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BASIC AREA - Article

Ammonium excretion, auxin production and effects of maize inoculation with ethylenediamine-resistant mutants of *Pseudomonas* sp.

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ABSTRACT: Plant growth-promoting bacteria (PGPB) comprise part of plant microbiome of biotechnological interest due to their potential to decrease the use of agrochemicals in agriculture. Among the commonly found PGPB species, the *Pseudomonas* genus is known for high competitiveness and efficiency in expressing growth-promotion traits. To increase the contribution of diazotrophic *Pseudomonas* sp. to the plant nitrogen nutrition, the strain AZM-01 was chemically mutagenized with methyl methanesulfonate (MMS), following the selection for resistance to ethylenediamine (EDA). From the 13 EDA-resistant mutant strains selected, four showed increased the ammonium excretion, with the highest value reaching up to 284% increase as compared to the wild strain, and six strains were found to produce significantly more auxins than the wild strain. Two independent inoculation trials with the wild and EDA-resistant *Pseudomonas* were performed on maize, with the objective to study the influence of bacteria on seed germination and its potential to promote maize growth under N-limiting condition. In general, *Pseudomonas* inoculation modified the root architecture of germinating seeds, and increased biomass of maize plants grown under N-limiting conditions. Shoot dry weight of maize was increased by inoculation with several EDA-resistant mutants as compared to the strain AZM-01, with emphasis on the EDA-5 strain which supports biomass accumulation at equivalent amount of plants grown under full N supply. Significant correlations between *in vitro* and *in vivo* parameters were found although low coefficient values predominate. The strategy of random mutagenesis was found suitable to develop PGPB strains with higher potential to supply maize plants with nitrogen.

Key words: biofertilizer, plant inoculation, methyl methanesulphonate, plant growth-promoting bacteria.

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INTRODUCTION

Representative strains of the plant microbiome with positive activity on growth and productivity of plants (called PGPB, or plant growth-promoting bacteria) have been studied as alternative biological input able to decrease, at least partially, the use of agrochemicals (Pieterse et al. 2016). The growth promotion through PGPB inoculation can occur by direct mechanisms, such as the nutrient provision, or by indirect mechanisms, such as the mitigation of stresses imposed to the host plant (Glick 2012). When the beneficial effects of PGPB inoculation imprints a better development of root system, the plants are thought to present a higher capacity to absorb water and nutrients from the soil. If the promotion of root growth comes together with the biological nitrogen fixation (BNF) ability, such microorganisms become extremely important for development of biofertilizers (Cassán et al. 2014).

Although the identification of diazotrophic bacteria in association with non-leguminous plants is common, the ability of these microorganisms to contribute effectively to nitrogen nutrition of the plant is still controversial (Santi et al. 2013; Cassán and Diaz-Zorita 2016). While the transfer of N fixed by symbiotic bacteria to the legume plants is a well-documented event, the mechanisms for N transfer involving non-symbiotic bacteria and non-leguminous plants are poorly understood. Regardless of the ecological condition assumed by diazotroph (symbiotic or non-symbiotic), the capacity to convert N_2 into NH_4^+ results from the nitrogenase enzymatic complex, whose activity is energetically costly and so, finely regulated at different levels, mainly by the availability of N.

The increase in BNF activity of diazotrophic associative bacteria to provide more N for the host plant can be achieved by the inhibition of the assimilation of NH_4^+ and/or the interference in the negative regulation of the nitrogenase enzymatic complex by ammonia. Previous studies reported that mutants with alterations in these functions excrete greater amounts of NH_4^+ and are more efficient at improving plant growth than their respective wild strains (Machado et al. 1991; Van Dommelen et al. 2009). The present work describes the random mutagenesis of *Pseudomonas* sp. strain AZM-01, a diazotrophic PGPB associative of maize, and the characterization of mutant strains resistant to ethylenediamine, aiming to obtain an improved strain with higher potential to promote the growth and the N-nutrition of maize.

MATERIAL AND METHODS

Bacterial strain and Molecular identification

The *Pseudomonas* sp. strain AZM-01 (formerly strain 9AGR01) was originally isolated from the maize rhizosphere and showed high potential to improve maize growth, as defined in a previous work (Costa 2013). This strain is available at the Plant Growth-Promoting Bacteria (PGPB) Collection of the Londrina State University, Brazil. This bacterium AZM-01 was cultivated in Dygs or JMV (Baldani et al. 2014) medium at 28 °C. Liquid cultures were incubated at 180 rpm in an orbital shaker, when needed.

For molecular identification, AZM-01 cells were cultivated Dygs liquid medium and concentrated by centrifugation. DNA was extracted according to the CTAB/NaCl method (Wilson 1997) and analyzed by 0.8% agarose gel electrophoresis. The DNA of the 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R (Lane 1991). The reaction mix contained 50 ng DNA, 1· PCR buffer, 20 pmol of each primer, 200 µM of dNTP, 2.0 mM of $MgCl_2$ and 1.5 U of Taq DNA polymerase. PCR thermal cycling consisted of initial denaturation for 2 min at 96 °C, followed by 35 cycles of denaturation (1 min, 94 °C), annealing (1 min, 58 °C) and extension (2 min, 72 °C). The 16S rRNA PCR products were electrophoresed in agarose gel and then purified with ExoSap PCR cleanup kit (Invitrogen), according to fabricant's recommendations. Partial 16S rRNA sequence was obtained on an ABI3500 automated sequencer (PE-Applied Biosystems) using the Big Dye kit (Invitrogen) and the 27F primer, according to fabricant's recommendations.

The phylogenetic identification of AZM-01 was performed with the Classifier and Sequence Match tools available in the Ribosomal Database Project (RDP, https://rdp.cme.msu.edu/). The phylogenetic relationships among the AZM-01 strain and the representative species of the 19 phylogenetic groups of *Pseudomonas* genus, as defined by Gomila et al. (2015), was performed with the program Mega 6 (Tamura et al. 2013). The GenBank accession number of 16S rRNA partial sequence of the *Pseudomonas* sp. AZM-01 is MF074244.

