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# Antagonism between *Azospirillum brasilense* Az39 and *Pseudomonas oryzihabitans*, a seed-borne endophyte, in growing rice plants

## Antagonismo entre *Azospirillum brasilense* Az39 y *Pseudomonas oryzihabitans*, una endófita de semilla, en plantas de arroz

G. Rariz; L. Ferrando; N. Echegoyen; A. Fernández Scavino*

Área Microbiología, Departamento de Biociencias, Facultad de Química, Universidad de la República. General Flores 2124, Casilla de Correo 1157, (11800), Montevideo, Uruguay. *E-mail: afernand@fq.edu.uy, ana.fernandez.scavino@gmail.com

## Abstract

The interactions between native endophytic bacteria and inoculated beneficial bacteria in plant tissues are relevant to guarantee the success of commercial inoculants assuming that benefitial bacteria must persist associated to the plant for a certain period of time. In this study we examine whether *Pseudomonas oryzihabitans* is able to antagonize *Azospirillum brasilense* Az39, a Plant Growth Promoting Bacteria widely used in South America. Surface-sterilized rice seeds inoculated with high amounts of *P. oryzihabitans* G16 or *A. brasilense* Az39 or with both strains were grown under hydroponic conditions. Shoot and root biomass of 15 days-old inoculated-seedlings were compared to those of uninoculated seedlings. In addition, enumeration and identification of endophytic bacteria were performed on these seedlings. Heterotrophic and siderophore-producing bacteria isolated from seedlings were identified by 16S rRNA gene partial sequencing. *A. brasilense* and *P. oryzihabitans* were able to colonize rice plants, being predominant in the roots and shoots of the respective inoculated seedlings. In co-inoculated plants, only *P. oryzihabitans* was recovered. The siderophore-producing *Sphingomonas* sp. was predominant in uninoculated plants and was detected in all inoculated plants. The vegetal biomass was the lowest in *P. oryzihabitans*-inoculated plants and the highest in *A. brasilense* inoculated plants.This work shows that *P. oryzihabitans* antagonizes *A. brasilense* in plant tissues and decreases rice plant yield. Other seed-borne endophytes, particularly siderophore-producer bacteria of the genus *Sphingomonas*, are not outcompeted by *P. oryzihabitans.*

**Keywords:** Plant growth promotion; Co-inoculation; Plant colonization; Siderophore production.

## Resumen

Las interacciones entre las bacterias nativas endófitas y bacterias benéficas inoculadas en los tejidos vegetales son relevantes para garantizar el éxito de los inoculantes comerciales asumiendo que las bacterias benéficas deben persistir asociadas a la planta durante un cierto período de tiempo. En este estudio se analiza si *Pseudomonas oryzihabitans* es capaz de antagonizar *Azospirillum brasilense* Az39, una bacteria promotora del crecimiento vegetal ampliamente utilizada en América del Sur. Las semillas de arroz esterilizadas superficialmente, inoculadas con altas cantidades de *P. oryzihabitans* G16 o *A. brasilense* Az39 o de ambas cepas, se cultivaron en condiciones hidropónicas. Se comparó la biomasa de brotes y raíces de plántulas inoculadas con la de las plántulas no inoculadas al cabo de 15 días de crecimiento. Se cuantificaron e identificaron las bacterias endófitas presentes en esas plántulas. Las bacterias heterótrofas y productoras de sideróforos aisladas se identificaron mediante secuenciación parcial del gen 16S rRNA. *A. brasilense* y P. *oryzihabitans* fueron capaces de colonizar las plántulas de arroz, siendo predominantes en las raíces y brotes de las plántulas inoculadas con cada una de estas cepas. En plantas co-inoculadas sólo se recuperó *P. rysihabitans*. Las bacterias productoras de sideróforos pertenecientes al género *Sphingomonas* predominaron en plántulas no inoculadas y se detectaron en todas las plántulas inoculadas. Los valores más bajos de biomasa vegetal se encontraron en plántulas inoculadas con *P. oryzihabitans* y los más altos en plántulas inoculadas con *A. brasilense*. Este trabajo muestra que *P. oryzihabitans* antagoniza *A. brasilense* en tejidos vegetales y disminuye el rendimiento de la planta de arroz. Otras endófitas asociadas a semilla, particularmente las bacterias productoras de sideróforos del género *Sphingomonas*, no fueron antagonizadas por *P. oryzihabitans.*

**Palabras clave:** Promoción del crecimiento vegetal; Co-inoculación; Colonización de plantas; Producción de sideróforos.

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## Introduction

Cultivated rice (*Oryza sativa*) is the most important staple crop in the world. Irrigating rice is a highly water-consuming agronomic process that affects water quality due to the use of agrochemicals. To satisfy the increasing world demand for sustainable rice production, biotechnological alternatives have been proposed. Among of them, inoculation with Plant Growth Promoting Bacteria (PGPB) is a valuable tool to improve crop yield, minimizing the use of fertilizers and pesticides. This technology has been expanded worldwide, particularly in Latin American countries (Valverde *et al.,* 2015).

