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# In vitro PGPR properties and osmotic tolerance of different *Azospirillum* native strains and their effects on growth of maize under drought stress

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**Abstract**

Osmotic variations in the soil can affect bacterial growth diminishing the number of inoculated bacteria. In a scenario of water deficit having tolerant bacteria would be beneficial to achieve a better response of the plant to stress. Thus, selection of more resistant bacteria could be useful to design new inoculants to be used in arid zones. In this sense, a group of *Azospirillum* isolates deposited in INTA collection was characterized in order to select strains tolerant to osmotic stress. The results obtained demonstrated that

Az19 strain has similar in vitro PGPR characteristics to Az39, the most used strain in Argentina for inoculants industries, with the advantage of a better tolerance to osmotic and salt stress. Inoculation of maize plants with this strain resulted in a better response against water deficit compared to Az39 strain, encouraging us to further study the behavior of this strain in greenhouse and field trials in view of developing new inoculants suitable for areas with water deficit.

**Abbreviations:**

αKB: α-ketobutyrate,

**Keywords:** *Azospirillum*; growth promotion; osmotic tolerance; maize; droughtPGPR: plant growth promoting rhizobacteria

PEG: polyethylene glycol

ACC: 1-aminocyclopropane-1-carboxylate

IAA: inodoleacetic acid

EMS: Ethyl methanesulfonate

## Introduction

In the last years, the growing interest in the development of a sustainable agriculture has led producers to reduce the use of chemical fertilizers by increasing crop inoculation with plant growth promoting rhizobacteria (PGPR). Within PGPR, *Azospirillum* is worldwide used to improve the yield of several crops as wheat, corn, rice, sugarcane leading to increments up

to 30 % (Vogel et al., 2013 and references therein). The mechanisms by which this PGPR improve plant growth includes nitrogen fixation, phytohormones, polyamines and trehalose production among others (Cassan et al., 2009; Rodríguez-Salazar et al., 2009; Bashan and de-Bashan, 2010). It is considered that the mode of action of *Azospirillum* is multiple and the importance of each of the mechanisms involved may vary depending on the soil and climate conditions imposed by the environment, highlighting the concept of "additive effect" in this interaction (Bashan and Holguin, 1997).

In Argentina, *Azospirillum brasilense* Az39 strain isolated from roots of wheat grown in soils of Marcos Juarez, Córdoba, was selected for its efficiency to promote growth of this cereal (Diaz Zorita and Fernández Caniggia, 2009, Rodríguez Cáceres et al., 2008) and it is recommended for use in commercial formulations (Cassán et al., 2009). Bacterial inoculants are generally applied on the seed prior to planting. Therefore, for optimal interaction with the plant, a bacterium must be able to survive on the surface of the seed and then carry out the colonization of the root system (Bloemberg and Lugtenberg, 2001). Drought stress is a limited factor for crop development causing several agricultural losses (Pereyra et al., 2006). The use of PGPR inoculation in plants growing in these extreme situations could help the plant to face, at least in part, this stress by increasing root length, which allows a better access to water (Kang et al., 2014; Cohen et al., 2015; Rodríguez Salazar et al., 2009). In the same way as plants suffer hydric stress when the availability of water is low, bacteria can also be affected by this stress. Changes in soil salt concentration, which is sensed by microorganisms as osmotic variations, may impact in bacterial growth. In a scenario of water deficiency, adaptation of diazotrophs to osmotic stress is of great importance, thus selection of more resistant bacteria could be useful to design new inoculants to be used in arid zones. Although some researchers informed that plants

inoculated with *A. brasilense* show a better response against stress conditions, there is no information about the survival of this bacterium when it is grown in adverse environmental situations. However, it has been reported that *Azospirillum* accumulate compatible solutes as proline, glycine betaine, glutamate and trehalose to cope with osmotic stress (Tripathi et al., 1998; Yuwono, 2005; Rodriguez Salazar et al., 2009).

In this sense, the purpose of this work was to characterize a group of *Azospirillum* isolates deposited in INTA collection in order to select strains tolerant to osmotic stress and evaluate their performance in increasing maize stress tolerance.

## Materials and methods

### Strain selection and culture conditions

Within the PGPR Collection of IMyZA INTA Castelar are deposited 35 isolates of *Azospirillum* spp. from rhizosphere soil or surface sterilized roots taken from different parts of the country (Rodríguez Cáceres et al., 2008). In order to work with strains of different origins, *Azospirillum* isolates Az3, Az8, Az19, Az63 from soil of diverse regions and from maize roots were included (Table 1). *A. brasilense* Az39, which is used in commercial inoculants produced in Argentina, was used as the reference strain.

Bacteria were cultured in Nfb liquid medium supplemented with 0.3 g $l^{-1}$ $NH_4Cl$ (Piccoli and Bottini, 1994) at 30º C in a rotary shaker set to 150 rpm.

### Genotyping of *Azospirillum* isolates

Extracts used as PCR templates were obtained by suspending freshly streaked bacteria in 50 µl of ultrapure water and boiling for 10 min. PCR amplification of the 16S rRNA was performed with universal primers 27F and 1492R (Frank et al., 2008) in a final volume of 40 µl containing: 1 µl of DNA extract as template, 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 µM primers, 0.2 mM dNTP and 1 U of Taq Polymerase. Reagents were purchased from

Invitrogen™ (Thermofisher Scientific, USA). Temperature cycling was carried out in a thermocylcer eppendorf Mastercycler ® and consisted of an initial 2 min denaturation step at 94°C followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 1 min and 30 sec. PCR products were analyzed by TAE-agarose electrophoresis, excised and purified with QIAEX II gel extraction kit (Qiagen) and sequenced at the Genomics Unit of the Biotechnology Institute of CNIA-INTA (http://www.inta.gov.ar/biotec). The resulting sequences were deposited in the Genbank database (Table 2). Phylogenetic analyses using the obtained 16S rRNA sequences and available *rpo*D sequences (Maroniche et al., 2017) were carried out with MEGA 6 software (Tamura et al., 2013) using the maximum likelihood method, Tamura-Nei substitution model and a bootstrap phylogeny test of 1000 iterations. Sequences of other *Azospirillum* strains were retrieved from Genbank.