Random Mutagenesis

For the mutagenesis assays, the *Pseudomonas* sp. AZM-01 strain was grown in 10 mL of liquid JMV medium for 24 h at 28 °C and 180 rpm. The cells were counted in a Neubauer chamber and normalized to 5×10^9 cells·mL^{-1}. To determine the tolerance to UV radiation, 1 mL of cell suspension was transferred to a petri dish placed at 40 cm of distance from UV radiation source ($\lambda \sim 253$ nm) and exposed to increasing time periods (0, 5, 10, 20, 30 and 40 seconds, corresponding to a fluency of 0, 318, 636, 1272, 1908 and 2544·Jm^{-2} respectively). The methyl methanesulfonate (MMS) tolerance was determined by exposure of cell suspension to increasing concentrations (0, 2, 4, 6, 8 and 10 mM) of MMS. After 30 min at 28 °C, the MMS was removed by two washes with phosphate buffer solution (NaCl 137 mM phosphate, 10 mM KCl, 2.7 mM, pH 7.4). The survivors' percentage to mutagenesis treatments was determined by counting of colony-forming units (CFU) in solid JMV culture medium after incubation for 72 h at 28 °C. The assays were performed in triplicate. The tolerance to ethylenediamine (EDA), used as a selection agent of mutants of interest, was assessed by plating of the cell suspension (5×10^4 cells·mL^{-1}) in JMV solid medium added with increasing concentrations of EDA (0, 1.5, 7.5, 11.25, 15 and 30 mM), in triplicate for each concentration. The survivor's percentage to EDA treatments was determined as described above.

The mutagenesis of *Pseudomonas* sp. AZM-01 was performed through physical agent (UV radiation), chemical agent (MMS), or a combination of both methods (UV followed by MMS) in the conditions established in the tolerance tests described above. Colonies of EDA-resistant mutants were selected after 72 h of growth in JMV medium with EDA and then transferred to liquid JMV medium. After growth for 24 hours (28 °C, 180 rpm), cell suspension was homogenized with sterile glycerol (1:1, v/v) and stored at –20 °C.

Assessment of ammonium production

The ability of EDA-resistant mutants to excrete ammonium was determined by the indophenol colorimetric method (Chaney and Marbach 1962). The bacteria were cultivated in 10 mL of JMV liquid medium with 10 mM of glutamic acid for 120 h at 28 °C and 180 rpm. Although the JMV medium was designed as an N-free culture medium for diazotrophs, EDA-resistant mutant strains grew poorly in the absence of glutamic acid, suggesting changes in the ammonium assimilation pathway ruled by the enzymes GS and GOGAT and the loss of their prototrophic capacity when subjected to aerobic growth. After bacterial growth, the culture supernatants were obtained by centrifugation (8000 g, 10 min) and used for the ammonium quantification. The cellular biomass was resuspended in 1 mL of NaOH 0.1 M, incubated for 30 min at 90 °C for cell lysis and was then centrifuged at 8000 g for 10 min. The Bradford protein assay was used for determination of protein content of cell suspensions. All assays were performed in triplicate for each strain.

Auxin production

The production of auxins by EDA-resistant mutants of *Pseudomonas* sp. was determined by Salkowski colorimetric method. Even though the Salkowski reagent is not specific to auxins, its use has been regularly performed to screen IAA-producing strains and to express the amount of IAA produced by plants, fungi and PGPB from several phylogenetic groups. In this sense, the results presented as auxin production may include indoleacetic acid, indolepyruvic acid and indoleacetamide (Glickmann and Dessaux 1995).

The cells were cultured for 48 h at 28 °C in 5 mL of liquid Dygs medium added with 100 µg·mL^{-1} of DL-tryptophan. After the growth, cells were centrifuged (8000 g, 5 minutes) and the supernatant (0.5 mL) was homogenized with 1.0 mL of Salkowski's reagent (40 mM $FeCl_3$; 7.9 M H_2SO_4). The reaction was incubated for 30 min in the dark, followed by the absorbance reading at 540 nm. The auxin quantity was determined by a calibration curve prepared with increasing concentrations of indole-3-acetic acid (IAA; Sigma). The cellular biomass was used for protein determination as described previously. All assays were performed in triplicate for each strain.

Germination test of inoculated maize seeds

The influence of the EDA-resistant mutants of *Pseudomonas* sp. in the initial growth of maize plants was determined by a germination test and analysis of development of maize seedlings at seven days post-inoculation (DPI). For →

the preparation of the inoculant, bacteria were cultivated in Dygs liquid medium for 12 hours at 30 °C and 180 rpm and normalized to a concentration of 1.0×10^6 cells·mL^{-1}. The maize seeds (AG 2040 hybrid, Monsanto) were disinfested (alcohol 95% for 30 s, H_2O_2 for 10 min and two washes with sterile water) and then immersed for 5 min in the inoculant suspension (48 seeds per strain). Inoculated seeds were spread over the wet germination papers (28·38 cm), which were divided in four germination sets with 12 seeds each. Each germination set was an experimental unit replication, where biometric values were taken as means of the 12 plantlets germinated. The germination sets were stored in plastic bags to avoid desiccation and then incubated for seven days under a photoperiod of 12 hours, temperature of 28 ºC and humidity of 40%. As control, the seeds were immersed in Dygs liquid medium. The dry weight of roots and shoots was assessed by gravimetric analysis and the architecture of the root system (length, diameter, area and volume) was determined by the GiA Roots software (Galkovskyi et al. 2012).

Effectiveness of wild and mutant strains in maize growth promotion

A completely randomized experimental design was conducted to investigate the effectiveness of mutant and wild *Pseudomonas* in promoting maize growth under nutritional N-limiting conditions. Pots filled with 2 kg of an unsterilized substrate, prepared by mixing sand and oxisol with high clay content (78%) in a proportion of 2:1 (v:v), were seeded with three seeds of the maize hybrid 2B610 (Dow AgroSciences) and placed in a greenhouse. The substrate presented the following composition: pH (in H_2O), 5.3; H + Al (cmolc·dm^{-3}), 11.02; K (cmolc·dm^{-3}), 0.51; Ca (cmolc·dm^{-3}), 5.4; Mg (cmolc·dm^{-3}), 2.1; Al (cmolc·dm^{-3}), 0.43; P (mg·dm^{-3}), 16.0; and organic matter (%), 2.42. Inoculants were prepared as described above, and plants were inoculated with 1 mL of bacterial suspension five days after sowing. The experimental design comprehended 16 treatments with five replicates, distributed as follow: 15 treatments where plants grown under N-limiting conditions (inoculation with the wild and the 13 different EDA-resistant strains, plus an uninoculated control), and a single control treatment with uninoculated plants grown under full N supply. The different doses of nitrogen were obtained by weekly application of Hoagland's nutrient solution (Hoagland and Arnon 1950) depleted (2 ppm N) or not (20 ppm of N) in nitrogen, supplemented as KNO_3. The plants were grown for 30 days with substrate held at field capacity during the growth period by applying 100 mL of distilled water to each pot every two days. The growth-promotion effects were evaluated by the following biometric parameters: root volume, root dry weight, shoot dry weight and plant dry weight.