*Azospirillum* spp. is one of the PGPB most widely used by farmers to increase the yield of several crops. The ability of the species *Azospirillum* to stimulate plant growth has been attributed to the production of the auxin indole-3-acetic acid (IAA), gibberellins, polyamines and amino acids (Bashan and de-Bashan, 2010). *Azospirillum brasilense* and *A. lipoferum* have been commonly used in commercially available inoculants for a variety of crops, with strain *A. brasilense* Az39 being the most frequently employed in biofertilizers. Several reports indicate that inoculation with Az39 significantly improved the yields of many cereals (Fulcheri and Frioni, 1994; Cassán *et al.*, 2009; Zawoznik *et al.*, 2011; García de Salamone *et al.*, 2012), although other authors did not find such auspicious results (Piccinin *et al.*, 2011; Araújo *et al.*, 2013; Hungria *et al.*, 2013).

The observed variability in the crop responses to the use of *Azospirillum* spp. as biofertilizer can be attributed to the differences in the conditions that the strain has to overcome to be installed successfully in the plant. The plant-PGPB interaction is essential for an effective improvement in vegetal yield, and it could be influenced by biotic and abiotic parameters. Among the biotic parameters that affect the inoculation efficiency, the interaction between PGPB and the naturally occurring endophytic bacteria has rarely been studied. Endophytes are defined as microorganisms that can be isolated from surface-sterilized plant materials, inhabiting plant tissues without causing harm to them (Hallmann *et al.*, 1997). The competition between native endophytic bacteria and inoculated bacteria may reduce the activity of PGPB and consequently diminish crop yield. Thus, the success of the endophytic PGPB and the persistence of the plant-bacteria association depends on the ability of the inoculated bacteria to grow inside the plant tissues, where native endophytes can also grow.

The main sources of endophytes are the seeds and the rhizospheric soil from where certain bacteria can be attracted towards the roots by plant exudates. Seed-borne endophytes have been examined in different rice varieties and regions (Okunishi *et al.*, 2005; Mano *et al.*, 2006; Loaces *et al.*, 2011; Ruiza *et al.*, 2011). It was shown that endophytes are able to inhibit beneficial bacteria in a dual plate antagonistic assay. Such is the case of *Pseudomonas oryzihabitans*, a common species detected in rice (Cottyn *et al.*, 2009; Ruiza *et al.*, 2011; Hardoim *et al.*, 2012), for which at least one strain (G16) has been able to inhibit several species of *Azospirillum* spp. and *Herbaspirillum* spp. employed in commercial biofertilizers (Loaces *et al.*, 2011). It has been suggested that inside the plants there may exist antagonistic interactions among endophytic bacteria like to those described among the rhizospheric community. (Muñoz Rojas *et al.*, 2005). Moreover, *A. brasilense* was excluded from the rhizoplane of rice plantlets by endophytes of the genera *Bacillus* and *Corynebacterium* (Bacilio-Jiménez *et al.*, 2001). However, antagonism between PGPB and endophytes into vegetal tissues,including shoots, has not been explored.

The aim of this work was to determine if the seed-borne strain *Pseudomonas oryzihabitans* G16 is able to antagonize *A. brasilense* Az39 into plant tissues of rice. The seeds were inoculated or co-inoculated with similar amounts of these strains, and 15 days-old seedlings were analysed for endophytic bacterial abundance, and the predominant isolated bacteria were further characterized. Vegetal yield parameters were compared for plants with different inoculation treatments. In addition, seeds from other rice varieties were examined to determine whether *P. oryzihabitans* is a ubiquitous endophyte.

## Material and methods

### *Seeds sterilization*

Three different rice (*Oryza sativa*) varieties were used in this study: INIA-Olimar, El Paso 144 and INIA-Tacuarí. Seeds (5 g) of each variety were peeled and hydrated in 80 mL of sterilized distilled water for one hour. The seeds were surface-sterili-

zed by shaking in a solution of NaClO 1.7% for 5 min, followed by four, 3 min washes with 200 mL of sterile water. Sterility was tested by rolling the seeds on R2A plates, where no growth was observed after incubation at 30ºC for 48 h.

*Experimental design of the plant growth assay under hydroponic conditions*

The variety Olimar was used for this assay. Plants were grown in a climate-controlled chamber with 16 h of daylight at 25ºC and 80% relative humidity. A randomized complete block design with two blocks (or replicates) per treatment was used. Each replicate was composed of 14 tubes containing 2 plants per tube. The treatments consisted of seeds inoculated with: *Azospirillum brasilense* Az39 (Az39), *Pseudomonas oryzihabitans* G16 (G16), or co-inoculated with *A. brasilense* Az39 and *P. oryzihabitans* G16 (Az39+G16). Uninoculated seeds were used as control treatment (C). The blocks were distributed completely at random in the chamber.

*Inoculation of seeds*

Strains of *A. brasilense* Az39 and *P. oryzihabitans* G16 were grow by 18 h in Tripticase Soy Broth (TSB) at 30ºC. The inocula were diluted with fresh medium to 0.1 $OD_{600}$. The bacterial density was verified by plate counting in R2A as described below. The bacterial suspensions contained per mL: 2.4 x $10^7$ CFU (Az39), 1.3 x $10^8$ CFU (G16) and, for the co-inoculation treatment, 2.0 x $10^8$ CFU of Az39 and 1.0 x $10^8$ CFU of G16. These values are in the range of the bacterial density recommended for the application of commercial products with *A. brasilense* AZ39 (http://www.calister.com.uy/producto/bioprom/).

