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Plant-Growth Promotion of Argentinean Isolates of *Azospirillum brasilense* on Rice (*Oryza sativa* L.) Under Controlled and Field Conditions

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Abstract: *Azospirillum* sp. has been shown to be a valuable resource to stimulate the growth and yield of diverse crop species. However, successful plant growth promotion (PGP) upon inoculation often depends on combining proper bacterial strains and plant genotypes. In this study, we isolated and characterized four new *A. brasilense* strains from diverse regions of Argentina and evaluated their PGP effect on a rice commercial local genotype under both controlled and field conditions. Under controlled conditions, most of the *Azospirillum* strains analyzed promoted plant growth, as evidenced by a significant increase in shoot height and root length and in some cases in dry weight. Strains Az24 and Az72 were the most effective. Unexpectedly, strain Az23, which was recovered from a rice productive field was a poor growth promoter of rice seedlings. Under field conditions, none of the parameters measured were statistically different from those of the control group. This lack of statistical significance might be attributed to the high dispersion of the data, which is expected in field experiments. In sum, our results suggest that *Azospirillum* promotes rice growth as a result of a non-specific plant-bacterium interaction.

Key words: *Azospirillum* · Rice · PGPR · Inoculation

INTRODUCTION

The genus *Azospirillum* is composed of rod-shaped Gram-negative diazotrophic bacteria able to colonize the rhizosphere and roots of plants. Members of this genus can be found in diverse geographic regions, associated with plants of many families [1], including cereals, forage grasses and cactuses [2]. Due to their nitrogen-fixing and plant growth-promotion (PGP) capability, *Azospirillum* species have long been studied as potential agricultural bio-fertilizers [3-7]. This has led to the biotechnological development of commercial inoculants for the agro-industry [8-10]. In Argentina, most of the *Azospirillum*-based commercial inoculants are composed of the *Azospirillum brasilense* strain Az39, isolated from the rhizosphere of wheat (*Triticum aestivum*) grown in the province of Córdoba [11].

It has been demonstrated that the synthesis and/or metabolization of plant growth regulators is an important mechanism underlying *Azospirillum* PGP effects [3]. These compounds include indole-acetic acid (IAA), gibberellins, cytokinins, ethylene [9, 12-16], abscisic acid and the diamine cadaverine [17]. However, many other factors may influence PGP effects during the plant-bacterium interaction. For example, the host genotype is an important factor determining the positive influence of *Azospirillum* on plant growth [18-19]. Some authors have stated that successful PGP upon inoculation depends on combining proper bacterial strains and plant genotypes [20-24], whereas others have proposed that *Azospirillum* PGP is non-specific since it positively influences the growth of a great diversity of plant species, but that particular bacterial strains may show greater affinity for certain plant host genotypes [1, 25].

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Rice (*Oryza sativa* L.) is one of the most important cereal crops worldwide. In Argentina, the main rice paddies are in Corrientes, Chaco, Formosa, Entre Ríos and Santa Fe provinces. Several studies on plant-associated microbial diversity have shown that rice rhizosphere and tissues contain a huge variety of heterotrophic bacteria including *Azospirillum*, *Herbaspirillum*, *Burkholderia, Bacillus* and *Pseudomonas* species [26-28]. When used for inoculation, some of the *Azospirillum* strains isolated from rice tissues or other plants have led to increased growth and yield of different commercial genotypes of rice. However, the results obtained in the field have been variable and dependent on the correct combination of rice genotype and bacterial strain [29].

In the present work, we isolated and characterized five *A. brasilense* strains from diverse regions of Argentina and evaluated their PGP effect on a rice commercial local genotype under both controlled and field conditions.

MATERIALS AND METHODS

Isolation and Molecular Characterization of *Azospirillum* Sp. Strains: New *Azospirillum* sp. strains were isolated either from soil samples collected from different regions of Argentina or from alfalfa (*Medicago sativa* L.) nodules (Table 1). The *A. brasilense* reference strain Az39 was deposited at the Instituto de Microbiología y Zoología Agrícola (IMYZA) INTA PGPB Collection, INTA Castelar, Buenos Aires, Argentina.