**Nitrogenase activity**

Nitrogen fixing ability of *Azospirillum* strains was estimated by the acetylene reduction assay according to Hardy et al. (1968). Briefly, 100 µl of a culture (CFU=$10^9$) were inoculated into 4 ml of semisolid Nfb medium (Döbereiner and Day, 1976) in 10 ml vials and incubated at 30 °C for 24 h. Vials were hermetically sealed with rubber stoppers and aluminum seals and 10% of the atmosphere of the vial was replaced by acetylene. After incubating 20 hours at 33 °C, ethylene production was measured in a gas chromatograph 5890 Series II (Brand Hewlett Packard) using nitrogen as a carrier gas (Laboratorio de servicios analíticos especiales, FAUBA). The activity is expressed as ethylene production (nmol $ml^{-1}$ $h^{-1}$).

**Phosphate solubilization**

Each bacterial strain was spot inoculated on SMRS1 medium containing tricalcium phosphate as inorganic phosphorus source, glucose as carbon source, bromocresol purple as a pH indicator, ammonium sulphate and yeast extract as nitrogen source (Otalora et al., 2003). After 7 days at 28 ° C the phosphate solubilizing ability was checked by the presence of a transparent halo around the colonies. *P. fluorescens* BNM233 (Rubio et al., 2013) was used as a positive control.

**Indol production**

*In vitro* indolic biosynthetic capacity of the isolates was estimated by the Salkowski colorimetric technique (Glickman and Dessaux, 1995) in supernatant aliquots (1 ml) of 5-days old cultures grown on NFB medium (Döbereiner and Day, 1976) amended with 100 $\mu g\ ml^{-1}$ of L-tryptophan. Two supernatant aliquots of each bacterial culture were processed. Indolic compound production was determined by extrapolating to a standard curve constructed with IAA and was expressed as $\mu g\ ml^{-1}$.

**Siderophore production**

Siderophore production was investigated using the O-CAS assay (Pérez-Miranda et al., 2007). An overlay of the CAS medium (Schwyn and Neilands, 1987) without nutrients was applied on top of Nfb agar plates that had been spot-inoculated with the *Azospirillum* strains and incubated at 30 ºC for 48h After incubation at 30 ºC, siderophore production was estimated by the development of Purple (catechol type) or orange (hydroxamate type) halos surrounding the colonies.

**ACC deaminase activity**

ACC deaminase activity was determined according to the method of Penrose and Glick (2003) adapted to 96-well microplates as described by Maroniche et al. (2016). Protein concentration for all samples was determined by the method of Bradford (1956) using

bovine serum albumin as a standard. *Pseudomonas brassicacearum* DBK11$^T$ (Long et al., 2008) was used as a positive control. ACC deaminase activity was calculated by measuring the amount of α-ketobutyrate produced by the deamination of ACC. ACC deaminase activity was expressed in mmol of α-ketobutyrate $mg^{-1}$ protein $h^{-1}$.

**Tolerance to osmotic and salt stress**

*Azospirillum* strains were cultured in Nfb medium supplemented with 0.3 g $l^{-1}NH_4Cl$ with or without the addition of PEG (20% w/v), sorbitol (0, 0.2, 0.4, 0.6, 0.8, 1 and 2M) or 200 mM NaCl. Samples were taken at different times (24 h for PEG and NaCl treatment and 0, 2, 6, 24 y 48 h for sorbitol treatments) and the number of viable cells was estimated by CFU count with the drop method (Herigstad et al., 2001) in RC media (Rodríguez Cáceres, 1982).

**Estimation of proline content**

Proline was measured by a modified ninhydrin method (Bates et al., 1973). Harvested bacterial cells were extracted overnight in 1 ml 3% (w/v) aqueous 5- sulphosalicylic acid. Precipitated protein and other debris were removed by centrifugation at 13,000 × g for 5 min. Proline content in the supernatant was measured by reaction with ninhydrin and glacial acetic acid after 1 h incubation at 100º C. The optical density was measured at 520 nm. The amount of proline produced by bacteria was calculated from a standard curve.

**Trehalose production**

Trehalose determination was performed following the protocol described by Rodriguez-Salazar et al. (2009). Trehalose determination was done only to Az39 and Az19 strains in the presence of NaCl.

Briefly, strains were grown during 24h at 30 °C in liquid Nfb with the addition of $NH_4^+$. The cultures were centrifuged and washed with water and resuspended in ethanol. After

incubation at 85 °C for 15 min, were centrifuged at 13,000 cells rpm for 5 min and the supernatant was recovered. After evaporation of excess ethanol, samples were resuspended in ultrapure water and analyzed by HPLC using a Supelcosil LC-$NH_2$ Column (SIGMA-ALDRICH) eluted with acetonitrile: water (80:20 v/v). Purified trehalose (Sigma Chemical Co.) was used as standard to determine the concentration.

**Inoculation assay in growth chamber**

Seeds of a commercial maize hybrid (DOW 510 PW), surface disinfected with 80% (v / v) ethanol and sodium hypochlorite (4% active chlorine) (Lupo et al., 2001) were pre-germinated in a wet chamber for 24 h at 25 °C. Then, they were planted in plastic pots using a mixture of sterile sand, soil, vermiculite and perlite (3: 3: 3: 1) as substrate. The assay was conducted in a growth chamber at 26 °C and a photoperiod of 16 h light: 8 h dark.

**Azospirillum culture and inoculation**

Az39 strain was used as the reference strain and Az19 strain was selected for their greater tolerance to osmotic stress in the in vitro tests. The strains were cultured in liquid Nfb medium supplemented with $NH_4^+$ and incubated for 48 h in a rotary shaker at 180 rpm and 30 °C. Cells were harvested by centrifugation at 8,142 g for 10 min and suspended in saline solution (Creus, 2004). Uniform seedlings with 1 cm radicles were inoculated by immersion for 2 hours in an inoculum containing $5x10^7$ CFU per seedling (Casanovas et al., 2000).

**Treatments and trial design**

The treatments resulted from the combination of two AXB factors where A) is the treatment with *Azospirillum* and whose three levels correspond to the treatments inoculated with the strains Az39, Az19, and to an uninoculated control and B) corresponds to the

water stress factor whose two levels are with stress and without water deficiency. Twelve pots per treatment were planted and arranged in a completely randomized design.