Approximately 100 seeds (2.5 g) were suspended in 50 mL TSB of each bacterial suspension. In the co-inoculation treatment the culture suspensions of Az39 and G16 strains were mixed immediately before the seeds were soaked into the mixed bacterial suspension. Non-inoculated seeds were suspended in sterile TSB. The seeds were shaken for 100 min to allow the penetration of bacteria into the seeds. The medium was removed and seeds were transferred to sterilized wet gauze pads in Petri dishes and incubated in the dark for 5 days at 30ºC for germination.

*Plants growth and yield*

Two germinated seeds were transferred aseptically to a 100 mL culture tube containing sterilized expanded perlite (Perliv®) and 30 mL of 1:2 diluted Yoshida medium (modified from Gregorio *et al.*, 1997). Hydroponic cultivation of plantlets was performed under gnotobiotic conditions for 10 days. After incubation, between 13 and 19 seedlings of each block were harvested, pooled and analysed. The dry weight of the entire plant was determined. Additionally, the fresh weight and length of the roots and aerial portions were measured.

*Counts of endophytic, heterotrophic, siderophore-producing and diazotrophic bacteria*

The roots and shoots of four seedlings from each replicate were analysed separately. Two hundred milligrams of each plant tissue was surface-sterilized by shaking for 5 min in 100 mL of NaClO 1.7 %. Four washes in 200 mL of sterilized distilled water were used to remove the disinfectant.

Each plant tissue sample was transferred to a mortar and homogenized in 4 mL of saline solution. The suspensions were serially diluted with sterile 0.9% NaCl and plated onto R2A for total heterotrophic bacteria (TH) counts or in R2A-CAS media (Schwyn and Neilands, 1987) modified by reducing the PIPES final concentration to 50 mM for siderophore-producing heterotrophic bacteria (SPH) counts. Plates were incubated at 30ºC for 72 h. Counts were expressed as the number of colony-forming units (CFU) per gram of fresh weight. The average number of bacteria among duplicated blocks and the standard deviation were calculated.

The density of endophytic diazotrophic bacteria was determined using the most probable number (MPN) counts in RMR medium (Elbeltagy *et al.*, 2001) without vegetal extract. Culture tubes showing turbidity and acetylene reduction (Loaces *et al.*, 2011) were considered positive for diazotrophs.

*Isolation, identification, and physiological characterization of endophytic bacteria*

*Endophytes from seedlings.* To identify the endophytes recovered from inoculated and non-inoculated seedlings, bacteria obtained from plate

counts on different media were isolated. Colonies with different morphology from the higher dilution counts in CAS-R2A and R2A media were purified and identified by 16S rRNA gene sequencing as described below.

Several seed-borne endophytes were further characterized. The isolates were tested for their ability to fix nitrogen, solubilize phosphate, and produce siderophores and indol acetic acid (IAA). Nitrogen fixation was evaluated by the acetylene reduction assay in 27 mL vials containing 18 mL of RMR semisolid medium. After incubation at 30ºC for 7 days in the dark, acetylene gas was added to the headspace atmosphere of the vials at a final concentration of 10% (v/v) and incubated at 30ºC. After 5 days of incubation, ethylene was determined by gas chromatography as described by Loaces *et al.*, (2011). *A. brasilense* Az39 was used as a positive control, and non-inoculated tubes were used as negative controls. Inorganic phosphate solubilization was confirmed using Pikovskaya agar (Pikovskaya, 1948) with cycloheximide (50 mg/l) after incubation at 30°C for 7 days. Siderophore-producing activity was detected on R2A-CAS medium by the orange-yellow halo around the colony. Indole acetic acid production was determined in medium containing 20 g/l of bacteriological peptone (Oxoid, Basingstoke, UK), 1.15 g/l $K_2HPO_4$, 1.5 g/l $MgSO_4.7H_2O$ and 2.5 mM of tryptophan (Merck, Darmstadt, Germany). After 12 h of incubation, 1 ml of supernatant was mixed with 1 ml of Salkowski reagent and analysed as it was described in a previous work (Loaces *et al.*, 2011). The analyses were performed in duplicate.

*Endophytes from seeds*

To examine the presence of *P. oryzihabitans* in other rice varieties relevant to Uruguay, 5 g of seeds of *Oryza sativa* varieties INIA-Tacuarí and El Paso 144 were peeled and surface-sterilized as described above. After maceration using a mortar, saline ten-fold dilutions were prepared and plated in R2A and R2A-CAS. Plates were incubated at 30ºC for 5 days. Colonies with a similar morphology to *P. oryzihabitans* G16 were purified, and DNA was extracted for further identification using 16S rRNA gene sequencing as described below. The isolates were tested for antagonistic activity against *A. brasilense* Az39 as it was described in a previous work (Loaces *et al.*, 2011).