For soil bacteria isolation, 10 g of the sample and 90 mL of sterile saline solution were mixed in 500-mL flasks and incubated at room temperature with shaking at 150 rpm. After 15 minutes, 100 µl of ten-fold serial dilutions of the samples in sterile saline solution were spread on *Azospirillum* selective RC medium [30]. After incubation for 6 days at 30°C, dishes were examined for colonies.

Soil samples were recovered from a productive rice field of Concepción del Uruguay city (Entre Ríos province), from the rhizosphere of Bahía grass (*Paspalum notatum*) grown in Mercedes city (Corrientes province) and from an agricultural field of Chaco province subjected to rotation of wheat (*Triticum aestivum* L.) and cotton (*Gossypium hirsutum* L.). The fourth sample consisted of nodules of alfalfa grown in Tilcara city (Jujuy province). After tissue homogenization, *Azospirillum* sp. strains were isolated in selective RC medium and purified through successive culturing. Four of the isolates recovered, named Az23, Az24, Az72 and Az74 (Table 1), were used for further characterization along with the reference strain Az39 [17].

The isolation from nodules was carried out according to Vincent [31]. Plant tissue was superficially disinfected by treating with calcium hypochlorite for 1 minute and washing with sterile distilled water four times. The disinfected tissue was homogenized in 9 mL of sterile distilled water and spread on yeast-mannitol-agar (YMA) plates, which were then incubated at 28°C. After 7 days, dishes were examined and *Azospirillum*-like colonies were subcultured in RC medium for further selection and purification.

The isolates were genotyped by 16S rDNA sequencing. A 16S rDNA gene fragment was PCR-amplified using the fD1short (5'-agagtttgatcctggctcag-3') and rD1short (5'-aaggaggtgatccagcc-3') primers [32], separated by agarose gel electrophoresis, purified with QIAEX II gel extraction kit (Qiagen) and sequenced. The resulting sequences (GenBank accession codes KC920686 to KC920689) were compared to other sequences available in the database using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/). Further phylogenetic analyses were carried out with the software MEGA 5.2 [33] by the Maximum Likelihood method using the Tamura-Nei substitution model and a bootstrap phylogeny test of 1000 repetitions.

Bacterial Growth and Seed Inoculation: Bacteria were grown in N-free malic acid broth [34] at 30°C and shaking at 180 rpm. After 48 hours, the number of viable cells of each culture was quantified by colony count in RC media seeded with 100 µl of 1:10 serial dilutions and incubated at 34°C for 6 days. Afterwards, all the cultures were adjusted to $1 \cdot 10^9$ CFU•mL^{-1}.

Rice (*Oryza sativa* L.) cv. Cambá INTA Proarroz seeds were combined with the inoculant in a non-porous container and mixed with a plastic spatula until absorption. The inoculum dose used in all treatments for both growth chamber and field trials was 10 mL inoculant per kg of seeds. An additional set of seeds were mock-inoculated with sterile distilled water as a negative control.

Growth Chamber Trial: Following inoculation, 25 seeds were placed on absorbent paper in a plastic box and moistened with sterile distilled water. Four replicas were used for each treatment. The boxes were arranged using a completely randomized block design in a growth chamber and incubated under controlled light and temperature conditions. Seven days after sowing, the plants were harvested and shoot height, root length (cm) and shoot and root dry weights (g) were measured.

Table 1: Origin of the *Azospirillum brasilense* strains used in this work.