**Irrigation and water stress imposition**

Pots of all treatments were filled with a certain weight of the substrate. The pots without water deficit were irrigated at field capacity one day before sowing and were kept under irrigation on demand throughout the assay. Those subjected to stress were watered with 50% of the field capacity only once, the day before sowing. After a week, all treatments were watered with 5 mL of nutrient solution per plant (Hoagland and Arnon, 1950). Fifteen days after sowing, the plants were harvested and the following parameters were measured:

*Growth Parameters:* Root height and length were measured and the fresh weight (FW) and dry weight (DW) of the shoot and the root system were determined.

*Water status of plants:* Relative water content (RWC) was determined on the last expanded leaf according to the method described by Barrs and Weatherley (1962) based on the following formula RWC% = (fresh weight-dry weight) * 100/ (turgor weight-dry weight).

*Proline content in leaves and roots:* The technique described by Bates et al. (1973) based on the reaction of proline with the ninhydrin reagent was used. Plant material (0.3 g) was homogenized with 3 mL of 5% (w / v) sulphosalicylic acid. The proline concentration was calculated using a standard curve.

**Count of viable cells in seeds prior to planting**

After inoculation, counts of viable cells recovered from seeds, were made through serial dilutions in saline solution and plated in RC medium.

**Colonization**

This parameter was measured 7 days after sowing for which 1 g of surface washed roots were ground in a sterile mortar and homogenized with 10 mL of saline solution. Serial

dilutions were made up to a $10^{-9}$ dilution. The dilutions were seeded in triplicate in semisolid Nfb medium to estimate the most probably number of bacteria (MPN) (Döbereiner and Day, 1976) per root fresh weight.

**Statistical analysis**

Data shown in tables and figures are mean values of two independent experiments with three repetitions.

Differences among treatments were analyzed by unpaired t test or one-way ANOVA followed by Tukey's multiple range or Duncan test using Infostat software taking $p< 0.05$ as significant.

**Results and discussion**

***Genotyping and characterization of Azospirillum strains***

In order to work with strains from different origins four isolates (Az3, Az8, Az19 and Az63, see Table 1) deposited in INTA *Azospirillum* collection were selected to evaluate their tolerance to osmotic stress with prospects of designing inoculants with improved drought-resistance strains compared to the main used in commercial formulations. In this sense, molecular identification and evaluation of *in vitro* plant growth promotion abilities were performed.

Molecular identification of the selected *Azospirillum* strains was performed using the 16S rRNA and *rpo*D gene sequences. The 16S rRNA gene data was obtained by PCR-amplification with universal primers 27F and 1496R and sequencing, while *rpo*D sequences already available from a previous characterization of the IMyZA-INTA collection (Maroniche et al., 2017) were retrieved from Genbank. The obtained sequences indicated that strains Az3, Az19 and Az63 are closely related to *A. brasilense* Az39 according to both 16S rRNA (99.8-99.9 % identity) and *rpo*D (99.6-99.9 % identity) genes

(Fig. 1A). Contradictory results were obtained for strain Az8, which was close to *A. formosense* CC-Nfb-7$^T$ according to 16S rRNA (99.3% identity) but identical to *A. doebereinerae* GSF71$^T$ according to *rpo*D. For a deeper phylogenetic analysis, both sequence sets were processed, along with data from *Azospirillum* type strains, to obtain multiple-alignments that were then concatenated and used for inferring a Maximum Likelyhood phylogenetic tree. This analysis indicated that strain Az8 belongs to the *A. doebereinerae* species since it formed a solid clade with type strain GSF71$^T$ (Fig. 1B). As expected, strains Az3, Az19 and Az63 grouped with the reference strain Az39, forming a solid clade adjacent to *A. formosense*, *A. brasilense* and *A. himalayense* species (Fig. 1B). Notably, previous results on whole genome comparisons indicated that Az39, which has been historically considered as an *A. brasilense* strain, might be part of a different species (Maroniche et al., 2017). Thus, taken together, all the evidences suggest that Az39, as well as strains Az3, Az19 and Az63, do not belong to *A. brasilense* species. New genomic information of *A. formosense* and *A. himalayense* will help to determine if these strains should be considered as part of an undescribed *Azospirillum* species.

The analysis of the abilities to promote plant growth showed that, in general, all strains have the capability to behave as PGPR.

The capacity to solubilize phosphates of *Azospirillum* strains was evaluated by spot-inoculation on SMRS1 solid medium. Although all strains could grow in this medium, only strain Az19 produced a consistent solubilization halo. The solubilization activity of strain Az39 was weak since colonies produced a slight clarification of the medium without forming a transparent halo (Fig. 2A). These results are in concordance with those reported by other authors (Puente et al., 2004; Rodríguez et al., 2004; Perrig et al., 2007) who also detected the capacity to solubilize phosphates of this strain. The rest of the strains did not

show solubilization activity, as it was observed by Di Salvo et al. (2014) for other native strains.

There are controversial results with respect to siderophore production depending on the methodology used. For example, Perrig et al. (2007) stated that Az39 and Cd strains were not able to produce siderophores in opposition to the results of Di Salvo et al. (2014) that found that all the strains tested have this ability. All the strains tested in our work produced a color change of the CAS medium (Pérez-Miranda et al., 2007) from blue to orange indicative of hydroxamate type siderophore production (Fig. 2B). Similar results were reported by Carcaño Montiel et al. (2006) and Tortora et al. (2011), who found that various strains of *A. brasilense* and *Azospirillum* spp. were able to produce siderophores under conditions of limiting iron.

Atmospheric nitrogen fixation was confirmed in all the strains by the acetylene reduction method, with values ranging from 0.26 to 0.05 nmol $h^{-1}$ $ml^{-1}$ $C_2H_4$. The greater fixers were Az39 and Az3, while Az8 and Az63 fixed nitrogen in a minor extent. Az19 showed a nitrogenase activity of about 75% of that of Az39 (Fig. 3A). In addition, all the strains could grow in Nfb semisolid medium (Döbereiner, 1998) forming a sub-superficial veil-like pellicle and medium alkalinization. Accordingly, Venieraki et al. (2011) observed a considerable variability in nitrogen fixation between several *Azospirillum* isolates. Although Perrig et al. (2007) reported similar results for *A. brasilense* Cd and Az39 with 0.162 and 0.1 nmol $C_2H_4$ $h^{-1}$ $ml^{-1}$, respectively, other authors have presented much higher values for N8 and Sp7 strains of the same species, obtaining 7.95 nmol $C_2H_4$ $h^{-1}$ $ml^{-1}$ and 845.6 nmol $C_2H_4$ $h^{-1}$ $ml^{-1}$, respectively (Mehnaz and Lazarovits, 2006; Cardenas et al., 2010).

