen flame ionization detector. Values were expressed as nmol C_2H_4 h^{-1} $culture^{-1}$.

2.5. *Extraction of total DNA from leaves*

After superficial disinfection of the leaves, as previously explained, total DNA was extracted from 1.0 g of each sample by two methods. One, according to Doyle and Doyle [19] that includes the use of CTAB (cetyl trimethylammonium bromide), and another one the same as the first, but with a modification according to Khan et al. [23], which includes the use of CTAB and PVP (polyvinylpirrolidone).

2.6. *PCR-nifH*

To assess the presence of diazotrophs in the total DNA extracted from the different leaf samples, PCR amplification of the *nifH* gene involved in the nitrogen-fixing process was carried out. Primers 278R and 19F [18,44] were used for amplification of the *nifH* gene fragment as described by Direito and Teixeira [18].

2.7. *PCR-16S rDNA*

The amplification of bacterial DNA from plant tissues was performed as suggested by Chelius and Triplett [13] to avoid interference from chloroplasts and mitochondrial DNA. The PCR mixture was made in a final volume of 50 μl containing per reaction: 1 μl (0.5–20.0 ng) total DNA, 0.3 μM of each primer (799f and 1492r), 200 μM of dNTP, 2 mM $MgCl_2$, 0.5 mg ml^{-1} of BSA and 1 U Taq DNA polymerase (Invitrogen, Life Technologies) in 20 mM Tris–HCl, pH 8.4, and 50 mM KCl. To generate fragments of small size and include the GC clamp for DGGE analysis, products of the first PCR were used as substrate for the second amplification with primers 968GCf and 1401r [33]. The PCR mixture was made in a final volume of 50 μl containing: 0.5–20.0 ng total DNA, 0.3 μM of the primers 1401r and 968GCf, 200 μM dNTP, 3.75 mM $MgCl_2$ and 0.1 U Taq DNA polymerase (Invitrogen, Life Technologies) in 20 mM Tris–HCl, pH 8.4, 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 amplification was accomplished using the following 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 ×) followed by one round at 72 °C for 5 min as final extension step. Five microliters of the PCR product were analyzed by electrophoresis in a 1% (w/v) agarose gel with 1 × TAE buffer and stored at −20 °C for DGGE analysis.

2.8. *DGGE gel and statistical analysis*

DGGE was performed with the phorU 2 × 2 electrophoresis system (Ingeny, Goes, The Netherlands) as described previously [32]. PCR samples were loaded onto 6% (w/v) polyacrylamide gels with denaturing gradients ranging from 55 to 65% (where the 100% denaturant contained 7 M urea and 80% formamide) in 0.5 × TAE buffer. The gels were run in 0.5 × TAE for 16 h at 100 V and 60 °C and then the bands were identified after staining with $AgNO_3$ according to Sanguinetti et al. [39]. The marker contained seven 16S rDNA fragments from different diazotrophs with different mobility: *Herbaspirillum seropedicae* HRC54, *H. rubrisubalbicans* HCC103, *Burkholderia tropica* Ppe8, *Gluconacetobacter diazotrophicus* PAL5, *Azospirillum amazonense* CbAmc, and *A. brasilense* REC3 and 13-2C strains.

DGGE analysis of all samples was repeated twice. All gels were scanned at 400 dpi. The number of DGGE bands was calculated from the densitometric curves of the scanned DGGE profiles with GelCompar II software (version 4.2, Applied Maths, Kortrijk, Belgium). Similarity indices were calculated for DGGE profiles of each of the DNA samples obtained by the CTAB and CTAB-PVP methods. UPGMA cluster analysis and Jaccard and Sorensen's similarity index were calculated using GelCompar II software. Sorensen's index (S), calculated on the basis of DGGE profiles ($S_{1,2} = 2a/2a + b + c$, where "*a*" is the number of bands common to both samples, and "*b*" and "*c*" the number of bands in samples 1 and 2, respectively), was used as a parameter to evaluate the influence of each treatment on the genetic diversity of bacteria [16].

3. Results

The effect of *Azospirillum* on plant nutrition from available soil nutrients has been described many times [8], but in this work we present results on its interaction with rice under rainfed cropping conditions in Tucuman, Argentina.

