Effects of *Azospirillum brasilense* applied along with herbicides on maize

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Abstract

The aim of this work was a study of a native strain (Az39) and a reference strain (Sp7) of *Azospirillum brasilense*, evaluating their effects on commercial herbicides like glyphosate and atrazine applied in maize. *In vitro* assays were performed to determine the survival capacity of both strains by adding each herbicide with and without the addition of carbon or nitrogen. A growth chamber experiment was carried out with maize plants, and the treatments consisted on applications of atrazine and glyphosate, with and without foliar inoculations of each strain. Morpho-physiological variables were determined in V6. The *in vitro* assays determined that the survival capacity of both strains was different in the presence of herbicides; Az39 was the only one able to survive both to the application of glyphosate and atrazine and was able to use glyphosate as the sole source of carbon. In maize plants, both herbicides affected the foliar area, the aerial and the radical biomass negatively and increased the damage of cell membranes. Most of the negative effects of both agrochemicals were mitigated with Az39, while both strains ameliorated the damage at the radical biomass. Therefore, the native strain has better adaptive characteristics against herbicides widely used in maize. This highlights the importance of the use of native microorganisms for effective inoculations in crops.

Introduction

In recent years, the use of beneficial microorganisms has been widely recognized due to its positive effects on the yield of many crops and the feasibility of developing a sustainable agriculture (García *et al*., 2017). Studies carried out for more than 20 years indicate that bacteria of the genus *Azospirillum* have a strong affinity for the roots of *Poaceas* (Mehnaz, 2015), highlighting their ability to stimulate plant growth (Pedraza *et al*., 2010; Vacheron *et al*., 2013; Gomez *et al*., 2014), yield (Dobbelaere *et al*., 2001; Costa, 2015), nitrogen content in plants (Richard *et al*., 2018), nitrogen fixation (Bashan and De-Bashan, 2010), radical proliferation and development of hormonal activities (Bashan and De Bashan, 2010; Cassán *et al*., 2011; Cohen *et al*., 2015). In recent years, there has been a concern about this species regarding the capacity for survival and degradation of commercially used xenobiotics. Some authors have shown that *Azospirillum* can survive to the presence of herbicides and they can degrade xenobiotics (Travaglia *et al*., 2015; Singh and Singh, 2016). One of the most studied species corresponds to *A. brasilense*, particularly to the strain Az39 that is isolated from the rhizosphere of wheat in Argentina. This strain was selected for its efficiency to promote the growth of this crop and has been distinguished for its capacity to increase yield and productivity in wheat and maize. These results led to the

National Agricultural Health Service (SENASA) together with companies producing inoculants, to recommend its formulation to be applied in seeds of numerous cereals and other crops (Cassán *et al*., 2015). In relation to this, there are commercial biofertilizers based on *Azospirillum* spp. However, its application is not always effective, which is why the use of native microorganisms is preferred. They adapt to the climatic conditions and can compete successfully with the native biota. For this reason, in this work, this strain is evaluated in a comparative way with the Sp7 strain. DNA homology studies indicated that within the species of *A. brasilense*, the Sp7 strain was the first described and it is considered as a strain of reference (Tarrand *et al*., 1978; Lin *et al*., 2015).
One of the crops in which this species is used as an inoculant is maize, which represents one of the most important sources of income for the agricultural sector in Argentina (Hallauer *et al*., 2010). However, there are various factors that affect its production. One of them is the competition that weeds exert and the control of them, in the great majority of cases, is achieved with the timely application of herbicides, which is an extended practice (Sannino and Gianfreda, 2001; Petcu *et al*., 2015). The use of transgenic varieties of this glyphosate tolerant crop has helped certain regions in which this herbicide is transformed into the