The *Azospirillum* strains were assayed for ACC deaminase activity since it has been linked to plant-growth promotion by rhizobacteria, mainly when plants are stressed. Although it has been reported that most *Azospirillum* strains do not present this characteristic (Holguin and Glick, 2001; Blaha et al., 2006), the enzymatic activity of all the isolates evaluated were similar to that of Az39 (ranging from 0.117 to 0.890 µmol αKB $mg^{-1}$ $h^{-1}$), confirming the results obtained by Di Salvo et al. (2014) who stated for the first time the capability of *A. brasilense* to use ACC as nitrogen source. Although none of them reached the levels of activity of *Pseudomonas brassicacearum* DBK11, used as the positive control (Fig. 3B), a revision of Esquivel-Cote et al. (2013) considered that values lower than 0.020 µmol αKB $mg^{-1}$ $h^{-1}$ reflect low levels of ACC deaminase activity whereas values of about 0.400 µmol αKB $mg^{-1}$ $h^{-1}$ are considered high.

Since the production of IAA has been highlighted as an important trait for plant growth-promotion by *Azospirillum* (Cassán et al., 2013), *in vitro* indolic compounds production was analyzed by measuring their release in the supernatant of 5-days old cultures. All the strains were able to produce these compounds (Fig. 3C). Az3 strain showed the highest auxin production with a value of 23.8 µg IAA $ml^{-1}$ while strain Az8 showed the lowest value (5 µg IAA $mL^{-1}$). Az39 produced 12.6 µg IAA $ml^{-1}$ which is much higher than that observed by Perrig et al. (2007), who reported a production of 2.9 µg IAA $ml^{-1}$ for this strain quantified by gas chromatography mass spectrometry during the exponential phase of growth in a medium without tryptophan. Other authors reported that *A. brasilens*e Sp7 and Cd show higher production values (40.2 µg and 58.71 µg $ml^{-1}$, respectively) (Guzman et al., 2012; Radwan et al., 2005). Analysis with liquid chromatography (HPLC) revealed that the production of IAA in strains of *A. lipoferum* may vary from 0.04 to 4.1 mg $ml^{-1}$ and *A. brasilense* 0.01-4.5 mg $ml^{-1}$ (Crozier et al., 1988). The lack of consistency of the results

obtained by other authors may be due to the different methodologies and different growing conditions used. However, the data obtained in these conditions are similar to those reported by Cassán et al. (2013) for *Azospirillum*, who found IAA production ranging from 5 to 50 µg IAA $ml^{-1}$ depending on the strain and the culture conditions.

***Tolerance of the strains to osmotic stress***

In order to evaluate their tolerance against osmotic stress, the strains were grown in liquid Nfb medium supplemented with $NH4^{+}$ and sorbitol, PEG or NaCl.

All strains were able to grow on the medium supplemented with different concentrations of sorbitol. Concentrations between 0.2 and 0.8 M did not produce significant effects in growth, while 1M sorbitol induced a negative effect on growth starting at 6 h of incubation when compared to the control treatment without the osmotic agent (Fig. 4). Strain Az19 showed the best performance since the decline in growth was smaller than for the other strains, showing a tolerance of about 90%. The survival of the strains was also evaluated using a concentration of 2 M sorbitol to generate stress, confirming that strain Az19 is the most tolerant to osmotic stress (Fig. 5A).

When 20% PEG was used instead of sorbitol, both Az19 and Az39 showed higher growth than the other strains (Fig. 5B). Accordingly, Cesari et al. (2016) observed a similar behavior for Az39 growing in a medium with a water potential of -0.8 MPa generated by PEG. Notably, CFU count indicated again that strain Az19 is the most tolerant strain (90%) ($p < 0.05$) (Fig. 5B).

Tolerance to salt stress was evaluated with strains Az39 and Az19 because they had the best performance in trials with sorbitol and PEG. Both strains could grow properly in Nfb liquid medium with 200 mM NaCl (Fig. 5C), which agrees with Rodríguez-Salazar et al.

(2009) that reported that Az39 is able to tolerate up to 200 mM NaCl. Nevertheless, our results indicated that Az19 is also more tolerant to salt stress than Az39 (Fig. 5C). Taken together, these results demonstrate that closely related strains of the same species have different behavior against osmotic stress. These data are consistent with those obtained by Rodriguez Salazar (2011) who also observed that different strains of *A. brasilense* show differences in tolerance to osmotic stress generated by sorbitol. According to our results, Az19 strain showed a significant higher tolerance to osmotic stress than the reference strain Az39. A better understanding of the osmo-adaptive mechanisms of strain Az19 would help improve the interaction of this bacterium with plants growing under water stress conditions. In this sense, the production of compatible osmolytes such as proline and trehalose were measured. Although the presence of proline was not detected in any of the strains subjected to osmotic or saline stress, the production of trehalose by Az19 was 388% greater than that produced by the reference strain Az39 and even exceeded the values obtained with the strain Na5 which is a Az39-derived EMS mutant that overproduce trehalose (Fig. 6) (Personal Communication, Ramírez, Trujillo). These data demonstrate a positive association between osmotic stress-tolerance and trehalose content in strain Az19. The similar behavior of strains Az39 and Az19 under mild stress conditions (tested with PEG) may be due to other mechanisms different to trehalose production, such as glutamate (Hartmann et al., 1991) and glycine betaine accumulation (Csonka, 1989) which can also act as osmoprotectants of the cell in situations of stress.

**Inoculation assay**

In order to evaluate the "*in vivo*" effect of the Az19 strain, the most tolerant strain in the "*in vitro*" tests, an inoculation assay of maize plants subjected to water stress was carried out. Az39 was used as a reference strain.

Table 3 shows the number of CFU present in the seeds at the time of planting and the number of diazotrophic bacteria obtained from roots after 7 days from inoculation. As it is shown in the table the initial dose of bacteria per seed was similar for the two strains inoculated and it was close to the theoretical dose (see Materials and methods).