After 120 days from sowing, rice grains were collected and weighed. Different yields were obtained, depending on the treatment applied (Fig. 1). The highest yield was obtained when S2 of *A. brasilense* and urea were applied in the same treatment (35.25 ± 10.17 kg $block^{-1}$), overcoming the values as

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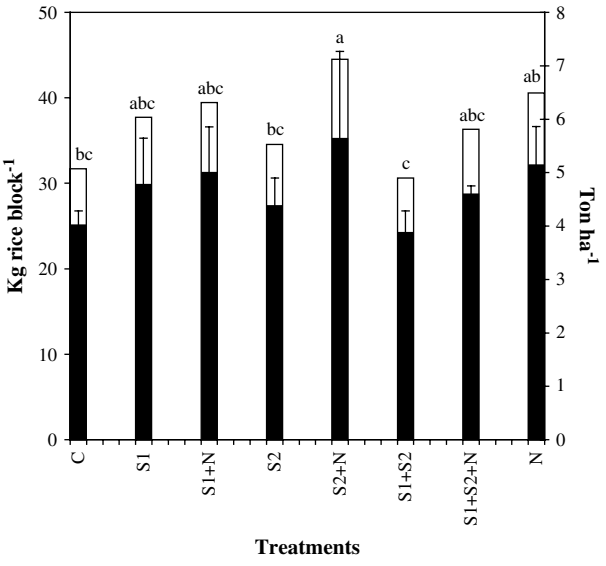

Fig. 1 – Grain yield obtained from a rainfed rice crop carried out in Tucumán, Argentina, considering *Azospirillum* inoculation and/or N-fertilization. Data shown as black columns are the means of four determinations and the error bars indicate SD (left axis). Different letters indicate significant differences at $P = 0.05$. The white part of each column represents estimated grain yield (right axis) in tons per hectare.S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; C, treatment without inoculation or N-fertilization; N, nitrogen applied as urea (50 kg N ha^{-1}).

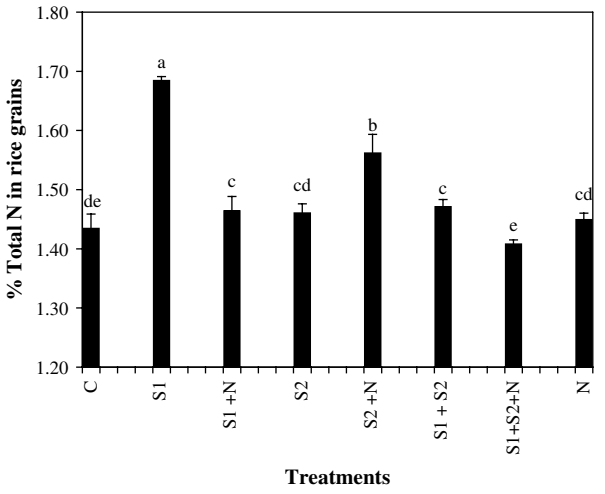

Fig. 2 – Total nitrogen content in rice grains, collected from a rainfed crop carried out in Tucuman, Argentina, determined by the micro-Kjeldahl method. Data are the means of three determinations and the error bars indicate SD. Different letters indicate significant differences at $P = 0.05$. S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; C, treatment without inoculation or N-fertilization; N, nitrogen applied as urea (50 kg N ha^{-1}).

when they were applied independently (27.37 ± 3.25 and 32.12 ± 4.49 kg block^{-1}, respectively). Both *A. brasilense* strains used separately as inoculum produced higher values of grain production compared to the control, without bacterial inoculation or N-fertilization, but when both strains were applied together a slight reduction in grain yield was observed (Fig. 1). Grain yield, expressed as tons per hectare, is also presented in Fig. 1.

Total nitrogen content in rice grains from every treatment was determined by the micro-Kjeldahl method, and results are shown in Fig. 2. The highest content was obtained when seeds were inoculated with *A. brasilense* S1 (1.68 ± 0.01%), with statistic significance ($\alpha = 5$%). When seeds were inoculated independently with both bacterial strains, the nitrogen content in grain was different, showing a better performance with S1 than S2 (1.68 ± 0.01 and 1.46 ± 0.01%, respectively). However, a reduction of nitrogen content was observed when S1 and urea were included together as treatment (1.46 ± 0.01%). Fertilization with urea did not improve the nitrogen content compared to the control, but when applied together with independent strains of *Azospirillum* improvement in nitrogen content was observed (Fig. 2). Curiously, when the treatment consisted of nitrogen fertilization and inoculation with both bacterial strains, the nitrogen content in grain was lower than the control (1.40 ± 0.01 and 1.43 ± 0.02%, respectively).