Strain	Origin	Plant	Location
Az39	Rhizosphere	Wheat	Marcos Juárez city, (Cordoba province)
Az23	Soil	Rice	Concepción de Uruguay city, (Entre Ríos province)
Az24	Rhizosphere	Bahia grass	Mercedes city, (Corrientes province)
72	Soil	Wheat/cotton	Villa Angela city, (Chaco province)
74	Nodules	Alfalfa	Tilcara city, (Jujuy province)

Field Trial: The field trial was carried out in an experimental field located in Concepcion del Uruguay, Entre Rios. The plot was 5 m long and composed of eight furrows separated 0.20 m from each other. Seeds were inoculated as explained and sown with a seeding machine at a seed density of 170 kg•ha^{-1}. Water-inoculated seeds were used as the control group. Each treatment consisted of five replications arranged in a completely randomized block design. Phosphate fertilization was applied at sowing using 70 kg•ha^{-1} of diammonium phosphate. N-fertilization with urea was supplied at two stages: 100 kg•ha^{-1} before the first flooding and 23 kg•ha^{-1} manual application plus 23 kg•ha^{-1} aerial application at reproductive differentiation. The plants were harvested at maturity and several parameters were estimated based on a 0.1m^2 area: number of spikes/m^2, number of panicles/m^2, number of spikes per panicle, % of barren seeds and 1000-grain weight. Finally, yield (kg•ha^{-1}) was estimated after manual harvest based on a 3 m^2 area of the central rows with a humidity correction of 14%.

Statistical Analysis: The data obtained from the growth chamber and field experiments were analyzed by the Kluskal-Wallis non-parametric test and the Repeated Measures ANOVA test plus Tukey´s post-test, respectively, using the GraphPad Prism 5 software. Differences were considered significant at $p<0.05$.

RESULTS

Isolation and Characterization of the *Azospirillum* Strains: New *Azospirillum* sp. strains were isolated from soil and plants from diverse locations of Argentina. The isolated strains were genotyped by 16S rDNA sequencing. The 16S sequences of the new isolates were closely related (>98% nucleotide identity) to sequences of bacteria annotated as *Azospirillum brasilense* strains in the GenBank database. A posterior phylogenetic analysis was carried out using sequences of members of different species of the *Azospirillum* genus. As a result, the isolates grouped with the *A. brasilense* strains in a common branch of the phylogenetic tree, indicating that they belong to this species. Within this branch, Az23 and Az72 were closer to the type strain sp7, while Az24 and Az74 clustered in a separate sub-group along with *A brasilense* strains Az39 and sp245 (Figure 1).

Isolates were genotyped by partial sequencing of the 16S rDNA gene. The resulting sequences were subjected to a multiple alignment with sequences of other members of the *Azospirillum* genus and a subsequent phylogenetic analysis by the Maximum Likelihood method. *Rhodospirillum centenum* was used as an out-group. Distances were calculated by the Tamura-Nei model. The lower bar indicates sequence divergence. Bootstrap values resulting from 1000 repetitions are indicated at the nodes. GenBank accession number for the 16S sequence is denoted for each strain (underlined).

Effect of the *A. Brasilense* Strains Isolated on Early Rice Growth: Inoculation with strains Az39, Az23, Az24 and Az72 resulted in a significant increase in shoot and root length compared to the control group (Fig. 2a). The most significant increase in both parameters ($p<0.0001$) was observed in treatments with Az39, Az24 and Az72. Analysis of the plant biomass showed that Az39, Az24 and Az72 induced a significant increase in shoot dry weight, whereas Az24 and Az72 also significantly increased root dry weight (Fig. 2b). Treatment with strain Az74 did not significantly modify any of the growth parameters examined when compared to the control group.

Rice seeds were inoculated with *A. brasilense* strains Az39, Az23, Az24, Az72 and Az74, or mock-inoculated as a control and grown under controlled environmental conditions. After 7 days, early plant growth was estimated by measuring shoot height, root length (a) and shoot and root dry weights (b). Treatments significantly different from the control group are denoted by asterisks (*).