When colonization of diazotrophs was evaluated by the most probable number method, no significantly differences were observed between the strains, both in control and stressed plants. However, water deficit reduced the number of diazotrophs per gram of root fresh weight in about 3 orders of magnitude for both strains.

In watered plants, both strains, Az39 and Az19, increased the height and the fresh and dry weight of the shoots of maize plants in a similar proportion (Fig. 7A, 7B and C, as it was reported by Casanova et al. (2003).

In plants subjected to water deficit, inoculation with any of the strains significantly increased the height of the plants and Az19 strain also increased the dry weight of the aerial part compared to the uninoculated control. Although in irrigated plants no significant differences of the dry weight of the roots between inoculation treatments were observed, inoculation of stressed plants with any of the strains significantly increased (Fig. 7D) this parameter. In this case, Az19 caused the maximum increment reaching values similar to that observed in non-stressed plants.

Water stress significantly decreased the relative water content determined on the last expanded leaf, except in those plants inoculated with Az19 which presented RWC levels closer to the controls (Fig. 8).

The positive effects of the inoculation with *Azospirillum* strains were more significant on plants under stress conditions than under normal irrigation (Fig. 7). For example, in watered plants Az19 produced an increase of 38% in dry weight of shoots while under

water deficit the increase obtained was of 80%. Similarly, inoculation of maize plants with *Bacillus* sp. or *Azospirillum* (Vardharajula et al., 2011 Bano et al., 2013) caused higher protection in plants subjected to water deficit. In concordance, Egamberdiyeva and Höflich (2004) reported that the greatest benefits from inoculation were obtained in crops under long periods of stress. Similar results were obtained in wheat plants (Chakraborty et al., 2013).

As it was expected, water stress significantly increased proline content in roots and leaves (Fig. 9). The accumulation of proline under stress conditions has been widely documented (Silveira et al., 2003; Valentovic et al., 2006; Bano et al., 2013) acting as an intercellular solute for osmotic adjustment. Increases in proline levels can be attributed to an increase in synthesis and a decrease in degradation under conditions of saline or water stress (Szabados and Savoure, 2010). In this sense, Hamdia et al. (2004) reported increases in both stem and leaves of maize when grown under salinity conditions.

Although in the irrigated plants there were no differences in proline content between inoculation treatments; in plants subjected to stress proline concentration increase 2.6 times in roots of uninoculated plants indicating that proline was produced by plants in response to stress. Inoculation with Az19 improved the plant response to stress increasing 11.6 times the proline content of roots compared with uninoculated controls. These data are consistent with those published by Kandowangko et al. (2009) and Casanovas et al. (2002) who observed that the inoculation with *Azospirillum* sp. improved the proline content of maize subjected to drought. The same effect was observed by Vardharajula et al. (2011) in maize inoculated with *Bacillus* sp.

**Conclusions**

This work showed that strain Az19 from the PGPR Collection of IMyZA INTA Castelar has similar *in vitro* PGPR characteristics than Az39, the most used strain in inoculants industries of Argentina. Nevertheless, the former displayed a higher tolerance to osmotic and salt stress. In addition, inoculation of maize plants with strain Az19 helped maize seedling tolerate drought stress to a higher level as compared to uninoculated plants and it resulted in a better response against water deficit compared to Az39. Further studies will be needed to determine the behavior of this strain in field trials in view of developing new inoculants suitable for areas with water deficit.

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Legends to figures

**Fig. 1** Molecular identification of the *Azospirillum* isolates. Isolates Az3, Az8, Az19 and Az63 were genotyped using 16S rRNA and *rpo*D sequences. *A. brasilense* Az39, which is a native strain used in most of the *Azospirillum*-containing inoculant formulations in Argentine, was included as a reference. (A) The four isolates were compared to all *Azospirillum* type strains by pairwise alignment using both gene sequences. Nucleotide identities obtained for 4 close type strains using 16S rRNA (upper table) and *rpo*D (lower table) genes are compared and the highest values are highlighted in bold. (B) Multiple-alignments obtained for both genes with sequences of the isolates and *Azospirillum* type strains were concatenated, and a phylogenetic tree was inferred by the Maximum Likelihood method, Tamura-Nei substitution model and a Bootstrap testing of 1000 iterations. Bootstrap values ≥ 50 are shown in the corresponding nodes. Other members of the *Rhodospirillaceae* family and *Bradyrhizobium japonicum* E109 were included as outgroups. Genbank accession codes for 16S rRNA and *rpo*D sequences used in the analyses are detailed in Table 2 or elsewhere (Maroniche et al. 2017).

**Fig. 2** (A) Phosphate solubilization test in SMRS1 medium. (B) Siderophore production in O-CAS medium. Images are representative of three replicated experimental units, obtained from two independent experiments.

**Fig. 3** (A) Determination of nitrogenase activity, (B) ACC deaminase and (C) IAA production of the strains as described in Materials and methods. Two independent experiments were carried out. Different letters indicate significant differences ($p < 0.05$) by Tukey test.

**Fig. 4** Growth curves of *Azospirillum* strains grown with different concentrations of sorbitol as described in Materials and methods. Two independent experiments were carried out. Bars represent standard error of the mean (SEM).

**Fig. 5** (A) Log of the average cell count (CFU $ml^{-1}$) graphed vs. time (hours postinoculation) for the different *Azospirillum* strains grown in NFB liquid media with 2 M of sorbitol. (B) Percentage of tolerance of *A. brasilense* strains grown in a medium with 20% PEG. (C) Percentage of tolerance of *A. brasilense* strains grown in a medium with 200 mM NaCl addition. Two independent experiments were carried out. Different letters indicate significant differences ($p < 0.05$) by Tukey test.

**Fig. 6** Trehalose production of strains Az19, Az39 and Na5 as described in Materials and methods. Two independent experiments were carried out. Different letters indicate significant differences ($p < 0.05$) by Tukey test.

**Fig. 7** Effects of *Azospirillum* inoculation on the growth of 15-day maize plants under controlled environmental conditions for the different hydric treatments (W: watered and S: stressed). Height (A), shoot fresh (B) and dry weight (C) and root dry weight (D) were measured in the control non-inoculated (C) and inoculated plantlets. Values are mean of 2 replicate set and 12 plants per set. Different letters mean significant differences between treatments by Duncan test ($p < 0.05$).