At grain filling stage, leaves were collected and the MPN of endophytic diazotrophic bacteria in different culture media was determined (Table 1). Aliquots from different samples were plated onto TSA medium to check the disinfection process and no bacterial growth was observed.

In general, the lowest MPN values were detected in control and nitrogen treatments (e.g., 0.7×10^1 and 2.5×10^1, respectively), whereas the highest values of cell counts were observed when rice plants were inoculated with S1 in absence or presence of urea as N-fertilizer (e.g., 9.5×10^4 and 4.5×10^5, respectively). The MPN varied according to the treatments and

Table 1 – MPN of endophytic bacteria from the rice phyllosphere grown in different N-free semisolid culture media

Treatments	MPN and growth culture media				
	NFb	JNFb	LGI	LGI-P	JMV
C	9.5×10^1Fa	2.1×10^1Gb	0.7×10^1Gd	1.2×10^1Gc	n.d.
S1	4.0×10^4Bb	9.5×10^4Ba	2.5×10^4Bc	2.5×10^4Bc	n.d.
S1 + N	9.5×10^4Ac	2.5×10^5Ab	9.5×10^4Ac	4.5×10^5Aa	n.d.
S2	9.5×10^2Db	9.5×10^2Db	1.5×10^3Da	2.5×10^2Ec	n.d.
S2 + N	2.5×10^2Ea	0.7×10^1Hb	0.4×10^1Hc	2.5×10^2Ea	n.d.
S1 + S2	2.5×10^2Eb	2.5×10^2Eb	4.0×10^2Ea	4.0×10^2Da	n.d.
S1 + S2 + N	4.0×10^3Cb	2.5×10^3Cc	9.5×10^3Ca	9.5×10^3Ca	n.d.
N	2.5×10^2Ea	9.5×10^1Fb	9.5×10^1Fb	2.5×10^1Fc	n.d.

MPN of bacterial cell per gram of fresh leaf was determined with McCrady's table for three replicates. The different growth culture media correspond to those explained in Section 2. Uppercase letters represent statistical differences in the column. Lowercase letters represent statistical differences in the row. n.d., bacterial growth not detected. S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; C, treatment without inoculation or N-fertilization; N, nitrogen applied as urea (50 kg N ha^{-1}).

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the differences in cells counts, observed by the growth in several culture media, reflects a response of diazotrophic endophytic bacteria populations present in tissues of rice leaves mediated by the inoculation and N-fertilization treatments. In Table 1, a great degree of statistical significance ($P \leq 0.05$) among treatments can be observed when analyzing the MPN in each culture medium, while this significance is minor when analyzing the MPN for each treatment among the different culture media.

Following bacterial purification from the highest dilution obtained with the MPN determination, nitrogenase activity was assessed by the reduction of acetylene to ethylene. As shown in Table 2, different values were obtained from cultures according to the N-free semi-solid growth media used, considered as semi-selective for different diazotrophs associated with inner tissues of rice leaves.

After superficial disinfection, total DNA from samples of leaf tissues was obtained by two protocols and quantified. The electrophoresis of DNA derived from both protocols showed that when PVP was present in the extraction buffer, a reduction of total DNA amount obtained from fresh leave tissues was observed (not shown). However, independent of the protocol used, amplification with primers for part of the *nifH* gene produced a single band of expected size (260 bp) from all tested samples and the positive control DNA from *G. diazotrophicus* (data not shown). These data confirm the presence of nitrogen-fixing bacteria associated to inner tissues of rice leaves, as previously indicated by bacterial growth in semi-solid N-free media (MPN determination) and ARA, with the exception of JMV medium where bacteria growth was not detected.