Statistical Analysis

The results from ammonium and auxin production by the wild and mutants strains, as well as the results from the germination test and the maize growth-promotion were subjected to Shapiro-Wilk's test to verify the normal distribution and variance of the data and the mean values were compared by the Scott-Knott test ($p < 0.05$). A Pearson's correlation analysis and respective p-value of significance was calculated from the mean values of the bacterial biochemical characteristics and the growth-promotion of seedlings and plants to explore the relationships between those parameters. All analyses were performed with the aid of the software R (http://www.r-project.org).

RESULTS

Phylogenetic relationships of AZM-01 strain were examined by comparing their partial 16S rRNA gene sequence with the RDP database. According RDP analyses, AZM-01 is a member of the genus *Pseudomonas* and had greater similarity to sequences of *P. mosselii* and *P. guariconensis*. The reconstruction of phylogenetic relationships among AZM-01 and *Pseudomonas* type strains representatives of 19 phylogenetic groups of *Pseudomonas* genus (Gomila et al. 2015), positioned AZM-01 in the same branch of the species *P. mosselii*, *P. guariconensis* and *P. entomophila*, with high bootstrap value (Fig. 1). *P. mosselii* was originally isolated from clinical specimens and some isolates have biotechnological importance, including for agriculture (Dabboussi et al. 2002; De La Torre-Ruiz et al. 2016). *P. guariconensis* was identified colonizing the rhizosphere of leguminous *Vigna unguiculata* and has potential to solubilize inorganic phosphates and to promote plant growth (Toro et al. 2013; Patel et al. 2015). →

P. entomophila was originally isolated as a entomopathogen of *Drosophila melanogaster*, with no report of its occurrence in clinical samples so far (Mulet et al. 2012).

The tolerance of AZM-01 to UV radiation and to the chemical compounds MMS and EDA are shown in Table 1. Based on the results obtained, the exposure to UV radiation for 5 s and 4 mM of MMS were established for the induction of physical and chemical mutagenesis, respectively. The concentration of 30 mM EDA was established as appropriated for screening of EDA-resistant mutants (Table 1). After selection, thirteen EDA-resistant strains were obtained, four from mutagenesis with MMS (EDA-1 to EDA-4) and nine from the combination of UV radiation and MMS (EDA-5 to EDA-13). No mutant was obtained from exclusive treatment with UV radiation, indicating that the use of chemical agent, isolated or in combination with UV radiation, was more effective for mutagenesis induction in AZM-01.

The ammonium excretion capacity by the EDA-resistant mutants was indirectly determined by measuring the NH_4^+ concentration in the supernatant of wild-type and mutant strains (Table 2). Mutant strains presented variable NH_4^+

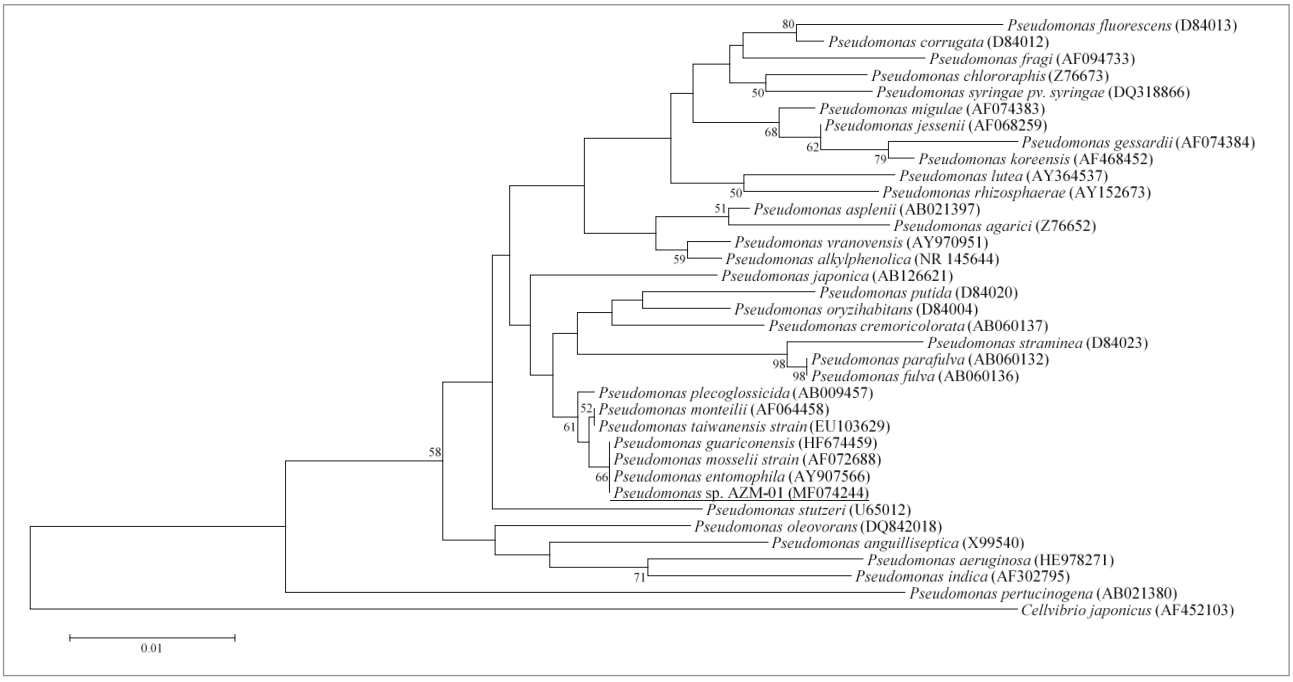


Figure 1. Phylogenetic tree of the 35 type strains of representative species of *Pseudomonas* phylogenetic groups and the strain AZM-01 (shown underlined) used in this study. The respective GenBank access numbers are presented in the brackets. The Jukes-Cantor method was applied to construct distance matrices followed by generation of dendrogram by neighbor-joining. The outgroup is represented by *Cellvibrio japonicus* Ueda107, and bootstraps values higher than 50% after 1000 replicates are indicated at branches.

Table 1. Tolerance of *Pseudomonas* sp. strain AZM-01 to ultraviolet radiation (UV) and to chemical compounds methyl methanesulfonate (MMS) and ethylenediamine (EDA).