Plant Growth Promotion of the *Azospirillum* Isolates on Field-Grown Rice: Next, the efficacy of the strains to increase rice growth and yield was tested in the field. Rice seeds were inoculated and sown in an experimental field as explained in methods. Plants were grown to maturity, harvested and examined for growth and yield estimation. Overall, grain production of the rice plants grown in the field was high, with a mean grain yield of 10,631 kg•ha^{-1}.

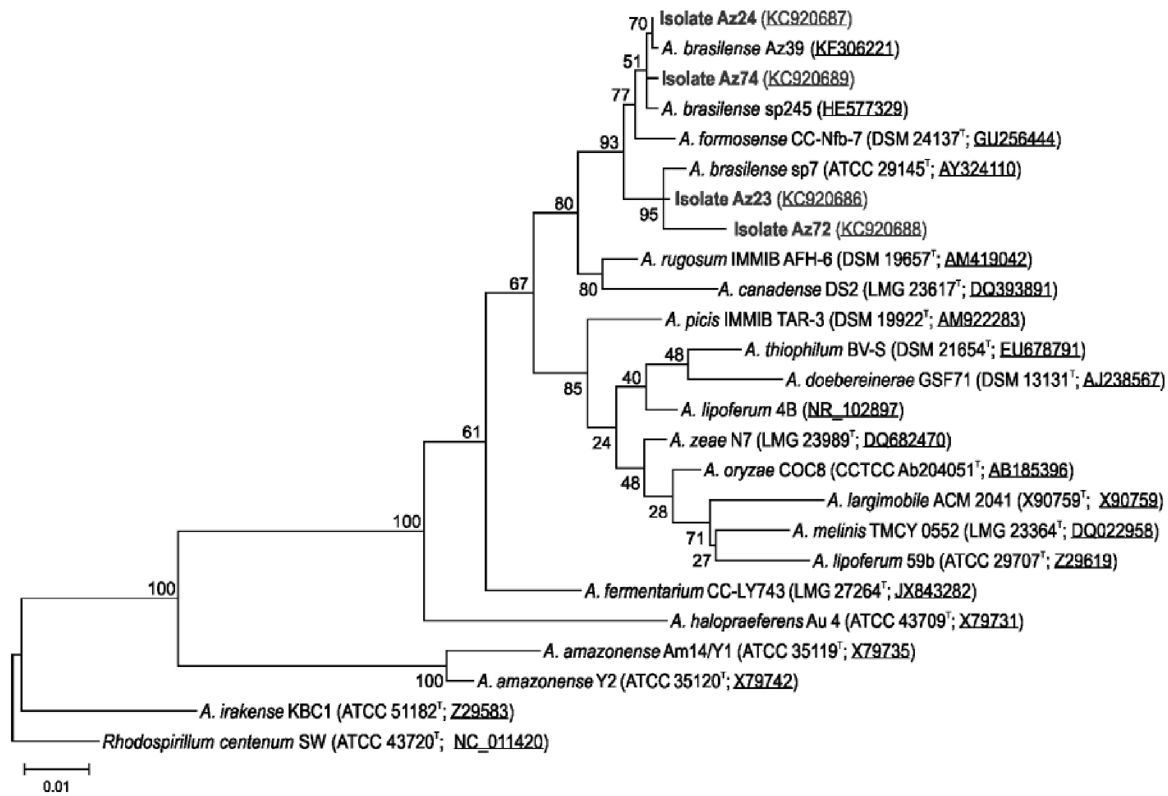

Fig. 1: Phylogenetic analysis of the *Azospirillum* isolates

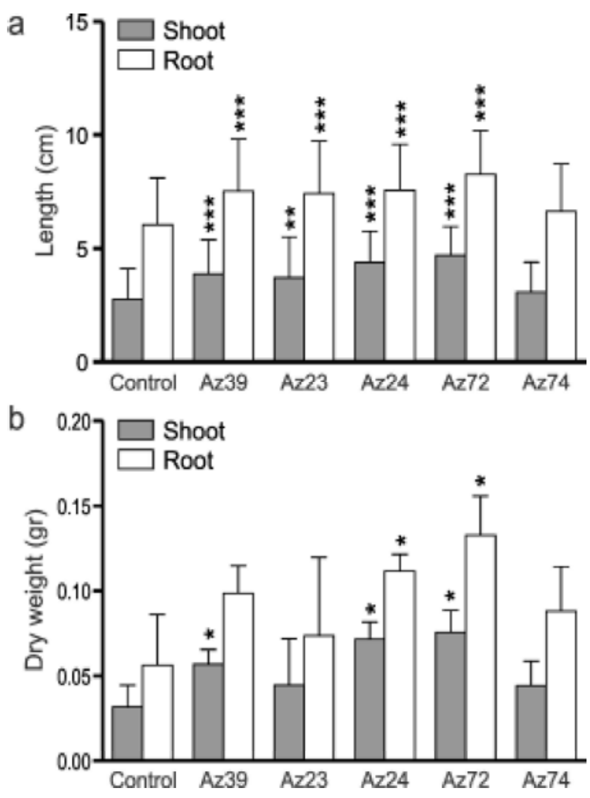

Fig. 2: Effect of the new *Azospirillum* strains on early rice growth

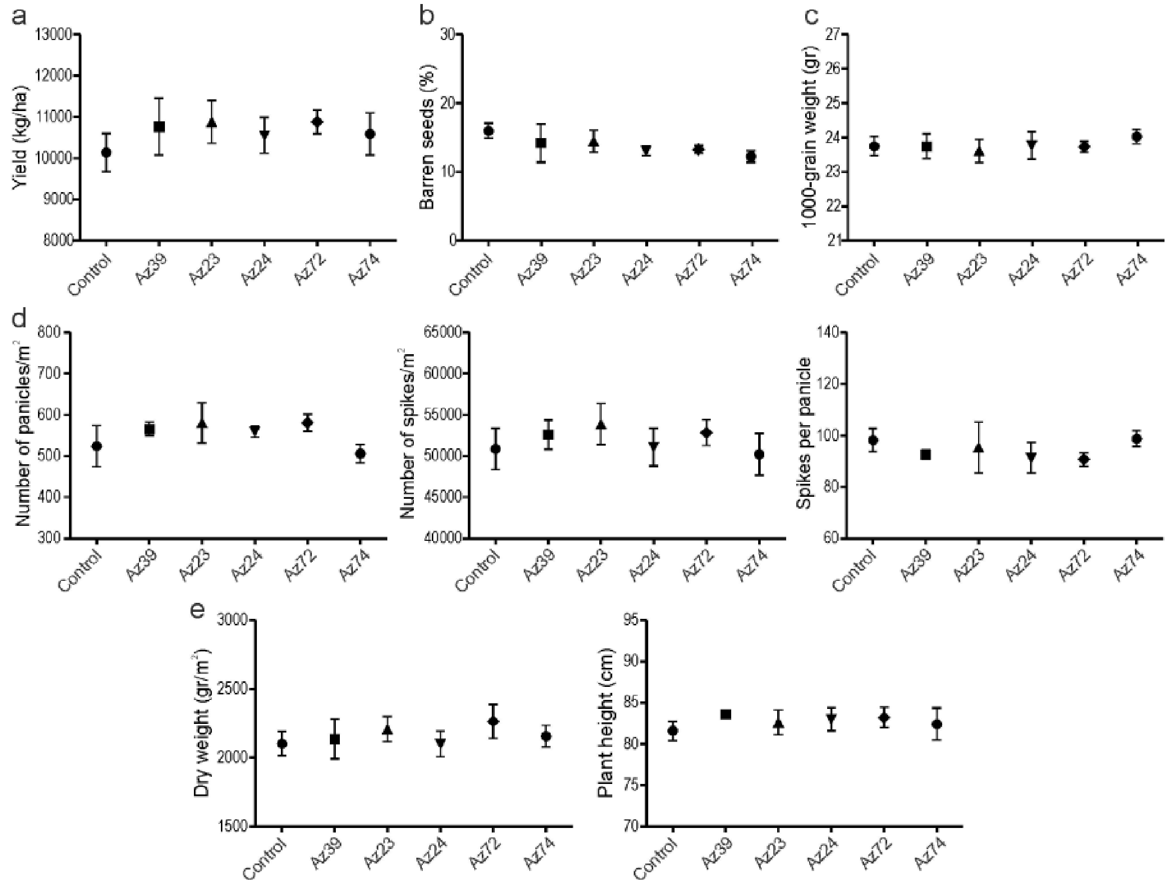

Fig. 3: Effect of the new *Azospirillum* strains on field-grown rice

The growth and yield parameters of the *Azospirillum*-inoculated plants showed no statistically significant differences when compared to the control group. In contrast, the yield average of the *Azospirillum*-treated plants was 5.84% higher than the control (Fig. 3a). Moreover, all the inoculated plants showed a lower percentage of barren seeds (Fig. 3b). In turn, only inoculation with Az74 induced a higher 1000-grain weight mean (Fig. 3c) while all the other treatments showed a higher number of spikes and panicles per m^2 even when the number of spikes per panicle was slightly lower (Fig. 3d). Finally, inoculation with Az72 increased total dry weight by 7.71% when compared to the control group (Fig. 3e).