The DGGE patterns of the 16S rDNA PCR products derived from DNA extracted from rice leaves revealed different bacterial communities, according to the treatments and the method of DNA extraction. Some band patterns corresponded to the gel position of the controls used as reference (e.g., *Herbapirillum*, *Azospirillum*, *G. diazotrophicus* and *Burkholderia tropica*). Cultivable putative species isolated with the semi-selective N-free media previously used, seem to correlates with these data. However, any further conclusion will depend of sequencing data from each 16S rDNA fragment isolated from the gel. We also observed that the number of bands representative of bacterial communities detected by DGGE analysis were more abundant when the DNA was extracted only with CTAB (method A), while a reduction in the number of bands was observed when it derived from DNA extracted by the CTAB + PVP protocol (method B), as shown in Table 3. When DNA was extracted by the CTAB protocol, 188 bands could be observed on the gel, while when it was extracted with CTAB + PVP only 91 bands could be discriminated by DGGE analysis. The difference between the numbers of bands generated from both methods of DNA extraction corresponds to 97 bands. This represents an overall reduction of 51.6% of the DGGE potential to reveal the endophytic bacterial diversity from samples of rice leaves when the protocol using CTAB + PVP was used for DNA extraction. The percentage of common bands observed when DGGE profiles derived by amplification of DNA from both protocols varied among treatments. When DNA was derived from the CTAB + PVP protocol, the overall number of common bands observed in DGGE profiles represented almost 77% of the total bands generated by DGGE analysis of 16S rDNA from those DNA samples. Similarly, bands that were observed exclusively in DNA profiles derived from DNA extracted with the CTAB protocol (A) also varied among treatments, indicating that the percentage of bands exclusive to these DGGE profiles represents the potential of DGGE analysis to characterize the potential diversity associated within the phyllosphere of rice plants of all samples (Table 3).

The dendrogram generated by DGGE analysis showed that treatments with inoculation and inoculation plus N-fertilization influenced the diversity of endophytic bacteria

Table 2 – Nitrogenase activity of bacterial isolates obtained from inner tissues of rice leaves and grown in different N-free semisolid culture media

Treatment	ARA (nmol C_2H_4 h^{-1} culture^{-1})			
	NFb	JNFb	LGI	LGI-P
C	n.d.	12.7 ± 1.34	n.d.	n.d.
S1	27.08 ± 2.36	n.d.	23.38 ± 2.29	76.63 ± 5.56
S1 + N	n.d.	31.53 ± 6.27	n.d	11.55 ± 0.71
S2	22.95 ± 2.64	n.d	23.01 ± 3.08	17.12 ± 1.36
S2 + N	17.01 ± 3.61	18.65 ± 1.59	n.d.	13.91 ± 1.47
S1 + S2	n.d.	32.95 ± 6.43	30.93 ± 3.63	83.64 ± 0.38
S1 + S2 + N	19.91 ± 2.48	n.d.	n.d.	12.57 ± 2.44
N	n.d.	22.14 ± 2.85	n.d.	18.44 ± 2.63

Each value of ARA is the average of three replicates and its standard deviation. n.d., bacterial growth was observed but nitrogenase activity was not detected. S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; C, treatment without inoculation or N-fertilization; N, nitrogen applied as urea (50 kg N ha^{-1}).

Table 3 – Number of bands derived from DGGE profiles of 16S rDNA products, considering the two methods of obtaining total DNA from rice leaves

Treatments	A	B	No. of common bands[a]	% of common bands in B[b]	% of exclusive bands in A[c]
C	22c	11cd	8b	72.72e	63.63c
S1	25b	12bc	11a	91.66a	56.00d
S1 + N	22c	14ab	11a	78.57c	50.00e
S2	25b	16a	11a	68.75f	56.00d
S2 + N	25b	4e	3c	75.00d	88.00a
S1 + S2	28a	9d	7b	77.77c	75.00b
S1 + S2 + N	25b	14ab	12a	85.71b	52.00e
N	16d	11cd	7b	63.63 g	56.25d
Total values	188	91	70	76.92	62.76

DNA extraction methods: A, CTAB; B, CTAB + PVP. S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; C, treatment without inoculation or N-fertilization. N, nitrogen applied as urea (50 kg N ha^{-1}). Letters represent statistical differences among treatments.

a AB, number of common bands derived from amplification of DNA extraction methods A and B.

b Value calculated by (AB/B) × 100.

c Value calculated by (A − AB/A) × 100.