*Molecular characterization of the isolates*

*16S rRNA gene amplification and sequencing.* The isolates were screened and identified by 16S rRNA gene analysis (Loaces *et al.*, 2011). The colonies were suspended in sterilized water, and the suspension was centrifuged (10 min at 15,000×g, 4ºC). DNA was extracted from the pellets using the Wizard Genomic DNA Purification Kit (Promega).

16S rDNA amplification was performed in 25 µL of reaction mixture that contained: 0.48 µM of primers (IDT, USA) 27f (5-AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5′-GGTTACCTTGTTACGACTT-3´), 0.2 mM of each dNTP (Fermentas), 2.5 mM $MgCl_2$, 0.25 mg/ml of bovine serum albumin (Roche), 2.5 µL of Taq buffer and 1 U of Taq DNA polymerase (Fermentas). PCR amplification was performed on 1 µL of DNA template in an automated thermal cycler (2720 Thermal Cycler, Applied Biosystems) with an initial denaturation (94ºC for 5 min) followed by 30 cycles of amplification (94ºC for 1 min, 55ºC for 1 min, and 72ºC for 3 min) and a single final extension (72ºC for 7 min).

16S rRNA gene amplicons were screened by ARDRA (Amplified Ribosomal DNA Restriction Analysis) to select representative ribotypes of all the isolates. PCR products were digested with 3 units each of *Msp*I (Fermentas) and *Rsa*I (Fermentas) at 37ºC for 10 h. The restriction fragments were separated on a 3.5% agarose (Methaphor, FMC, USA) gel running in 0.5 × TBE buffer for 1 h at 100 mV and stained with ethidium bromide. A computer-assisted evaluation of the ARDRA patterns generated was made using the GelCompar 4.2 program (Applied Maths, Kortrijk, Belgium). Restricted amplicons were grouped into operational taxonomic units (OTUs) based on these restriction patterns.

Representative ribotypes according to the ARDRA profile were selected, and the 16S rRNA genes were partially sequenced using the primer sets described above. All sequencing reactions were performed by the Macrogen Sequencing Service, Korea using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, Foster City, USA). The sequences were submitted to the National Center for Biotechnology Information database, and the closest relative sequence was used for identification.

The sequences of the isolates obtained from

seeds of the varieties INIA-Tacuarí and El Paso 144 are available at the GenBank database with the accession numbers: KM225652 to KM225657.

*nifH gene amplification***.** All of the isolated strains were tested for the presence of the nifH gene. Primers previously described (Zehr and McReynolds, 1989; Poly *et al.*, 2001) were used for screening. The nifH gene amplification and PCR conditions for the primer set designed by Poly *et al.* (2001) were performed according to a previous work (Ferrando and Fernández Scavino, 2015). PCR reactions using the primer set from Zher and McReynolds (1989) (nifHZMRf, 5'-TGYGAYC-NNAARGCNGA-3´; nifHZMRr, 5'-ADNGC-CATCATYTCNCC-3´) were performed in 25 µL (total volume) mixtures containing approx. 100-200 ng of total DNA, 0.1 mmol/L of each primer, 2.5 mmol/L $MgCl_2$, Taq buffer, Bovine Serum Albumin 0.2 mg/mL (Roche®), 0.2 mmol/L of each dNTP and 1.2 U of Taq DNA polymerase (Fermentas©). The reactions were performed in an Applied Biosystems 2720 Thermal Cycler (Singapur) using the following program: initial denaturation step at 94ºC for 5 min, followed by 30 cycles at 94ºC for 60 s, 57ºC for 60 s and 72ºC for 60 s, with a final extension step at 72ºC for 10 min. The amplicons obtained were confirmed by electrophoresis on 1% agarose gel (Agarose I, Amresco) at 100 V.

*Statistical analyses*

Statistical analyses were performed using InfoStat (Di Renzo *et al.*, 2009). Plate counts (HB and HSPB) and vegetal yield data were subjected to analysis of variance (ANOVA). Differences were considered significant at the 95% (of higher) confidence level. Data related to the CFU were transformed into logarithmic values before statistical analysis.

## Results

*Effect of inoculation on plant yield*

Plant growth was affected by the inoculated bacteria. After 15 days of seed inoculation, shoot fresh weight was significantly higher in seedlings inoculated with strain Az39 than in seedlings inoculated with strain G16 (Table 1). Additionally, the same trend was observed for most of the parameters measured, with Az39 stimulating growth and G16-inoculated seedlings showing the lowest yield values. Non-inoculated or co-inoculated seedlings showed intermediate values for all of the parameters measured.

*Bacterial abundance in non-inoculated and inoculated plants*

Total heterotrophic (TH) and siderophore-producing heterotrophic (SPH) bacteria were counted in the shoots and roots of seedlings after 15 days of incubation to compare the population density of endophytic bacteria in different inoculation treatments.

The abundance of the total heterotrophic bacteria expressed as the log CFU/g fresh tissue was between 5.2 and 6.9 in shoots and between 7.5 and 9.2 in roots of all seedlings. Non-inoculated plants exhibited a high density of heterotrophic bacteria with counts that were not significantly different from the co-inoculated or G16-inoculated plants. These results indicate that seed-borne bacteria were able to grow in the roots and shoots of rice, reaching similar densities as those of inoculated plants.