Rice seeds were inoculated with *A. brasilense* strains Az39, Az23, Az24, Az72 and Az74, or mock-inoculated as a control and sown in the field as explained in methods. At harvest time, plant growth was estimated by measuring the grain yield (a), barren seed percentage (b), 1000-grain weight (c), number of panicles per m^2, number of spikes per m^2, number of spikes per panicle (d), dry weight and plant height (e).

DISCUSSION

Several experiments have been carried out to test the effect of *Azospirillum* inoculation on plant growth and yield [35-38]. Xie and Yakota [39] isolated the *A. oryzae* strain COC8 from paddy soil associated with *Oryza sativa* roots. In contrast, other authors have reported that *Agrobacterium*, *Burkholderia*, *Enterobacter*, *Pseudomonas* sp. and *Pantoea ananatis* strains are the most abundant isolates recovered from roots and rhizospheric soil samples of rice [40, 41].

In this study, four new isolates of *Azospirillum* were recovered from soil and plant samples from diverse locations of Argentina, including a rice productive field. Molecular genotyping by 16S rDNA sequencing showed that isolates Az23, Az24, Az73 and Az74 were strains of *A. brasilense*. When inoculated on rice under controlled conditions, Az39, Az23, Az24 and Az72 promoted plant growth by significantly increasing shoot height and root length and dry weight in some cases. Az24 and Az72 were the most effective strains in stimulating rice early growth in these experiments. An increase in rice biomass has already been observed by treatment with the *A. amazonense* strain AR3122 as compared to the absolute control under gnotobiotic conditions [42]. Rodrigues *et al.* [43] also observed that the inoculation with different strains of *A. amazonense* increases grain dry matter accumulation and the number of panicles of rice in greenhouse experiments. Other authors have observed different effects on plant growth in rice due to the inoculation with PGPR. De Souza *et al.* [40] reported that rice inoculated with the bacterial isolate identified as *Herbaspirillum* sp., *Burkholderia* sp., *Pseudoacidovorax* sp. and *Rhizobium* sp. significantly increased shoot length, dry shoot biomass and dry root weight.

Strain Az74 was the only one that did not promote rice growth. As this strain was recovered from plant tissue (i.e. alfalfa nodules), we hypothesize that it may be adapted to an endophytic life style. Thus, it is possible that the performance of strain Az74 was due to a poor competence with other seed and soil-borne bacteria and/or to a poor interaction with rice. According to Doi *et al.* [44] and Loaces *et al.* [41], rice has a particularly adapted microflora in the rizosphere.