UV			MMS			EDA		
T[(a)]	CFU[(b)]	%[(c)]	[mM]	CFU[(b)]	%[(c)]	[mM]	CFU[(b)]	%[(c)]
0	5.75×10^{10}a	100a	0	5.75×10^{10}a	100a	0	8.91×10^{3}a	100a
5	2.24×10^{9}b	3,9b	2	9.33×10^{9}b	16,2b	1.5	8.13×10^{3}b	91,9b
10	6.03×10^{8}c	1,1b	4	2.34×10^{9}c	4,1c	7.5	7.94×10^{3}b	88,9b
20	2.19×10^{8}d	0,4b	6	5.50×10^{8}d	1,0c	11.25	6.61×10^{3}c	74,0c
30	1.23×10^{7}f	< 0,1b	8	8.91×10^{7}e	0,2c	15.0	0,00d	< 0,1d
40	1.73×10^{7}e	< 0,1b	10	1.86×10^{6}f	< 0,1c	30.0	0,00d	< 0,1d

[(a)]Exposure time (s) to UV radiation. [(b)]Colony forming units (CFU mL^{-1}) were obtained after 72 h of growth in JMV solid medium at 28 °C. [(c)]Percent of CFU decrease in relation to controls. Means followed by the same letters within each column do not differ significantly according to the Scott-Knott test ($p < 0.05$) applied to $\log_{10}$ transformed values.

Table 2. Determinations of ammonium (NH_4^+) and auxins in cultures of the wild strain and EDA-resistant mutants of *Pseudomonas* sp. AZM-01.

Strains	NH_4^+/Protein (µM/mg)[(a)]	%	Auxin/Protein (µg/mg)[(a)]	%
AZM-01	1445.7 ± 53.9	100b	10.7 ± 1.6	100b
EDA-1	2129.7 ± 1376.6	147b	8.6 ± 2.4	80b
EDA-2	4104.0 ± 567.7	284a	25.3 ± 8.9	236a
EDA-3	1656.0 ± 449.8	114b	14.6 ± 3.3	136b
EDA-4	2093.8 ± 941.9	145b	12.4 ± 1.0	116b
EDA-5	2777.1 ± 311.6	192a	8.6 ± 2.3	80b
EDA-6	3899.3 ± 363.0	270a	19.1 ± 4.6	179a
EDA-7	934.5 ± 119.7	65b	6.5 ± 3.2	61b
EDA-8	960.8 ± 708.0	66b	6.2 ± 2.2	58b
EDA-10	3161.4 ± 28.3	219a	23.5 ± 9.5	220a
EDA-11	2055.9 ± 64.0	142b	4.0 ± 0.9	37b
EDA-13	757.6 ± 174.8	52b	20.7 ± 6.2	193a
EDA-14	1383.4 ± 243.2	96b	21.5 ± 5.4	201a
EDA-16	751.4 ± 151.4	52b	28.4 ± 9.7	265a
CV (%)	38.41		38.83	

[(a)]Ammonium and auxins (IAA-equivalent) were determinate by indophenol blue and Salkowski methods, respectively. Means followed by the same letters in the column do not differ significantly according to the Scott-Knott test ($p < 0.05$).

excretion capacities: five strains showed a decrease (down to 52%) and eight strains had increments (up to 284%) in the NH_4^+ concentration when compared to AZM-01 strain, which had 1445.7 µM NH_4^+ mg^{-1} of protein. Nonetheless, significant differences between the mutants and the wild strain AZM-01 values were markedly positive variations, and among all strains tested, four strains excreted significantly more NH_4^+ than wild-type strain: EDA-2 (284%), EDA-5 (192%), EDA-6 (270%) and EDA-10 (219%).

As observed for the ammonium excretion, the concentration of auxins in supernatant varied among the selected mutants, which showed a decrease (down to 37%) to an increase (up 265%) when compared to AZM-01 (10.7 µg of auxins·mg^{-1} of protein). Significant differences in production were also related to increased values and was observed in six mutants, as compared to AZM-01: EDA-2 (236%), EDA-6 (179%), EDA-10 (220%), EDA-13 (193%), EDA-14 (201%) and EDA-16 (265%).

The effects of inoculation of EDA-resistant mutants on initial growth of maize plants are displayed in Table 3. Significant differences in all growth parameters were observed between the non-inoculated plants (control) and the plants inoculated with any of the EDA-resistant mutants or the AZM-01 strain, indicating that the inoculation treatments affected, at different levels, the maize development. Interestingly, most inoculation treatments decreased significantly the shoot plant biomass, even in plants inoculated with the wild-type strain. Only plants inoculated with the mutants EDA-1, EDA-2, EDA-6 and EDA-7 showed no significant differences in the shoot dry weight in comparison to the control treatment. Regarding the root biomass, the plants inoculated with AZM-01, EDA-3, EDA-5 and EDA-16 exhibited values significantly lower than the control treatment, while the other inoculation treatments did not affect the root biomass significantly.

However, the architecture of the root system of maize plants was strongly affected by inoculation of AZM-01 or EDA-resistant strains, as shown in Fig. 2. The maximum length of the root system was significantly increased by inoculation of all strain tested, with exception of EDA-16 strain, which did not differ from the control treatment. A similar effect was observed for the mean root diameter: the roots were significantly wider (at different extents), except when plants were inoculated with the mutant EDA-10; meanwhile the inoculation treatments with the mutant strains EDA-11, EDA-13, EDA-14 and EDA-16 resulted in thinner roots than those observed in control plants. Most inoculation treatments also increased the area and volume of the root system, with exception of two treatments, which resulted in similar values to the control in both (EDA-8 inoculation) or just one (EDA-16 inoculation) of these parameters.

Table 3. Initial development of maize plants (AG 2040 hybrid, Monsanto) as affected by inoculation of wild and EDA-resistant mutant strains of *Pseudomonas* sp. AZM-01. All parameters were determined seven days after inoculation (DAI).