compound used mainly to control the growth of weeds during the crop cycle (Bonny, 2016). It is one of the most mobile herbicides through the phloem and is especially effective against perennial weeds, as it accumulates in the tissues of stems, roots and storage organs. Its co-metabolic degradation depends on the microbiological activity of the soil. Nevertheless, over the years, the incorrect use of glyphosate caused a large number of weeds to resist to it. Currently, the objective is to use agrochemical mixtures that include glyphosate as the main and broad-spectrum herbicide together with other active residual ingredients (Metzler *et al*., 2016). Among the herbicides, one of the most used is atrazine that is derived from triazines (Salazar-Ledesma *et al*., 2018). Like glyphosate, biodegradation is one of the main processes responsible for the elimination of atrazine (Sene *et al*., 2010). Some studies evaluate the effects of glyphosate on rhizobacteria (Zabaloy *et al*., 2008; Aguirre-Cadena *et al*., 2014; Barriuso *et al*., 2011; Arango *et al*., 2014; Newman *et al*., 2016), although there is no evidence regarding the effects of atrazine in relation to *Azospirillum*. In turn, in conventional agriculture, inoculation of plant growth promoting rhizobacteria (PGPRs) at seed level is carried out, although its effectiveness depends on its characteristics and its interaction with the soil. In this sense, we have begun to investigate the effectiveness of the foliar application of biological formulations, since the incorporation of the inoculant pretends to take advantage of the intrinsic capacity of the bacteria to produce compounds with high biological activity, independently of the edaphic conditions (Zanettini and Puente, 2017). Based on this, a new proposal of inoculation at the time of sowing (seedtime) could be the sprinkling of these bacteria at leaf level, which would represent a new technological proposal (Di Palma *et al*., 2017). In addition, the use of these biological compounds has also reduced the application of chemical fertilizers in various crops (Alori and Babalola, 2018), which can contribute to lower costs, as well as being an alternative to the contamination of soil and subsoil by chemical fertilizers. This background generates the following question: Can the environmental problem generated by inputs for maize cultivation be solved with the use of PGPRs and application strategies? Based on this, a comparative study between the native strain (Az39) and the reference strain (Sp7) of *A. brasilense* was proposed, evaluating their effects on commercial herbicides like glyphosate and atrazine, both *in vitro* and in maize plants.

Results

In vitro assays

The survival capacity of both strains was different regarding the presence of glyphosate. It affected the growth of the Sp7 strain negatively, observing significantly lower OD values (0.07 ± 0.05) with respect to the control (1.4 ± 0.005) as well as with the viable cell counts (0.63 x 10^4 and 1.3 x 10^8 CFU mL^{-1}, respectively) (Fig.1 a). On the contrary, in the case of strain Az39, this herbicide did not affect its growth when it was applied in its culture medium (OD control and glyphosate: 1.41 ± 0.005 and 96 ± 0.002). This strain was able to grow with the addition of glyphosate with a count of 1.2 x 10^{11} CFU mL^{-1}, achieving its maximum growth peak at 36 h of incubation, establishing a longer lag phase than the control treatment, although this treatment presented counts of the same order (Fig.1 b).

On the other hand, the use of the herbicide atrazine did not affect the growth of each strain, although strain Az39 achieved higher growth values with respect to Sp7. The reference strain showed an OD of 1.001 ± 0.2 with a count of 5.8 x 10^8 CFU mL^{-1} at 24 h of incubation, with similar values to the control, being the same as 0.9053 ± 0.05 and 2.8 x 10^8 CFU mL^{-1}. Contrarily, the native strain reached an OD of 1.22 ± 0.05 at 24 h incubation with a viable bacterial count of 1.9 x 10^{11} CFU mL^{-1}, whereas in the control, it was 1.4 and 2.1 × 10^{11} CFU mL^{-1}, respectively (Fig. 1).

Herbicides as nutritional source

In the assays carried out to evaluate whether this strain uses herbicides as a nutritional source, it was determined that *A. brasilense* Az39 was not able to use atrazine as a nitrogen source, given that after 70 h of incubation its growth in the liquid medium containing atrazine as the sole source of nitrogen was not observed. However, this strain was able to use the glyphosate-based herbicides as the sole carbon source, showing a slower growth compared to the control, with a longer lag phase of 12 h, achieving its maximum growth peak at 60 h of incubation with a count of 10^{11} CFU mL^{-1} (Fig. 1 b). The Sp7 strain was not able to survive neither use glyphosate as the sole carbon source nor atrazine as a nitroge source (Fig. 1 a).

Maize plants assays

In the assays carried out in plants under controlled conditions, glyphosate affected the aerial and root dry biomass negatively (Fig. 2), it affected the leaf area and the chlorophyll *a* content, and increased the cell membrane damage with respect to the control without application of the herbicide (Table 1). Some of these effects, such as radical dry weight (Fig. 2 b) and the content of chlorophyll *a* (Table 1), were ameliorated when they were sprayed with both strains, while in the measurements of the leaf area, only the native strain was able to increase significantly compared to the treatment with glyphosate without inoculation. In addition, Az39 was also able to diminish the membrane damage caused by this herbicide (Table 1).