Inoculation with *Azospirillum* significantly in-

**Table 1.** Effect of inoculation with different bacterial strains on the growth of *Oryza sativa* seedlings after 10 days of incubation under hydroponic conditions. Seeds were inoculated with *Azospirillum brasilense* (Az39), *Pseudomonas oryzihabitans* strain G16 (G16), *Azospirillum brasilense* + *Pseudomonas oryzihabitans* (Az39+G16) or were not inoculated (control). These values represent an average of two blocks (between 13 and 19 plants each) ± SD.

| Treatment | Root | | Shoot | | Plant |
|---|---|---|---|---|---|
| | Fresh weight (mg) | Length (cm) | Fresh weight (mg) | Length (cm) | Dry weight (mg) |
| Az39 | 57.27 ± 16.15 | 3.73 ± 1.33 | 48.36 ± 13.51 a | 12.62 ± 4.49 | 14.49 ± 5.47 |
| G16 | 41.67 ± 20.70 | 2.23 ± 1.79 | 27.83 ± 19.35 c | 8.19 ± 6.05 | 10.26 ± 6.25 |
| Az39 + G16 | 52.82 ± 16.43 | 3.10 ± 1.18 | 31.18 ± 15.55 b | 9.86 ± 4.37 | 12.81 ± 5.56 |
| Control | 54.15 ± 19.47 | 3.65 ± 1.69 | 37.46 ± 21.00 b | 10.28 ± 5.79 | 15.05 ± 4.27 |

Means followed with a different letter indicates significant differences ($p < 0.05$).

creased the heterotrophic bacteria in roots compared with non-inoculated plants (Figure 1), suggesting that Az39 could grow or increase the population size of seed-borne endophytes in roots. Strain G16 also increased the bacterial counts, although the values were not significantly different from other treatments. On the other hand, co-inoculated and non-inoculated seedlings showed a similar density of heterotrophic bacteria, indicating that mixed inoculation did not produce a significant increase of heterotrophic endophytes.

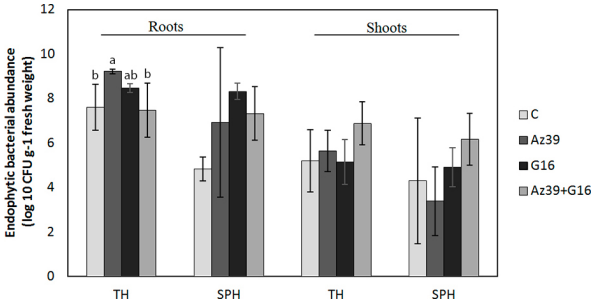


**Figure 1.** Abundance of total heterotrophic (TH) and siderophore-producing heterotrophic (SPH) bacteria in the roots and shoots of seedlings after 15 days of incubation. Error bars represent the standard deviation. Different letters indicate significant differences ($p < 0.05$).

R2A-CAS medium was used to improve the recovery of the inoculated strains, especially because non-siderophore producing seed-borne endophytes were abundant, even in inoculated plants (Fig. 1). Siderophore producing bacteria were in the range of 3.4 - 6.2 log CFU/g in shoots and 4.8 - 8.3 log CFU/g in roots. SPB were the lowest in non-inoculated plants, although no significant differences were observed among treatments in the roots or shoots. Since strains Az39 and G16 produce siderophores, the high density of SPB observed in the inoculated plants was not surprising.

Diazotrophic bacteria were below the detection limit of the method (60 MPN diazotrophs/g fresh weight) for non-inoculated and G16-inoculated plants. These results indicate that most of the seed-borne endophytes were not able to fix $N_2$. In Az39-inoculated seedlings, the abundance of diazotrophs (MPN/g fresh weight) was 4.6 x $10^2$ in the shoots and 4.8 x $10^4$ in the roots for both replicates. In co-inoculated plants, diazotrophs were only recovered from the shoots of one block (8.0 x $10^1$/g fresh weight), suggesting that inoculation with strain G16 decreased the density of Az39.

*Identification of endophytic bacteria*

The bacteria recovered from R2A and R2A-CAS count plates were purified and sixty isolates were selected for molecular characterization. The genetic profiles of these isolates were screened by ARDRA, and eight different OTUs were obtained. Several isolates representative of each OTU profile were identified by 16S rRNA gene partial sequencing.

*Endophytic bacteria from inoculated seedlings*

The predominant bacteria recovered from inoculated plants were the bacteria inoculated, except in co-inoculated seedlings. Table 2 shows the density of the inoculated bacteria, when recovered, in inoculated plants. In single-inoculated seedlings *A. brasilense* or *P. oryzihabitans* were the most abundant bacteria recovered. However, in co-inoculated plants, *A. brasilense* was not recovered from R2A or R2A-CAS count plates and *P. oryzihabitans* was the most abundant heterotrophic bacteria detected in both vegetal tissues.