Strains	Shoots (mg)	Roots (mg)	Lenght[a] (cm)	Diameter (µm)	Area (cm²)	Volume (mm³)
Control[b]	470.7 ± 15.2a	638.0 ± 27.2a	23.6 ± 0.12e	422 ± 2.5f	67.0 ± 2.5d	1011 ± 41.8d
AZM-01	420.0 ± 28.4b	585.3 ± 32.4b	25.7 ± 0.30c	467 ± 0.8a	77.3 ± 4.7b	1288 ± 76.2b
EDA-1	489.7 ± 38.0a	647.7 ± 43.4a	27.1 ± 0.32a	463 ± 2.8b	84.4 ± 2.5a	1388 ± 44.0a
EDA-2	469.0 ± 6.5a	662.0 ± 5.9a	26.5 ± 0.17b	453 ± 1.3c	80.8 ± 1.8a	1301 ± 27.6b
EDA-3	421.3 ± 30.2b	615.0 ± 50.2b	26.2 ± 0.24b	459 ± 0.9b	76.9 ± 0.2b	1257 ± 7.1b
EDA-4	406.0 ± 16.4b	694.0 ± 2.2a	26.6 ± 0.25b	450 ± 1.1c	70.8 ± 1.9c	1136 ± 30.9c
EDA-5	359.3 ± 3.1b	621.7 ± 12.4b	26.4 ± 0.27b	462 ± 0.7b	75.2 ± 0.8b	1235 ± 7.6b
EDA-6	432.3 ± 22.3a	648.3 ± 24.2a	26.9 ± 0.34a	452 ± 3.2c	75.2 ± 2.4b	1203 ± 16.3b
EDA-7	438.3 ± 43.3a	686.0 ± 58.3a	25.8 ± 0.48c	442 ± 3.9d	71.1 ± 0.4c	1118 ± 16.0c
EDA-8	380.3 ± 30.6b	660.3 ± 68.0a	25.2 ± 0.18c	434 ± 2.0e	66.5 ± 0.1d	1053 ± 15.6d
EDA-10	389.3 ± 10.5b	687.7 ± 20.2a	26.3 ± 0.20b	419 ± 3.0f	84.6 ± 1.2a	1275 ± 36.1b
EDA-11	382.7 ± 13.3b	707.3 ± 18.6a	26.3 ± 0.35b	414 ± 4.5g	83.4 ± 0.7a	1248 ± 16.0b
EDA-13	412.3 ± 15.2b	709.3 ± 27.7a	25.3 ± 0.17c	404 ± 1.3h	83.3 ± 0.8a	1232 ± 5.0b
EDA-14	365.7 ± 27.4b	670.7 ± 43.6a	24.3 ± 0.15d	414 ± 1.7g	74.6 ± 0.7b	1124 ± 10.9c
EDA-16	403.7 ± 40.9b	552.3 ± 13.0b	23.2 ± 0.43e	387 ± 2.2i	71.0 ± 1.8c	1042 ± 32.8d
CV (%)	7.58	6.61	1.35	6.8	3.08	3.23

[a]Root length, diameter, area and volume were determined using GiARoots software. [b]Non-inoculated control. Means followed by the same letters in the column do not differ significantly according to the Scott-Knott test ($p < 0.05$).

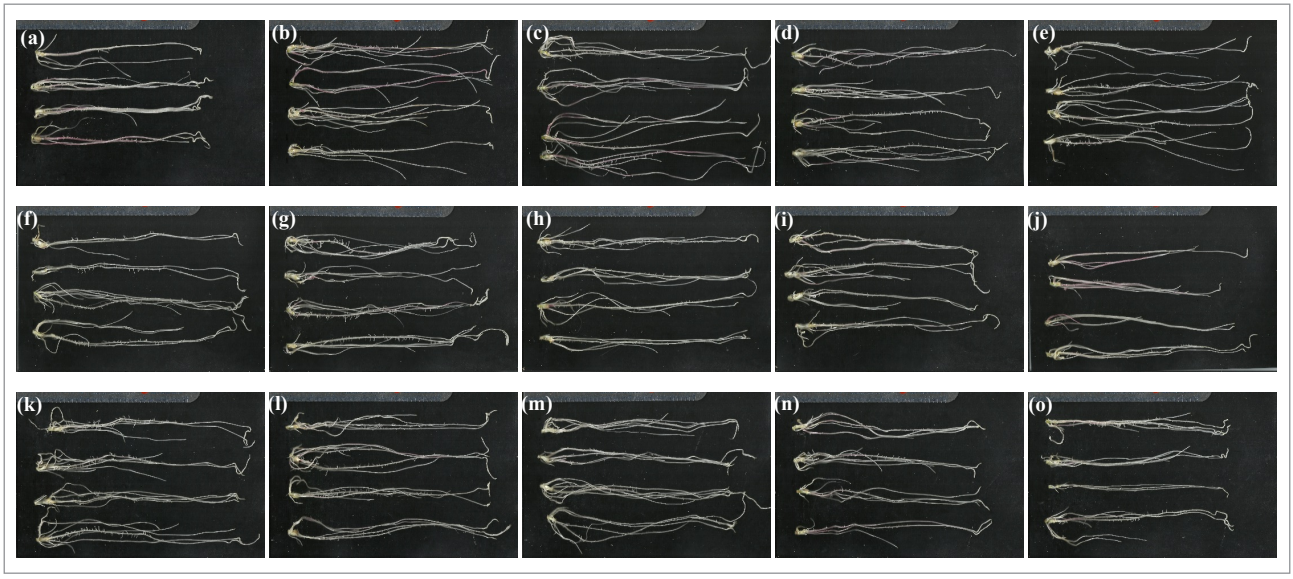


Figure 2. Representative images of maize seedlings (AG 2040 hybrid, Monsanto) as affected by inoculation of wild and EDA-resistant mutant strains of *Pseudomonas* sp. AZM-01 at seven days after inoculation.

The results of inoculation trial under greenhouse conditions is presented in Table 4, demonstrating that the nitrogen availability and the inoculation treatments imprint significant modifications of maize growth. Control (uninoculated) plants at N-limiting condition had lower biomass (shoots, roots and plant biomasses) and lower root volume than control plants growing at full N supply, reinforcing the need for N input in the experimental system to favor adequate plant growth. Furthermore, inoculation with the wild strain AZM-01 showed improved development when compared to control plants under same N availability (2 ppm N, limiting N supply), as a result →