With the application of atrazine, the maize plant decreased their radical dry weight (Fig. 2 b), the chlorophyll *a* content and the foliar area (Table 1) with respect to the control without the application of the herbicide. In those treatments, where both the reference strain and the native strain were applied, the radical dry weight variable increased significantly (Fig. 2 b), while in the chlorophyll content, only the native strain had significantly higher values compared to the treatment with the herbicide without inoculation (Table 1). None of the strains was able to reverse the negative effect of the herbicide caused on the foliar area and on the cell membrane damage measurements (RCR %) (Table 1).

Factorial design for both bacterial strains determined the absence of herbicide-bacterial strain interaction. In the plants inoculated with the reference strain Sp7, a negative effect was observed with the treatment of glyphosate compared with atrazine in all the variables presented (DWR (g): 0.08 ± 4.1 x 10^{-3} b: 0.09 ± 0.01 a; DWAP (g): 0.14 ± 0.01 b: 0.20 ± 0.01 a; FA (cm^2): 43.17 ± 1.5 b: 49.08 ± 1.5 a; RCR (%): 34.77 ± 2.36: 25.56 ± 3.34 b). Chlorophyll content (mg g$^-$^{1} FW) was higher in plants treated with glyphosate compared to those plants treated with atrazine (105.26 ±

Table 1. Content of chlorophylls *a* (mg g^{-1} FW), relative conductivity rate (% RCR) and foliar area (cm^2) of maize plants at 28 days post-sowing.

Treatments	Chlorophylls *a* (mg g^{-1} FW)	RCR (%)	Foliar area (cm^2)
Control	109.5 ± 23.76 [a]	15.10 ± 2.639 [c]	57.05 ± 2.724 [a]
G	84.27 ± 2.907 [c]	30.71 ± 4.892 [a]	42.90 ± 2.675 [c]
A	66.60 ± 6.198 [d]	21.31 ± 6.717 [ab]	48.10 ± 1.952 [b]
Sp7	150.0 ± 29.44 [a]	16.45 ± 3.530 [b]	55.78 ± 1.916 [a]
Az39	95.12 ± 5.152 [b]	24.26 ± 2.745 [b]	56.60 ± 2.031 [a]
G + Sp7	106.1 ± 17.74 [a]	33.00 ± 2.315 [a]	44.46 ± 1.629 [c]
G + Az39	137.1 ± 18.62 [a]	21.66 ± 2.571 [b]	53.74 ± 1.552 [b]
A + Sp7	70.63 ± 5.972 [d]	22.80 ± 3.191 [b]	51.16 ± 2.087 [b]
A + Az39	77.34 ± 8.883 [cd]	18.59 ± 2.827 [bc]	51.23 ± 1.099 [b]