**Fig. 8** Effect of *Azospirillum* inoculation on relative water content (RWC) of maize plantlets under different hydric treatments (W: watered and S: stressed). Values are mean of 2 replicate set and 12 plants per set. Different letters mean significant differences between treatments by Duncan test ($p < 0.05$).

**Fig 9** Effect of *Azospirillum* inoculation on leaf (A) and root (B) proline content of 15-day-old plantlets under different hydric treatments (W: watered and S: stressed). Values are mean of 2 replicate set and 12 plants per set. Different letters mean significant differences between treatments by Duncan test ($p < 0.05$).

Fig 1

A

| | 16S rRNA | | | | |
|---|---|---|---|---|---|
| | GSF71 $^{T}$ | Sp7 $^{T}$ | CC -Nfb -7 $^{T}$ | ptI -3 $^{T}$ | Az39 |
| Az3 | 96.5 | 97.8 | 98.8 | 99.1 | **99.9** |
| Az8 | 97.0 | 98.3 | **99.3** | 98.3 | 99.2 |
| Az19 | 96.3 | 97.7 | 98.8 | 99.0 | **99.8** |
| Az63 | 96.3 | 97.8 | 98.9 | 99.2 | **99.9** |

| | *rpoD* | | | | |
|---|---|---|---|---|---|
| | GSF71 $^{T}$ | Sp7 $^{T}$ | CC -Nfb -7 $^{T}$ | ptI -3 $^{T}$ | Az39 |
| Az3 | 95.9 | 99.0 | 98.5 | 97.3 | **99.6** |
| Az8 | **100** | 95.6 | 95.9 | 95.3 | 95.5 |
| Az19 | 95.8 | 98.8 | 98.4 | 97.2 | **99.9** |
| Az63 | 95.3 | 98.8 | 98.7 | 97.3 | **99.8** |