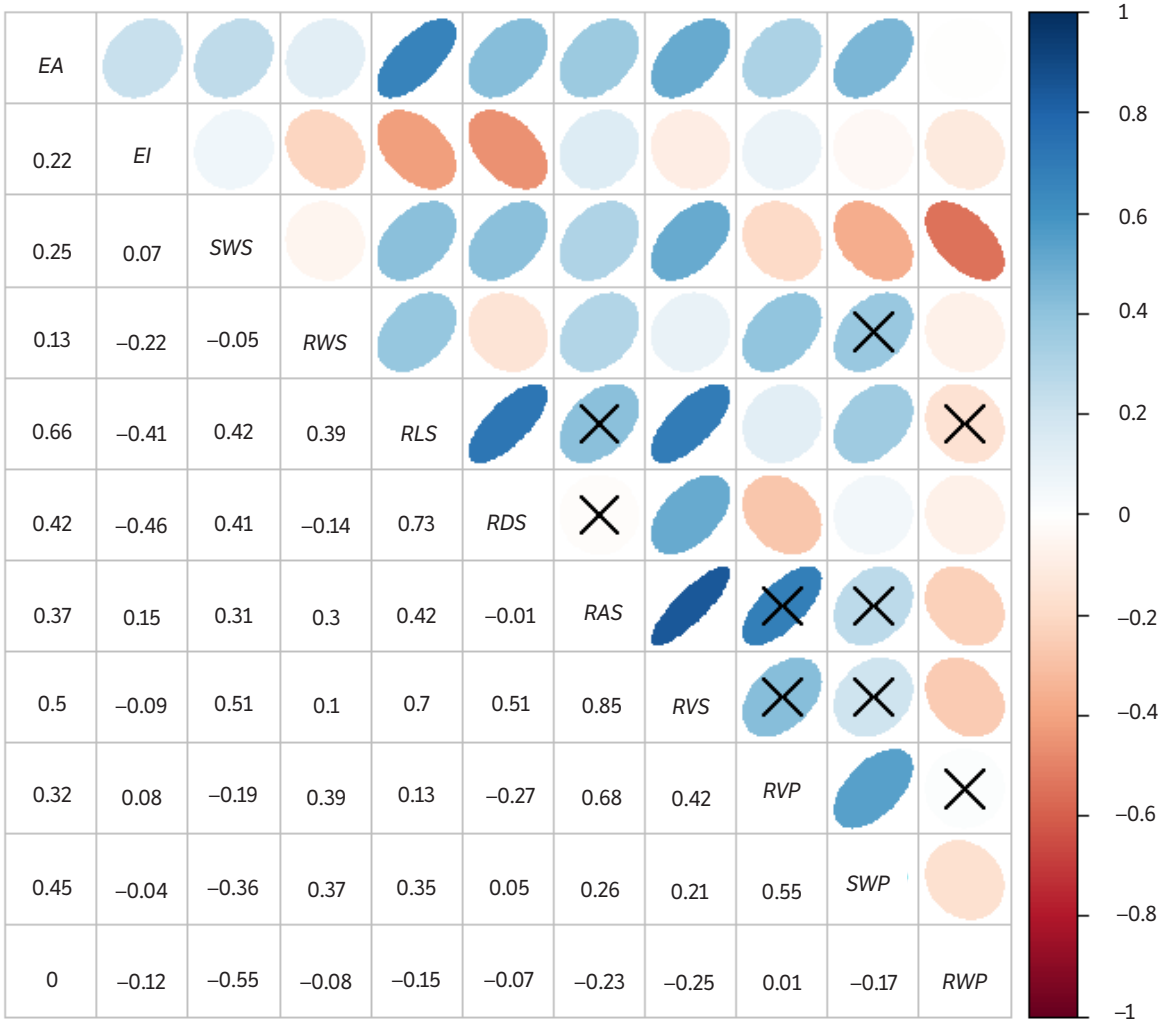
Table 4. Biometric variables of maize plants grown for 30 days in pots filled with sterilized sand as substrate under different nitrogen levels (20 and 2 ppm of N; N+ and N- respectively) and incoculated with wild (AZM-01) and EDA-resitant mutants of *Pseudomonas* sp. Control treatments are related to uninoculated plants and inoculation treatments were conducted at 2 ppm of N. Values are means ± standard errors.

Strains	Shoot dry weight (g)	Roots dry weight (g)	Plant dry weight (g)	Root volume (cm^3)
Control - N[(a)]	0.17 ± 0.10c	0.43 ± 0.06b	0.60 ± 0.13d	4.3 ± 0.58c
Control + N[(b)]	0.42 ± 0.06a	0.75 ± 0.15a	1.17 ± 0.05a	5.3 ± 0.55b
AZM-01	0.33 ± 0.04b	0.63 ± 0.03a	0.95 ± 0.07b	5.0 ± 0.50b
EDA-1	0.35 ± 0.02a	0.47 ± 0.07b	0.82 ± 0.27c	5.3 ± 0.82b
EDA-2	0.38 ± 0.07a	0.56 ± 0.15b	0.94 ± 0.05b	5.3 ± 0.96b
EDA-3	0.40 ± 0.07a	0.47 ± 0.26b	0.87 ± 0.10b	4.3 ± 0.96c
EDA-4	0.39 ± 0.02a	0.53 ± 0.13b	0.92 ± 0.10b	4.0 ± 0.74c
EDA-5	0.38 ± 0.05a	0.76 ± 0.13a	1.14 ± 0.16a	4.8 ± 0.50b
EDA-6	0.35 ± 0.04b	0.60 ± 0.06a	0.95 ± 0.11b	4.8 ± 0.96b
EDA-7	0.33 ± 0.05b	0.61 ± 0.13a	0.94 ± 0.10b	4.3 ± 0.82c
EDA-8	0.37 ± 0.05a	0.65 ± 0.02a	1.02 ± 0.18b	4.8 ± 0.50b
EDA-10	0.44 ± 0.10a	0.61 ± 0.05a	1.02 ± 0.02b	6.5 ± 1.26a
EDA-11	0.41 ± 0.03a	0.57 ± 0.05b	0.98 ± 0.05b	6.3 ± 0.50a
EDA-13	0.32 ± 0.03b	0.63 ± 0.13a	0.95 ± 0.13b	5.0 ± 0.50b
EDA-14	0.39 ± 0.03a	0.55 ± 0.14b	0.94 ± 0.19b	5.8 ± 0.96a
EDA-16	0.32 ± 0.02b	0.59 ± 0.07a	0.93 ± 0.09b	4.3 ± 0.96c
CV (%)	15.18	20.09	13.64	15.41

[(a)]Non-inoculated control plants grown at 2 ppm of N as KNO_3. [(b)]Non-inoculated control plants grown at 20 ppm of N as KNO_3. Means followed by the same letters in the column do not differ significantly according to the Scott-Knott test ($p < 0.05$).

of its growth-promotion characteristics. Nevertheless, the AZM-01 inoculated plants differ from fully N supplied control plants, presenting lower biomass accumulation for the shoot and plant dry weights parameters. Maize inoculation with EDA-mutant strains had, in general, a better development than control plants with limiting N-supply, except for the root volume, where the mutants EDA-3, EDA-4, EDA-7 and EDA-16 had no effect. None of these strains had an apparent improvement of the ammonium-excretion capability, and only the EDA-16 mutant strain showed higher production of indolic compounds than the wild strain, although no improvement on root development was observed in plants inoculated with this mutant strain. Considering the four EDA-mutants with increased ammonium excretion activity, only the EDA-5 inoculation resulted in plant biomass accumulation at equivalent amount of the control plants grown under full N-supply.