Treatments: C: distilled water applications; G: applications of glyphosate (1 L 100L^{-1}); A: applications of atrazine (1 Kg 100 L^{-1}); 7: foliar applications of Sp7; 39: foliar applications of Az39; G7: applications of glyphosate and Sp7; G39: applications of glyphosate and Az39; A7: applications of atrazine and Sp7; A39: applications of atrazine and Az39. Different letters on each column indicate statistically significant differences.
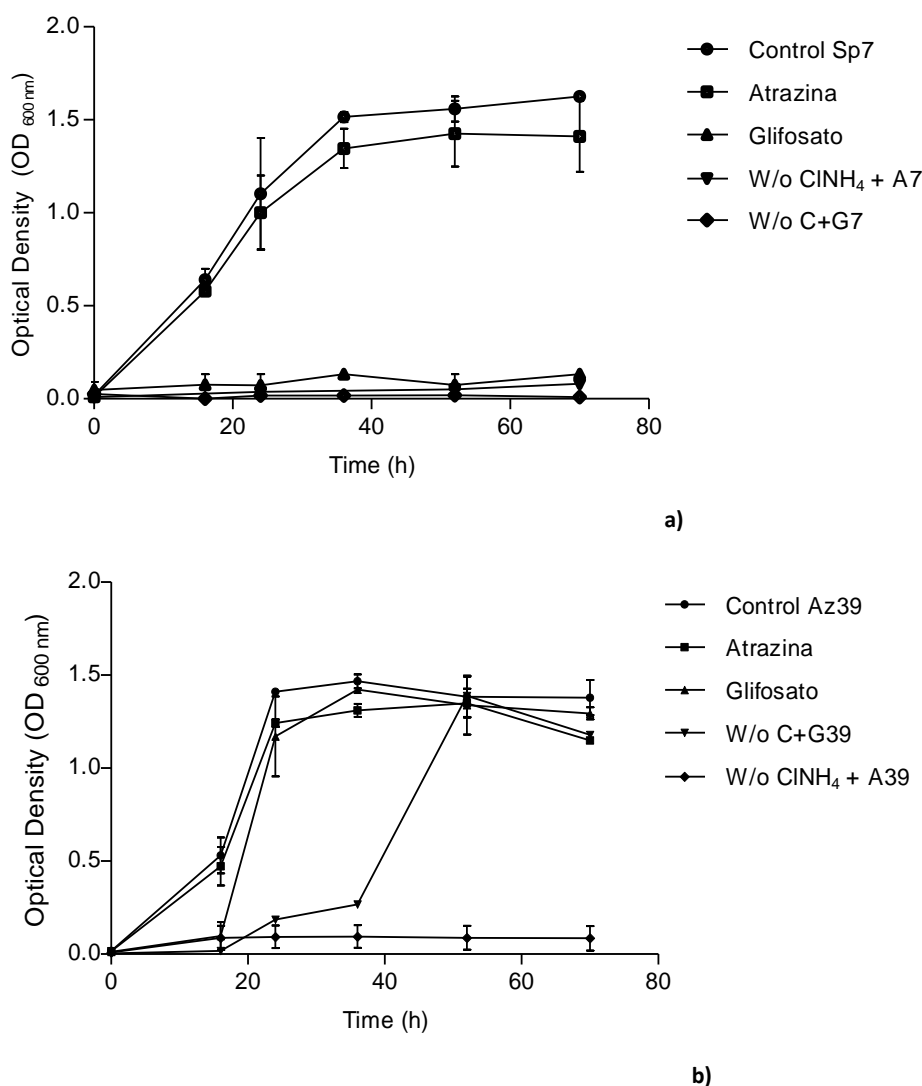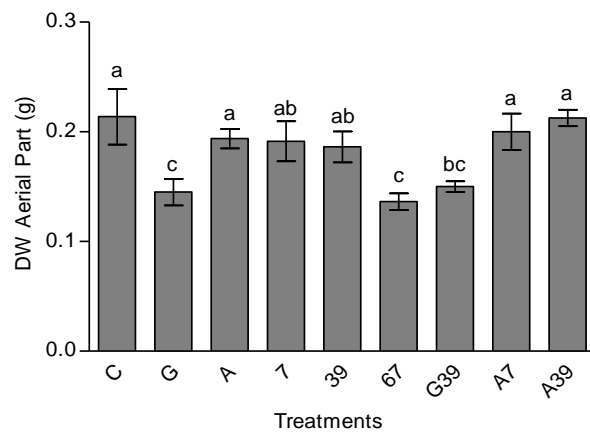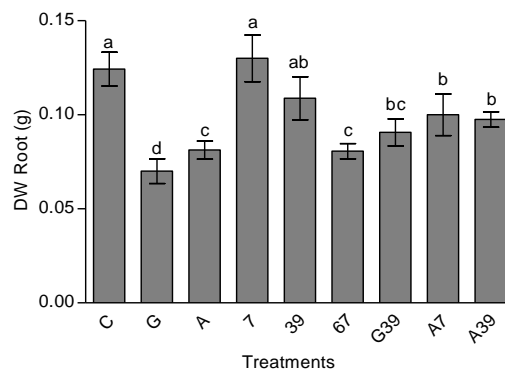

a)

b)

Fig 1. Growth curves of two strains of *Azospirillum brasilense*. a) Sp7 in Fructose culture medium. Treatments: Sp7: control without herbicides (○); A7: + atrazine (0.04 g L^{-1}) (▲); G7: + glyphosate (0.5 g L^{-1}) (▢); W/o ClNH$_4$ + A7: without addition of ClNH$_4$ as a source of N + atrazine (▼); W/o C + G7: without addition of malic acid as a source of C + glyphosate (◊); b) Az39 in NFb culture medium. Treatments: Az39: control without herbicides (○); A39: + atrazine (0.04 g L^{-1}) (▲); G39: + glyphosate (0.5 g L^{-1}) (▢); W/o ClNH$_4$ + A39: without addition of ClNH$_4$ as a source of N + atrazine (▼); W/o C + G39: without addition of malic acid as a source of C + glyphosate (◊).