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in the phyllosphere of rice plants in comparison with the control or N-fertilization (Fig. 3). The formation of two clusters showing around 55% of similarity was observed. One cluster revealed that when N-fertilization was associated with the inoculation of rice with each strain, but independently, the endophytic bacterial populations showed 70% of similarity between both treatments. On the other hand, independent of the association with N-fertilization, the presence of both strains in the inoculation treatment influenced the bacterial diversity of rice phyllosphere to 60% of similarity. In comparison with the control, different levels of similarities were observed among the bacterial populations present in the endophyllosphere of rice plants subjected exclusively to inoculation with each strain (S1 or S2) or N-fertilized (Table 4). From these data we can see that inoculation of S1 influenced the bacterial community diversity associated with the endophyllosphere, while according to the S index when S2 was used as inoculant only a slight difference could be detected in the bacterial community.

Table 4 – Influence of inoculation and N-fertilization on the genetic diversity of endophytic bacterial community present in the endophyllosphere of rice plants, based on DGGE profiles

Treatments	S1	S2	S1 + S2	N	S1 + N	S2 + N	S1 + S2 + N
Sorensen index (S)	0.30	0.47	0.28	0.32	0.23	0.34	0.30

S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; and N, nitrogen applied as urea (50 kg N ha^{-1}). $S = 0$ indicates that two samples are completely different, whereas $S \geq 0.5$ indicates identical samples.

4. Discussion

In this work we assessed the *Azospirillum* inoculation and nitrogen fertilization effect on grain yield and on the endophyllosphere diversity of nitrogen-fixing bacteria in a rice crop assay carried out in Tucumán, Argentina. We selected the inner tissues of rice leaves, based on previous data of dynamic movement of endophytic bacteria from roots toward leaves of rice plants [12]. Besides, we consider that leaves not only have the potential to guarantee a selective environment, but also are appropriate to evaluate bacterial genetic diversity, based on their role as the main bioreactors for C-fixation, energy generation, oxygen control and other metabolisms. Most of the literature regarding rice crops associated to diazotrophs relies on submerged soil systems, as 85% of the world's rice production takes place under those conditions [27]; however, here we present data of rainfed rice production, depending only on the rainfall received during the cropping period.

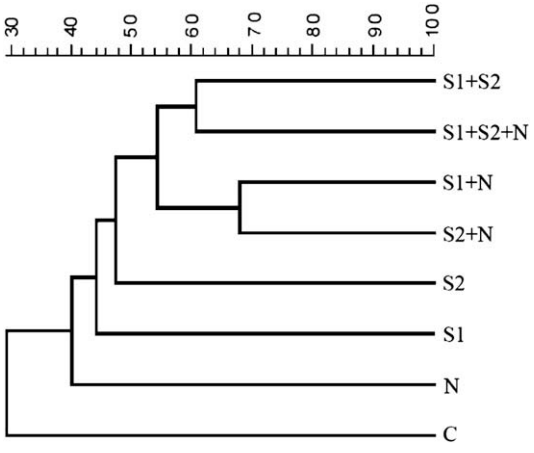

Fig. 3 – UPGMA dendrogram, based on the coefficient of Jaccard, illustrating the genetic relationships among the potential diversity of endophytic bacterial community of rice leaves according to *Azospirillum* inoculation and/or N-fertilization treatments. S1, *A. brasilense* REC3; S2, *A. brasilense* 13-2C; C, treatment without inoculation or N-fertilization; N, nitrogen applied as urea (50 kg N ha^{-1}).