Since some EDA-resistant mutants showed significant differences in relation to AZM-01 strain on most of the parameters analyzed (biochemical parameters, seed germination and plant growth; EDA-2, EDA-6 and EDA-10 mutants), a correlation analysis was performed to identify possible interactions among these variables (Fig. 3). Most of pairwise comparisons showed significant covariations patterns, although the respective correlation coefficients were usually low. Significant ($p < 0.05$) negative and positive correlations with Pearson's coefficients below $r = -0.40$ or above $r = 0.40$ (here considered moderate values for linear relationships) were found in 18 from the 55 possible interactions studied. Moderate negative correlations were observed between the amount of indolic compounds and the length ($r = -0.41$) and diameter ($r = -0.46$) of root seedlings, and between the shoot dry weight of seedlings and the root dry weight of plants ($r = -0.55$). On the other hand, positive linear correlations comprised the ammonium excreting capacity and root length of seedlings ($r = 0.66$), root diameter of seedlings ($r = 0.42$), volume of seedling's root ($r = 0.50$) and the shoot dry weight of plants ($r = 0.45$). Seven positive correlations with values above 0.40 were related to the growth parameters of seedlings among them (shoot dry weight versus the length, diameter and volume of roots; root length versus diameter and volume of roots; and root volume versus diameter and area of roots). A single positive correlation between the growth parameters of plants among them was also observed (root volume versus shoot dry weight). No significative correlations between growth parameters in seedlings and plants were evident.



Strong correlations are indicated by flat ellipses with higher color density whereas weak correlations are indicated by rounded ellipses with weak color density, as indicated in the scale bar

Correlation values between variables are indicated in the lower triangular portion of the matrix, and insignificant correlations ($p > 0.05$) are evidenced by "X" mark in the ellipses at upper triangular portion of the matrix

EA extracellular ammonium
EI extracellular indolic compounds
SWS shoot dry weight of seedlings
RWS root dry weight of seedlings
RLS root length of seedlings
RDS root diameter of seedlings
RAS root areas of seedlings
RVS root volume of seedlings
RVP root volume of plants
SWP shoot dry weight of plants
RWP root dry weight of plants

Figure 3. Correlation matrix of associations between biochemical and growth-promotion characteristics of seedlings and plants inoculated with the wild and EDA-resistant mutant strains of *Pseudomonas* sp. AZM-01.

DISCUSSION

The ability to fix atmospheric nitrogen is not common in species of *Pseudomonas* genus, which comprises more than 140 species with a great metabolic diversity (Venieraki et al. 2014). The diazotrophic capacity of *Pseudomonas* species seems to have been acquired through horizontal gene transfer, not being considered a determining taxonomic characteristic. The phylogenetic positioning of the AZM-01 strain within the *P. putida* group, where other plant growth promoting *Pseudomonas* sp. species are also classified, reinforces the potential use of that strain for biotechnological purposes. A clear identification of AZM-01 at specie level, however, requires further studies,

since most diazotrophic *Pseudomonas* sp. species are phylogenetically close to *P. stutzeri* (Venieraki et al. 2014).

Horizontal gene transfer is a common mechanism observed in bacteria, which allows them to improve metabolic fitness and endurance to outcompete other species or unfavorable conditions found in each habitat, in addition to its role in bacteria evolution. In general, genes acquired by horizontal transfer tend to be eliminated from the genome quickly, due to the high cost of energy and reducing power required for its maintenance and activity (MacLean and Millan 2015). Differently from other diazotrophs, where the nitrogenase genes are vertically transmitted due to chromosomal location, *Pseudomonas* are thought to acquire the biological nitrogen fixation genes by plasmid-mediated horizontal gene transfer, resulting in the need for a positive cost-benefit balance for its maintenance (Van Dommelen et al. 2003; Pascuan et al. 2015). However, the genetic engineering of *P. protegens* with nitrogenase genes from *P. stutzeri* has already been performed, resulting in modified strains that expressed the nitrogenase constitutively, excreted more NH_4^+ and had higher capacity to improve the plant growth (Setten et al. 2013). Thus, the improvement of plant growth-promoting strains by either molecular or classical methods is a highly promising field in microbiology, having potential to contribute to the development of highly efficient bioproducts and to the reduction of chemical inputs use in agriculture.

Inhibitors substances of the enzymes involved in nitrogen assimilation, like the compounds ethylenediamine (EDA) and L-Methionine-DL-Sulfoximine (MSX), have allowed to identify mutants with greater ability to excrete ammonia, higher diazotrophic activity and constitutive nitrogen fixation activity, even in presence of high levels of ammonium, which is a negative BNF regulator (Christiansen-Weniger and Van Veen 1991; Machado et al. 1991; Srivastava 2006). Here, random mutagenesis and screening for EDA-resistant mutants of *Pseudomonas* sp. AZM-01 enabled the selection of four mutant strains (EDA-2, EDA-5, EDA-6 and EDA-10) that excreted more ammonia (up to 284% on strain EDA-2) than AZM-01 strain. Such results indicate alteration in the ammonium assimilation and/or in the regulation of the nitrogenase enzymatic complex, whose activity is suppressed transcriptionally by ammonium availability (Setten et al. 2013).

As evidenced by the Salkowski analysis, some EDA-resistant mutants showed increases in the production of indolic compounds, although no correlation with the ammonium excretion capacity was observed. In *A. brasilense*, the production of indoleacetic acid was shown to be increased under impaired N_2-fixing condition by adding NH_4^+ to the culture medium (Hartmann et al. 1983), while *Rhizobium tropici* shown an inhibition of indoleacetic acid production by ammonium (Imada et al., 2017), suggesting some relation between the N-availability and the biosynthesis of indolic compounds. Regardless the apparent relationship on auxin production and nitrogen metabolism and in addition to this topic being neglected in the literature, is not clear if both reported traits are under coordinated expression. A recent study by Defez et al. (2017) reported increases in nitrogenase activity in mutant strains of diazotrophic bacteria transformed to increase the production of IAA, indicating co-regulation of both IAA and BNF traits in different bacterial strains. The study by Thuler et al. (2003) showed that *Beijerinckia derxii* can produce IAA at higher levels under BNF conditions. Nevertheless, the BNF activity and IAA biosynthesis were separated in time; while the BNF occur in early growth stage the IAA was increased at the stationary growth phase.