*Seed-borne endophytic bacteria*

The dominant species recovered in both replicates from uninoculated seedlings was *Sphingomonas yabuuchiae*. In these plants, the species reached counts of 7.5 ± 1.1 log CFU/g fresh weight of roots and 5.2 ± 1.4 log CFU/g fresh weight of shoots. Furthermore, this species was present in

**Table 2.** Abundance of inoculated bacteria in roots and shoots of inoculated plants with *Azospirillum brasilense* (Az39), *Pseudomonas oryzihabitans* (G16) and *Azospirillum brasilense* + *Pseudomonas oryzihabitans* (Az39-G16). Values are the mean of two replicated blocks ± SD.

| Inoculation treatment | Species detected | Abundance | |
|---|---|---|---|
| | | Roots | Shoots |
| Az39 | *Azospirillum brasilense* | 6.7 ± 0.1 | 2.4 ± 0 |
| G16 | *Pseudomonas oryzihabitans* | 8.4 ± 0.1 | 5.1 ± 1.0 |
| Az39-G16 [1] | *Pseudomonas oryzihabitans* | 7.5 ± 1.2 | 6.7 ± 1.2 |

[1] *Azospirillum brasilense* was not recovered in any media or dilution from the co-inoculated plants.

shoots and roots of all inoculated plants (Table 3), indicating that this seed-borne endophyte was not excluded by competition by *P. oryzihabitans* or *A. brasilense*.

The seed-borne endophytes isolated from inoculated seedlings were in lower densities than *A. brasilense* or *P. oryzihabitans*. Members of the genus *Pantoea* were less ubiquitous than *S.yabuuchiae*, being detected in non-inoculated (OTU B) or *P. oryzihabitans*-inoculated (OTU J) seedlings (Table 3). Bacteria from the genera *Methylobacterium*, *Microbacterium* and *Chryseobacterium* were also isolated, but usually they were restricted to one treatment or to one block of one treatment.

*Pseudomonas oryzihabitans* (strain ED1) was recovered from the roots of uninoculated plants in one of two replicated blocks with a density three orders lower than the predominant *S. yabuuchiae*.

*Physiological characterization of seed-borne endophytic bacteria*

Four strains of seed-borne endophytic bacteria were further characterized regarding their physiological traits relevant for plant promoting growth or ability to outcompete *A.brasilense* or *P.oryzihabitans*. None were able to fix $N_2$, and the nifH gene was not detected. However, most of the isolates exhibited other valuable properties, such as phosphate solubilization, siderophore production and IAA production (Table 4). Interestingly, the highest IAA producer was the ubiquitous endophyte: *Sphingomonas yabuuchiae*.

**Table 3.** Identification and localization of seed-borne endophytic bacteria. ARDRA profile group of each isolated strain (OTU); Treatments: Control (C), *Azospirillum brasilense* (Az39), *Pseudomonas oryzihabitans* (G16), *Azospirillum brasilense* + *Pseudomonas oryzihabitans* (Az39-G16).

| Strain | OTU | Identity | | Source | |
|---|---|---|---|---|---|
| | | Closest sequence (Accession number in GenBank) | % Similarity | Treatment | Plant tissue |
| A9, A13, B9, C2, D7, D11-D14, D16, DRII | A | *Sphingomonas yabuuchiae* GTC 868(T) (AB071955) | 99.4-100 | C, Az39, G16, Az39-G16 | Roots Shoots |
| B2, B3 | A | *Sphingomonas pseudosanguinis* G1-2(T) (AM412238) | 100 | Az39-G16 | Roots |
| C1, C10, C11, C13 | J | *Pantoea anthophila* LMG 2558(T) (EF688010) | 98.7 | G16 | Roots Shoots |
| D3, D8, D10 | B | *Pantoea eucrina LMG* 2781(T) (EU216736) | 100 | C | Roots Shoots |
| A11, A12, D4 | K | *Methylobacterium platani* PMB02(T) (EF426729) | 97.8 | Az39, G16 | Roots Shoots |
| D5, D15 | D | *Microbacterium testaceum* DSM 20166(T) (X77445) | 99.9 | C | Roots Shoots |
| ED1 | E | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 100 | C | Roots |
| A14 | H | *Chryseobacterium haifense* H38(T) (EF204450) | 98.1 | Az39 | Shoots |

**Table 4.** Plant growth promoting (PGP) properties of several seed-borne endophytes. Indole-3-acetic acid (IAA). IAA production was measured by duplicated, the mean value and SD are shown.

| Strain | Identification | PGP property | | |
|---|---|---|---|---|
| | | Phosphate solubilization | Siderophore production | IAA production (µg/mL.DO) |
| DRII | *Sphingomonas yabuuchiae* | + | + | 26.0 ± 1.7 |
| C10 | *Pantoea antophila* | + | + | 9.1 ± 0.6 |
| D10 | *Pantoea eucrina* | + | + | 15.7 ± 2.4 |
| ED1 | *Pseudomonas oryzihabitans* | + | + | 17.0 ± 9.1 |

*Detection of* P. oryzihabitans *in seeds of different rice varieties*

Seeds of *O. sativa* var. INIA-Tacuarí and El Paso 144 were screened for bacteria morphologically similar to *P. oryzihabitans* G16. The isolates obtained in R2A-CAS plates were purified and identified by 16S rRNA gene partial sequencing (Table 5).