Our results suggest that plant growth promotion is a result of a non-specific *Azospirillum*-plant interaction, since strain Az23, recovered from a rice productive field, was a poor growth promoter of rice seedlings. Our results are similar to those obtained by Pedraza *et al*. [45], who reported that the response of rice to inoculation was associated with the bacterial genotype used for the inoculation since the grain yield was different depending on the strain of *A. brasilense* applied. These observations highlight that a successful growth promotion of rice by *Azospirillum* will depend on a suitable combination of plant and bacterial genotypes. Hence, appropriate *Azospirillum* strains must be selected and evaluated when developing an inoculant for particular rice cultivars.

The results of our field trial showed that several growth parameters were increased with the inoculation. Some strains increased total dry weight, 1000-grain weight and number of spikes and panicles per m^2 and all the inoculated plants showed a lower percentage of barren seeds and an increased mean rice yield of approximately 5%, although the differences were not statistically significant. This is in accordance with that found by Kapulnik *et al.* [46], who observed that enhancement in vegetative growth of cereals attributed to *Azospirillum* inoculation was rarely observed under field conditions. In a review of the published data, Okon and Labandera-Gonzalez [3] stated that 60 to 70% of studies with *A. lipoferum* and *A. brasilense* had shown plant responses to inoculation, although the results were statistically significant in only 5–30% of the cases. Moreover, Banayo *et al.* [47] tested a biofertilizer in rice during four seasons and observed that the increase in grain yield was not always statistically significant and that the increase in yield varied considerably between seasons.

The response obtained in our field experiment can be attributed to the high dispersion of the data, which is expected in experiments that are under a great environmental influence. It has been pointed out that the variability is a major problem when analyzing PGPR field trials, resulting in erratic conclusions about the effectiveness of microbial inoculants [48-50]. Moreover, Dobbelaere *et al.* [49] proposed that the inconsistency of the response of amended crops to bacterial inoculants could be explained by varying environmental soil plant and microfloral components at any given experimental site.

The grain size of the plants treated with Az23, Az24, Az39 and Az72 seemed unaltered, as evidenced by a similar 1000-grain weight value when compared to the control. The yield increase observed might be a result of the lower percentage of barren seeds in the spikes and/or the higher number of panicles per plant. On the other hand, the higher 1000-grain weight value obtained with the Az74-treated plants indicates the occurrence of larger grains that in turn might be responsible for the yield increase observed. Therefore, these results suggested that the soil and rhizospheric *Azospirillum* strains promote rice growth by mechanisms different from those of the endophytic strain Az74.

Regarding the final growth parameters, plant mean height was considerably higher in all *Azospirillum* treatments than in the control group. According to different authors [4, 50-51], this may be attributed, in part, to the IAA production by the strains since auxins impact directly on plant elongation. Secondly, the mean dry weight was most notably increased in Az23- and Az72-treated plants, in coincidence with the two highest yield values obtained with these treatments. Also, strain Az72 induced the most significant increase in dry weight of rice seedlings in the growth chamber trial. In summary, *A. brasilense* Az72 was most effective strain to promote rice growth, both in chamber and field conditions, even when compared to the Az39 reference strain. Plants treated with Az72 showed a marked increase in early growth in the growth chamber trial and in final growth and yield in the field trial. Additional field experiments with a higher number of repetitions need to be conducted to confirm Az72 as a suitable rice growth promoter.

New experiments will be required to determine whether rice growth promotion is enhanced when using *Azospirillum* strains along with lower levels of fertilizers.

CONCLUSIONS

All four *A. brasilense* strains that were recovered from different ecological locations of Argentina were able to promote rice growth. Growth promotion was consistent in the field trial but, however, not significant upon statistical analysis. Overall, *A. brasilense* Az72 was the most efficient PGPR strain for stimulating rice growth both in controlled environmental conditions and in the field. A more comprehensive study of Az72 may lead to the development of a commercial inoculant for rice.

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