Plant inoculation with beneficial bacteria, as the *Pseudomonas* strain used in this study, are known to induce changes in plant architecture due to modification of the plant endogenous hormonal balance (Mosimann et al. 2016; Calvo et al. 2017). The potential of *Pseudomonas* AZM-01 and derivative EDA-resistant mutants to produce auxins was demonstrated in the presented study, and this trait should be related to the modifications on roots of plants inoculated with the wild and mutant strains. PGPB diazotrophic species of *Azospirillum* genus are the most studied and the most common bacteria in commercial inoculants (Cassán and Diaz-Zorita 2016). Ammonium-excreting mutants of *A. brasilense* improved the accumulation of biomass and N of wheat plants (Christiansen-Weniger and Van Veen 1991; Van Dommelen et al. 2009). Moreover, they altered morphology and growth of the root system, suggesting a phytohormonal effect on wheat growth. As reported earlier, ammonium-excreting mutants of *A. brasilense* can supply all the nitrogen requirement of *Setaria viridis* (Pankievicz et al. 2015). Besides the *Azospirillum* genus, diazotroph mutants of other PGPB species obtained either by induced mutagenesis or genetic transformation showed greater plant-growth promoting →

capacity than their wild-type strains. Genetically modified strains of *Pseudomonas* genus, for instance, had higher efficiency to supply the plant with BNF-derived N or to promote biomass accumulation, when compared to wild-type strain (Setten et al. 2013; Fox et al. 2016).

Beneficial mutualistic associations between the plant and their microorganisms occurs only when the environment imposes some limitation to plant development that can then be supplied by the association; otherwise, the high microbial populations can represent a significant drain of energy and result in inferior growth of the host plant (Partida-Martínez and Heil 2011). In the present study, the plant-growth promoting capacity was evaluated in two independent trials: a short duration assay where the plant developed at the expense of the seed reserves, and a greenhouse trial where plants needed to acquire nutrients from the soil. The seed-germination trial is expected to mainly point out the influence of inoculated bacteria on the plant hormonal balance, while the greenhouse trial was designed to highlight the potential of the wild and EDA-mutant strains to favor the maize growth under N-limiting conditions.

Although no significant increases of root and shoot biomasses were observed in seedlings germinated in the presence of the AZM-01 wild strain or any of the EDA-resistant mutants, some mutants had not reduced the plant biomass (shoot and/or root), as observed in the plants inoculated with the wild strain. In fact, the inoculation of wild *Pseudomonas* sp. or its EDA-resistant mutants had shown greater influence on the architecture of the root system of seedlings, as observed by the values of length, diameter, area and volume, which were mostly increased. These alterations reflect a higher proliferation of roots in maize seedlings in response to inoculation, confirming the influence of PGPB in the hormonal endogenous imbalance of plants, as observed in other studies (Cassán et al. 2014). This is further evidenced by the significant negative correlations, although under weak coefficient values, observed between the *in vitro* production of indolic compounds by the studied strains and the biomass, length, diameter and volume of root seedlings. For instance, plants inoculated with the EDA-16 mutant, which had the highest ability to produce indolic compounds according to the Salkowski assay, presented the lowest root biomass as compared to the other treatments. As reported earlier (Cassán et al. 2014), the plant response to auxin can vary from beneficial to harmful, depending on the concentration and sensitivity of plant tissue. It is interesting to note that ammonium excretion capacity showed positive correlations for the parameters related to root architecture of seedlings (includding two moderate coefficient values), in opposition to the effect of indolics compounds over these variables.

The identification of promising PGPB is time-consuming, due to the lack of a reliable biomarker that allows the selection of elite PGPB strains based on biochemical or genetic analysis, leading to the need to perform inoculation trials for the screening of isolate collections (Finkel et al. 2017). Nevertheless, since the *in vivo* screening of large microbial collections is not trivial, sequential approaches based on *in vitro* analysis of the expression of the interest growth-promotion traits, followed by inoculation experiments to validate PGPB strains, are the common strategy adopted even if there is no consensus on the application of such approaches to select for PGPB candidates (Smyth et al. 2011; Beneduzi et al. 2013; Barnett et al. 2017). This study presented the results of two independent inoculation trials, in which few variables have shown significant correlations coefficients at moderate linear interaction range among them. This corroborates the complexity developed in associative plant-bacteria interactions and the importance to execute *in vivo* assays to determine the effectiveness of a given PGPB. This is clearly demonstrated by the maize inoculation with *Pseudomonas* AZM-01 strain, which showed detrimental effect in plant growth at the germination assay, but was effective in promoting maize growth when plants were grown for a longer period of time under N-deficient condition. The greenhouse trial also suggests that the maize N-nutritional status was improved by inoculation of several of the EDA-resistant mutants that increased the shoot biomass as compared to the wild strain AZM-01, since the shoot biomass in maize is strongly affected by nutrient availability, specially nitrogen (Li et al. 2012). The improvement of maize growth by the EDA-resistant mutants is corroborated by the moderate correlation coefficient found for the shoot biomass accumulation and ammonium concentration, in agreement to the inoculation effect observed for the root architecture of seedlings.

Considering the set of data obtained, while the mutants EDA-2 and EDA-6 showed great potential for →

the development of bioproducts, according to the results from the germination trial, the performance of these strains in promoting maize growth did not differ from the wild strain performance, as observed in the greenhouse trial results. Although the higher capacity of EDA-2 and EDA-6 mutants to excrete ammonium to produce auxins and improve the root architecture of maize seedlings could favor the exploitation of soil and the absorption of nutrients and water and, consequently, improve plant growth (Bhattacharyya and Jha 2012; Cassán et al. 2014), these potentials were not fully expressed when plants were grown for a longer period of time. The EDA-5 mutant, which showed the lowest capacity to excrete ammonium among the four mutants that differed from the wild strain, however, showed great potential to increase maize growth under N-limiting conditions. Variability in the plant response to PGPB inoculation according to the period of plant development used to access the inoculation effects has been already reported (Smyth et al. 2012, Calvo et al. 2017), but reasons that lead for this variability are currently poorly understood. Further physiologic and genetic studies related to BNF and to biosynthesis of auxins, as well as studies on the plant-bacteria interaction are needed to offer a better comprehension of the mechanisms by which the *Pseudomonas* sp. strains promotes plant growth. The development of mutant strains by random mutagenesis is reinforced by the present study as an important tool to develop high-performance inoculant strains with increased potential to provide biologically fixed nitrogen to non-legume plants.

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