Several siderophore-producing bacteria similar to *P. oryzihabitans* G16 were isolated from seeds of both varieties. These strains could be identified as *P. oryzihabitans*, very closely related to the type strain (NBRC 102199). All of them, except strain 1B, were able to inhibit *A. brasilense* Az39 in agar plates.

## Discussion and conclussions

The development of inoculant technology is a valuable alternative to improve crop productivity and sustainability of agriculture since inoculants reduce the utilization of chemicals, minimizing pollution and the consumption of non-renewable energy sources. Moreover, the use of native PGPB contributes to the valorization of regional genetic resources and enhances the probability of adaptation of the local biota, especially to address the stress conditions imposed by climate change.

The challenges to expanding this technology lie in the better understanding of the influences of the biotic and abiotic parameters on the biological interactions involved. The success of the inoculation is mainly attributed to the early fitting of PGPB to the plant. However, this is not a simple two-partner relationship since a complex community of microorganisms from the seeds and soil influence the colonization and activity of the PGPB. Thus, the agronomic potential of PGPB depends on the density and activity of a diverse group of microorganisms that can be synergic, neutral or antagonistic to the inoculated bacteria.

In this work, a gnotobiotic experiment was set up to determine the effect of the inoculation of similar amounts of a seed-borne rice endophytic bacteria, *P. oryzihabitans*, on *A. brasilense*-inoculated seedlings. The effects of the inoculation or co-inoculation on plant yield and on the native endophytic bacterial community were also determined.

The impact of the inoculation on plant growth was studied for the whole plant, but significant differences were observed only in the shoot biomass. *A. brasilense* Az39 had a growth promotion effect, whereas *P. oryzihabitans* G16 was deleterious for plant development. Co-inoculated seedlings exhibited similar vegetal yield values as non-inoculated seedlings, suggesting that the opposite effects caused by strains G16 and Az39 were neutralized when both strains were inoculated at high densities. The ability of *A. brasilense* Az39 to promote vegetal growth has been well documented in field trials (Fulchieri and Frioni, 1994; Cassán *et al.*, 2009; Zawoznik *et al.*, 2011; Masciarelli *et al.*, 2013; Díaz-Zorita *et al.*, 2015; Pereg *et al.*, 2016), but the harmful effect of members of the

**Table 5.** Identification of seed-endophytic bacteria closely related to *Pseudomonas oryzihabitans.*

| Strain | GenBank accession number | Closest relative (GenBank accession number) | % Similarity | *Oryza sativa* variety |
|---|---|---|---|---|
| 1B | KM225652 | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 100 | INIA-Tacuarí |
| 2a | KM225653 | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 100 | INIA-Tacuarí |
| 4a | KM225654 | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 100 | INIA-Tacuarí |
| G1c | KM225655 | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 99.7 | El Paso 144 |
| G4 | KM225656 | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 99.9 | El Paso 144 |
| G7c | KM225657 | *Pseudomonas oryzihabitans* NBRC 102199(T) (BBIT01000012) | 99.9 | El Paso 144 |

species *P. oryzihabitans* on plant growth has not been reported. Although bacteria closely related to this species are common endophytes of rice seeds and plants (Cottyn *et al.*, 2009; Ruiza *et al.*, 2011; Hardoim *et al.*, 2012; Hameed *et al.*, 2015), the harmful effect may only be observed when this microorganism is a prominent member of the endophytic community.

Non-inoculated and inoculated seedlings harboured a high density of heterotrophic and siderophore-producing bacteria in the roots and shoots (Figure 1). The high density of endophytes in the control plants indicates that the seed-borne endophytes were able to grow *in planta*, reaching similar densities as in the inoculated plants. Moreover, these results showed that, irrespective of the inoculation, endophytes in seedlings incubated under gnotobiotic conditions can be as high as in seedlings cultivated in soil (Mano *et al.*, 2007; Ferrando *et al.*, 2012; Hameed *et al.*, 2015).

Inoculation with *A. brasilense* Az39 significantly increased heterotrophic counts compared with non-inoculated or co-inoculated roots (Figure1). *A. brasilense* was the predominant species identified in these seedlings (Table 2), suggesting that strain Az39 was able to overgrow the population of seed-borne endophytes in the roots and shoots. Similarly, *P.oryzihabitans* was predominant in the shoots and roots of G16-inoculated plants. These results confirm that both species are endophytes capable of root and shoot colonization and, when inoculated in high amounts, they can prevail over the seed-borne endophytes.