B

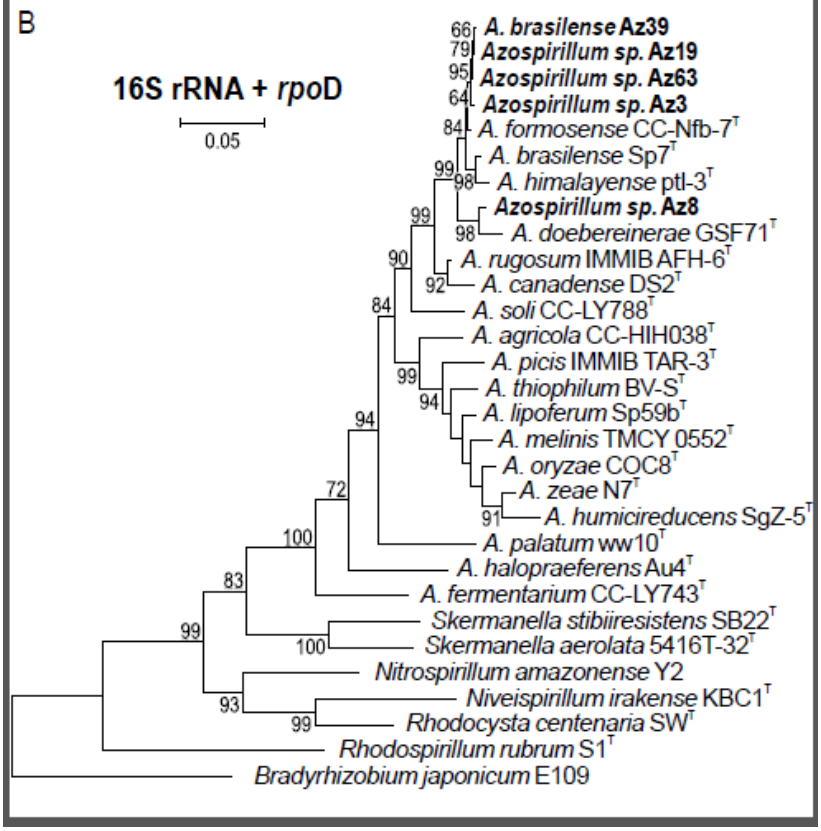

Fig 2

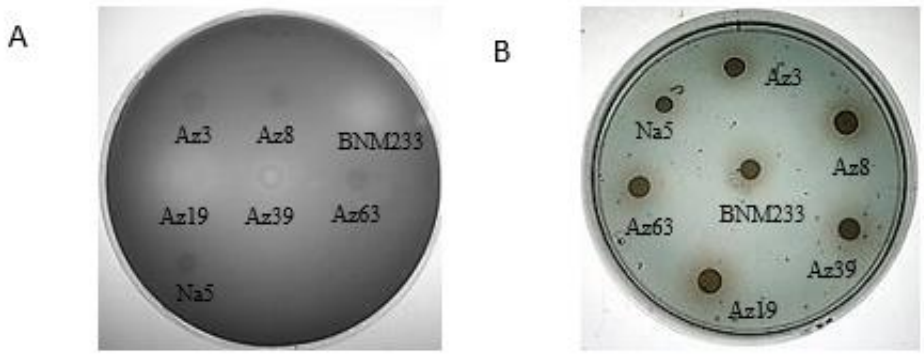


Fig 3

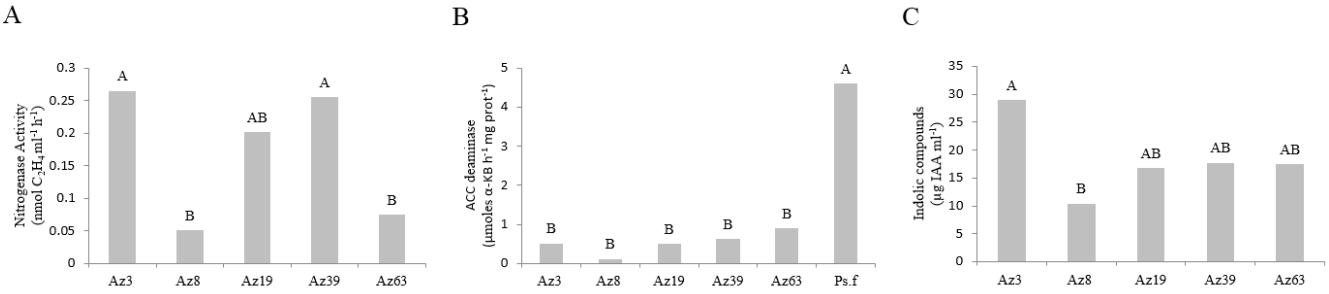

Fig 4

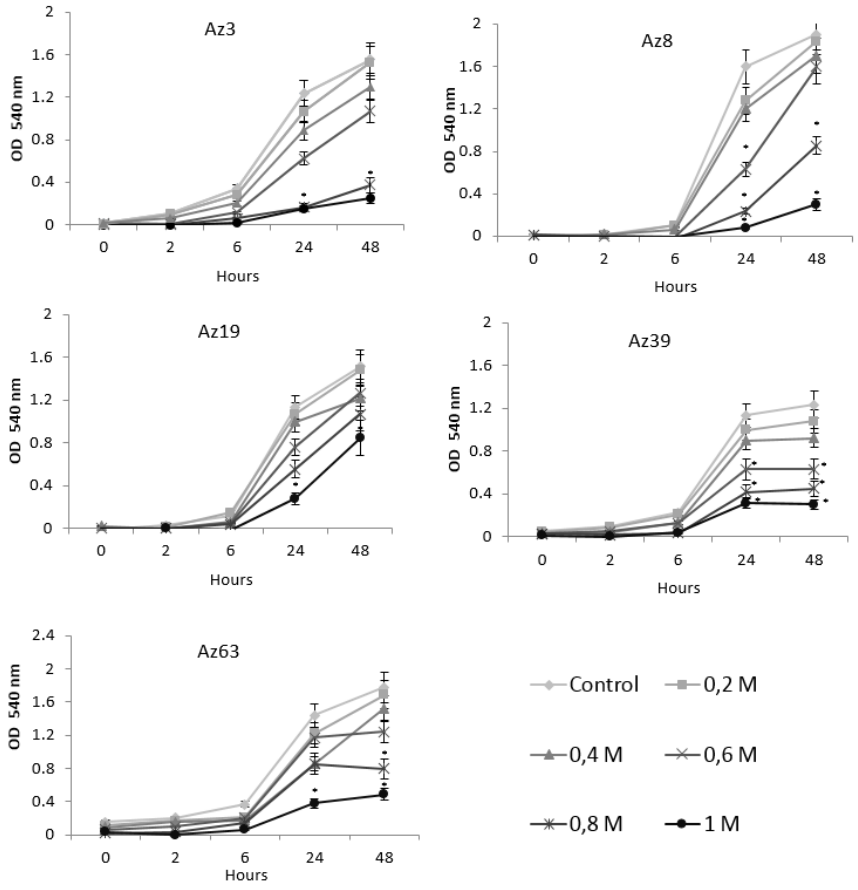

Fig 5

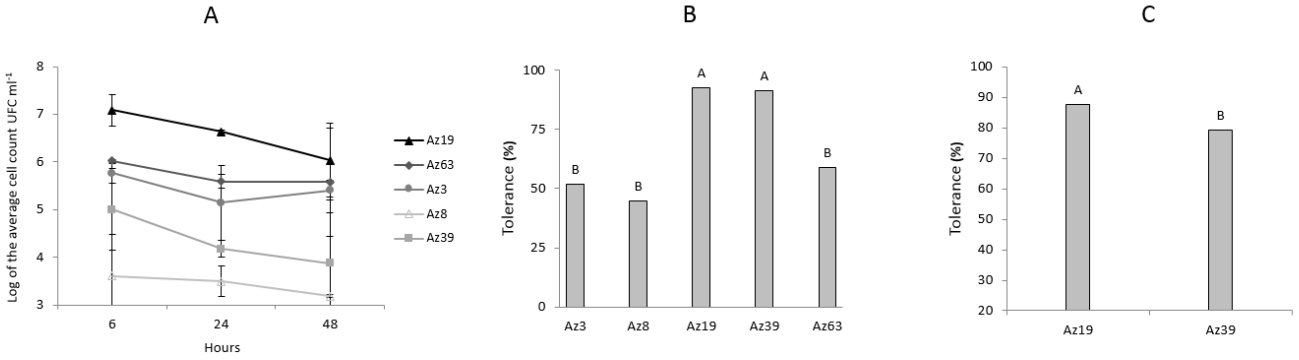


Fig 6

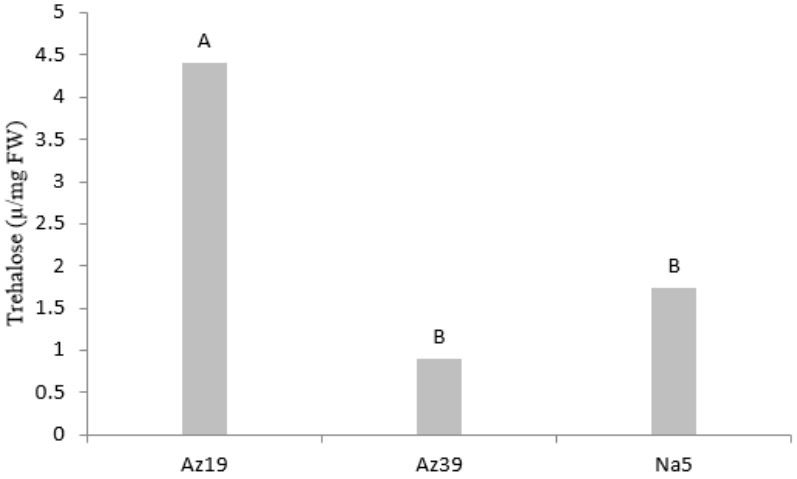

Fig 7

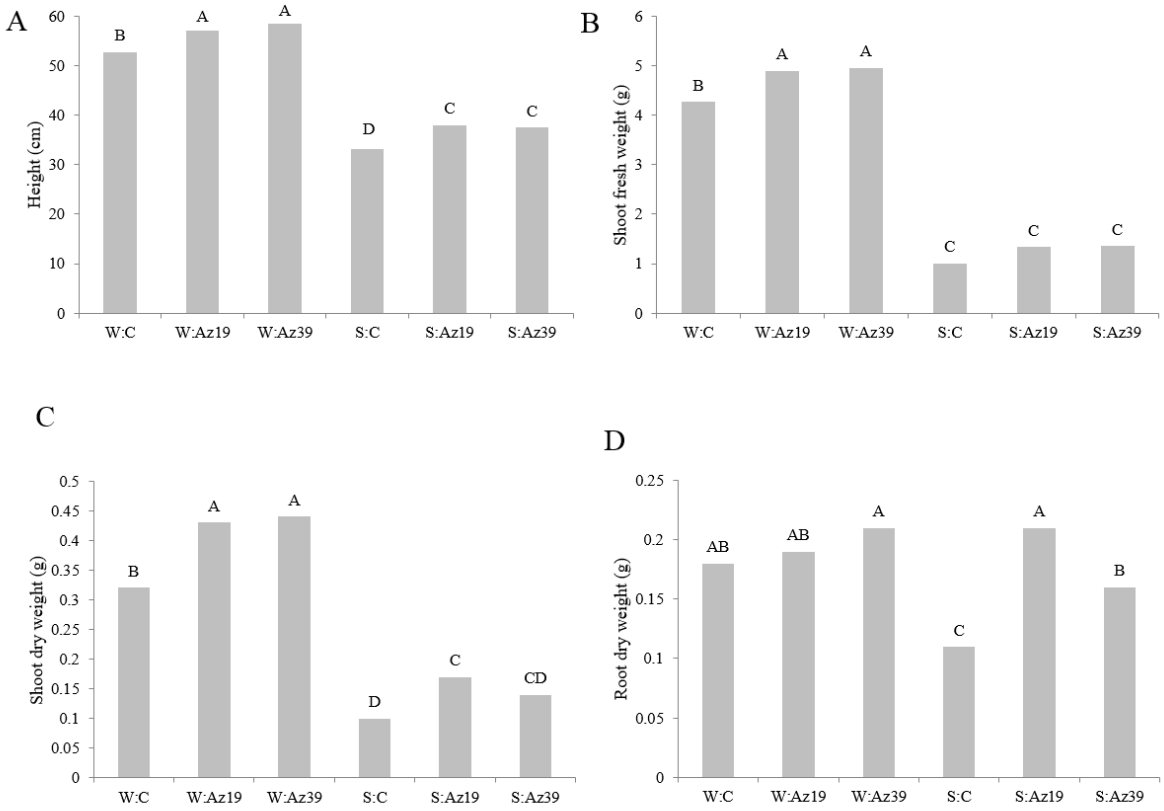

Fig 8

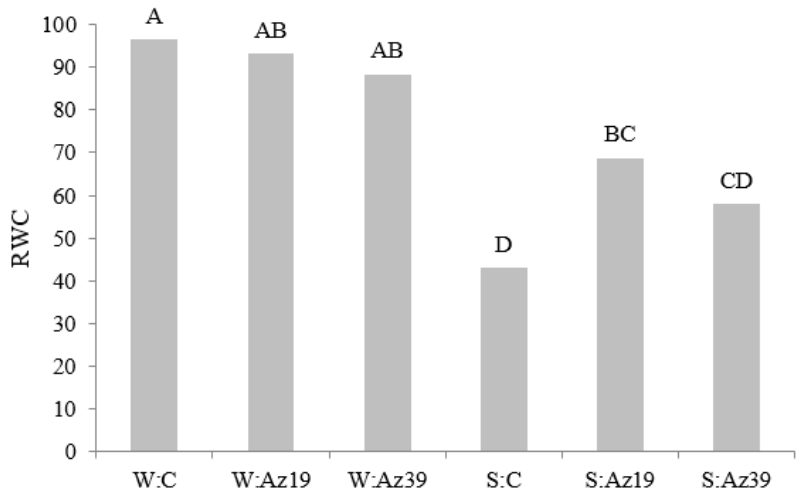


Fig 9

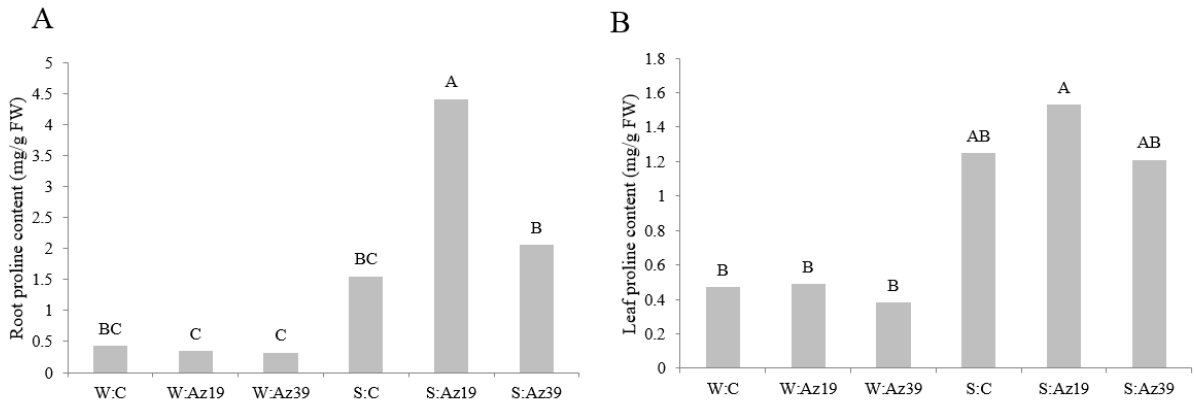

Table 1. Isolates origin

| Isolate | Origin |
|---|---|
| Az39 | Wheat roots, Marcos Juárez, Córdoba, Argentina |
| Az3 | Maize roots, Castelar, Buenos Aires, Argentina |
| Az8 | Soil of corn field, Villa Rumipal, Córdoba, Argentina |
| Az19 | Wheat fallow soil, Castelar, Buenos Aires, Argentina |
| Az63 | Soil from General Arenales, Buenos Aires, Argentina |

Table 2. Genbank accession numbers of isolated strains

| Isolate | Accession number |
|---|---|
| Az3 | KY399208 |
| Az8 | KY399209 |
| Az19 | KY399210 |
| Az63 | KY399211 |

Table 3. Number of colony forming units (CFU) per seed before sowing and per gram of fresh weight of maize roots after 7 days of inoculation

| Treatment | CFU per seed | Colonization | |
|---|---|---|---|
| | | Control | Stress |
| Uninoculated | | 1,10E+05 | 2,50E+02 |
| Az19 | 1E+07 | 2,50E+05 | 1,10E+02 |
| Az39 | 1,1E+07 | 1,10E+05 | 2,50E+02 |