In general, we have observed that grain yield was improved when inoculated with *Azospirillum* and/or fertilized with urea; although this value may not be very important when expressed as kg per block, its extrapolation to ton ha^{-1}, may be interesting in terms of economic value. For instance, the grain yield obtained under rainfed rice cropping in presence of N-fertilizer was 27.86% higher with respect to the control; however, the inoculation of S1 also contributed, with 18.92% improvement. This response depended on the bacterial genotype, as higher yield was obtained with S1 than with S2 when used independently. In combination with urea, S2 showed a better performance on grain yield, but for a still unknown reason, when both strains (S1 + S2) were inoculated simultaneously, and grain yield decreased, being lower than the control (without bacterial inoculation or N-fertilization). A similar behavior was observed in the highest total N-content in grain and the MPN of N_2-fixing bacteria when seeds were inoculated with S1 than with S2. In this context, we could speculate that inhabiting the interior of the rice leaves, these bacteria are thought to stay away from competition with bacteria of the rhizosphere and obtain nutrients directly from host plants [4,9,17,22]. In response, the plant interior (low in O_2 and relatively high in carbon source) provides an environment favorable to N_2-fixation, allowing the bacteria to transfer fixed N products efficiently to the host [22,40]. Considering the MPNs obtained herein, although showing statistical significance ($P \leq 0.05$), the contribution of inoculation to the amount of N-content present in rice plants could be interpreted as poor, although it must be taken into account that N_2-fixing bacteria could represent only part of the number and diversity generally reported by other authors [41,42]. Besides, considering that the bacteria associated to inner tissues of rice leaves could have contributed with part of the amount of fixed nitrogen in the grain N-content, under our experimental conditions the beneficial effect of inoculation could be due to the type of plant growth promotion associated with the strains inoculated. *A. brasilense* REC3 (S1) produces more phytohormones than *A. brasilense* 13-2C (S2), thus stimulating differentially the development of roots to explore a wider soil volume for obtaining nutrients, whether the soil was fertilized or not with urea. This latter is supported by data of dry root and shoot weight observed after 60 days of rice plant growth, using both strains [3], and by the root surface area enhancement of strawberry plants when inoculated with *A. brasilense* REC3 [43].

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For evaluating the impact of inoculation and N-fertilization over the genetic diversity, a fingerprinting approach was used to determine the communities associated to the rice endophyllosphere. The number and intensity of DGGE bands, used as a means to express the bacterial diversity associated to the endophyllosphere of rice, was higher when total DNA of the different treatments was obtained using only CTAB than when using CTAB + PVP. Perhaps, the use of PVP for DNA extraction from fresh leaf tissue interfered in the representativity of DNA from the bacterial community but not in the amplification process, affecting the presence of the amplified products but not its quality.

Comparing the community profiles using 16S rDNA-PCR DGGE from the different treatments and standards, bands putatively representing four genera of diazotrophs associated to inner tissues of rice leaves were detected (e.g., *Azospirillum*, *Herbaspirillum*, *Burkholderia* and *Gluconacetobacter*). Although bacterial growth was not detected in the semi-selective JMV culture medium used to isolate *Burkholderia*, in the DGGE patterns derived from total DNA of rice leaves we observed bands coinciding with the control, corresponding to *Burkholderia tropica*, although sequencing data are necessary to confirm this assumption. In this context, the 16S rDNA-PCR DGGE analysis proved to be a powerful tool for observing non-cultivable bacteria species, whether N_2-fixers or not, present in the bacterial community inhabiting inner tissues of rice leaves.

The DGGE profiles obtained revealed a depiction of stability in the dominating bands, which presumably represent numerically dominant species. This stability and genetic diversity measured by Sorensen's index was observed independently of the method used to extract total DNA from rice leaves. Interestingly, the genetic diversity associated with the endophyllosphere of rice plants was more affected in terms of S index when N-fertilization and S1 inoculation was associated with the treatment; on the other hand, it was less affected when S2 was applied, as compared with control. These data are related with the values of grain yield and N-content presented herein, suggesting that agronomic parameters of rice crop and the bacteria diversity are, indeed, affected by *Azospirillum* inoculation and/or N-fertilization.

The variability of patterns at the level of weaker bands might indicate that the underlying minority populations were more variable. However, the introduction of *A. brasilense* strains used independently as inoculants did not influence the dominant members of the endophytic microbial communities in the phyllosphere of the rainfed rice crop. It is known that microbial communities in active ecosystems may possess the capacity to maintain structural stability and consequently to blur effects of introduction of bacterial inoculant strains [20,29]. It was also reported that a comparison between bulk and rhizosphere soil revealed no significant differences in microbial community structure in rice fields, as evidenced by comparing groups and ratios of phospholipid fatty acids [37]. However, in the current study we could speculate that the dominating bacterial community living endophytically in the phyllosphere of rice is stabilized due to the apparent lack of competition, as can occur for, example, in the rhizosphere where root exudates play an important role in the bacteria–plant interaction, with consequences on the microbial community structure. However, this stability can suffer slight, but no less important, changes in microbial community structure associated with the endophyllosphere that can account for differences in the agronomic parameters of grain production in rice plants. Nevertheless, further studies on the bacterial genetic diversity in the endophyllosphere at different stages of rice growth, as affected by the environment, are necessary to gain new insights in understanding this complex interaction.

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