Unexpectedly, only *P. oryzihabitans* was retrieved as the predominant species in the shoots and roots of co-inoculated plants (Table 2). In agreement with these results, whereas diazotrophs were quantified in the shoots and roots of Az39 inoculated plants, only the shoots of plants from one replicate was positive for diazotrophs in co-inoculated plants. Since *P. oryzihabitans* G16 does not have the ability to fix nitrogen and the seed-borne diazotrophs were below the quantification limit, positive tubes in diazotrophs counts can be attributed to the presence of *A. brasilense*. These results indicate that *P. oryzihabitans* G16 overgrew *A. brasilense* Az39 in plant tissues when the seeds of rice were co-inoculated with similar amounts of both strains. Antagonism of *P. oryzihabitans* G16 towards PGPB has been previously observed in agar plates (Loaces *et al.*, 2011), but this activity has not been proved *in planta*. Noticeably, there are many commercial inoculants with combined formulations composed of *A. brasilense* and biocontrol agents belonging to the genus *Pseudomonas*, particularly *P. fluorescens* (Valverde *et al.*, 2015). The combination of phytobeneficial microorganisms with different metabolic capacities is frequent in commercial inoculants, but their mutual exclusion has been scarcely tested. This is particularly relevant since many mechanisms of biocontrol towards plant pathogens could be the same as those against beneficial bacteria. It has been shown that *Pseudomonas fluorescens* decreased the abundance of *A. brasilense* and its phytostimulation capability in wheat roots (Couillerot *et al.*, 2011). These authors found that the antimicrobial compound 2,4-diacetylphloroglucinol (DAPG) produced by *Pseudomonas* is responsible for such inhibition. It was also observed that different species of *Azospirillum* can be resistant to DAPG, although all of them decayed in the rhizosphere of maize when they were co-inoculated with *P. fluorescens* F113 (Couillerot *et al.*, 2013). Thus, the interaction between *Azospirillum* spp. and *P. fluorescens* could be complex and require more than one compound.

Although *P. oryzihabitans* G16 was isolated from seeds of the variety INIA-Olimar (type Indica) of *O. sativa*, strains of this species were isolated from seeds of a variety of the same type (El Paso 144) or of the type Japonica (INIA-Tacuarí) (Table 5). The six strains identified in this work produced siderophores, and five of them were able to antagonize *A. brasilense* Az39 in an agar plate assay. Additionally, *P. oryzihabitans* was detected in non-inoculated seedlings incubated under hydroponic conditions (strain ED1, Table 3), indicating that this microorganism can grow in plant tissues and can be translocated to new seeds and thus be preserved as endophyte with the reproductive cycle of the rice. It was postulated that seeds are a source of bacteria that can be dispersed into rhizospheric soil (Hardoim *et al.*, 2012). Our results showed that bacteria closely related to strain *P. oryzihabitans* G16 were widespread among rice varieties cultivated in Uruguay, kept in seeds and probably dispersed by rice-rhizospheric soil.

Antagonism between *A. brasilense* and seed-borne Gram positive bacteria in rice rhizoplane of 15 days-old plantlets has been observed by Bacilio-Jiménez *et al.* (2001), although the mechanism involved has not been explored. These authors observed that the root surface was coloni-

zed preferentially by endophytes from the genera *Bacillus* and *Corynebacterium*, which promoted the growth of roots, whereas *A.brasilense* inhibited the growth of rice. In our case, strains of *P. oryzihabitans* that antagonize *A. brasilense* in vitro showed a high ability to capture $Fe^{+3}$. Thus, as it has been reported for many bacteria, siderophore production could be a plausible mechanism to enforce biocontrol capabilities (Miethke and Marahiel, 2007; Fernández and Pedraza, 2013). Furthermore, we observed that the iron-mediated competence of strain G16 did not suppress the growth of endophytes with a noticeable ability to produce siderophores (Table 3 and Table 4). Members of the genus S*phingomonas*, prominent in non-inoculated plants, were found in all treatments, suggesting that these bacteria have the highest fitness to *O. sativa* during the early steps of plant growth, even when high amounts of other inoculated bacteria were present. Bacteria from the genus *Sphingomonas* have been reported previously as endophytes of rice (Ferrando *et al.*, 2012; Zhang *et al.*, 2013). *Pantoea* sp. were also present in uninoculated plants and able to thrive in plants inoculated with strain G16 (Table 3). This genus has been frequently found among the culturable endophytic bacteria in rice (Verma *et al.*, 2004; Mano *et al.*, 2006; Prakamhang *et al.*, 2009; Loaces *et al.*, 2011). Interestingly, bacteria from both genera also showed traits involved in vegetal growth promotion, such as the capability to solubilize inorganic phosphate or to produce IAA (Table 4). Therefore, siderophore-producing seed-borne bacteria with phytostimulatory properties were not excluded from plant tissues by inoculation with hign amounts of *P. oryzihabitans* G16.

In summary, the present work shows that high amounts of the strain G16 of *P. oryzihabitans*, an endophytic species common in rice seeds, can damage young plants when present at high densities. In these conditions, *P. oryzihabitans* outcompetes *A. brasilense* Az39, the most commonly used strain in commercial inoculants for cereals in South America. On the other hand, the antagonistic capability of *P. oryzihabitans* seems to be selective, since other seed-borne endophytes, such as *Sphingomonas* sp. or *Pantoea* sp., which are strong siderophore producers, were not inhibited *in planta*. Moreover, high amounts of *A. brasilense* Az39 stimulated the growth of young plants and prevailed over the harmful *P. oryzihabitans* present in low amounts in rice seeds. Therefore, the stimulation of *A. brasilense,* commonly attributed to phytohormone production, could also, or alternatively, be due to the modulation of the native, potentially detrimental, bacteria associated with the plant.

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