a)

b)

Fig 2. Dry weight (g) of aerial part (a) and root (b) of maize plants 28 days after sowing. Treatments: C: distilled water applications; G: applications of glyphosate (1 L 100L^{-1}); A: applications of atrazine (1 Kg 100 L^{-1}); 7: foliar applications of Sp7; 39: foliar applications of Az39; G7: applications of glyphosate and Sp7; G39: applications of glyphosate and Az39; A7: applications of atrazine and Sp7; A39: applications of atrazine and Az39. Different letters on each bar indicate statistically significant differences (p <0.05).

7.94 a: 70.10 ± 8.37 b, respectively). In radical biomass, a beneficial effect of the inoculation was observed (inoculated: 0.09 ± 4.9 x 10^{-3} a; uninoculated: 0.08 ± 4.9 x 10^{-3} b). In plants treated with the native strain Az39, a beneficial effect of inoculation was observed in DWR (g) (0.09 ± 0.01 a: 0.08 ± 0.01 b), chlorophylls *a* (mg g^{-1} FW) (104 ± 8.05 a : 76.89 ± 9 b), foliar area (cm^2) (51.74 ± 1.35 a : 45.04 ± 1.35 b) and RCR (%) (21.76 ± 2.85 b : 31.61 ± 2.79 a), compared to uninoculated plants.

Discussion

The ability of *A. brasilense* Az39 to survive at the presence of commercial herbicides like glyphosate and atrazine under the doses tested was demonstrated. The ability to use glyphosate as the sole source of carbon has also been proved. From the ecological point of view, this is an important factor taking into account that the use of these chemicals would not affect the population of the bacterial strain, and that glyphosate could be metabolized by the bacterium with its consequent removal from the environment. In relation to this, the experiments of Aguirre-Cadena *et al*. (2014) also adhere to this idea. They observed that when applying glyphosate, the population of *Azospirillum* in the rhizosphere of wheat plants did not decrease and was even higher in the presence of this herbicide. Similar results are cited by Zabaloy *et al*. (2008), affirming a stimulation of the microbial activity after the

application of glyphosate, mediated by carbon and nitrogen mineralization, without affecting the microbial biomass of the soil. Schlatter *et al*. (2017) found that repeated glyphosate applications had minimal impacts on soil and rhizosphere bacterial community composition and diversity. The *in vitro* capacity of the bacterium to tolerate the presence of this herbicide in its commercial (isopropylamine salt of N-phosphonomethyl glycine) and non-commercial formula (Isopropylamine salt) and the capacity to degrade it when it was introduced in their environment as an additional source of carbon in low concentrations was observed (Travaglia *et al*., 2015). However, many authors (Zablotowicz and Reddy, 2004; Zobiole *et al*., 2010; Martínez-Nieto *et al*., 2011; Nandula and Tyler, 2016) point out that the use of these chemicals is detrimental to the viability of rhizosphere bacteria, among them, those corresponding to the group of biological nitrogen fixers. In this sense, the results obtained in this study can be related to those obtained by the authors mentioned previously, indicating that the reference strain (Sp7) could be within that group in which its viability in the presence of glyphosate is affected. This highlights the importance of the use of native strains such as Az39 since it can survive the presence of both herbicides tested in this work. This gives an adaptive advantage over other strains, probably due to the region in which it was isolated with a history of application of these agrochemicals. This supports the assertion by Garcia *et al*. (2010), indicating that although there are commercial

biofertilizers of *Azospirillum* spp., its application is not always effective, highlighting the use of native microorganisms adapted to the conditions of the area. Similar studies carried out in the presence of glyphosate and herbicide tolerant *Burkholderia cepacia* PSBB1 when used as bioinoculant, safeguarded the plants from the inhibitory effect of glyphosate and concomitantly augmented the whole dry matter production, nutrient accumulation and seed attributes of chickpeas. Interestingly, the ability of strain PSBB1 to excrete such growth enhancers was not eliminated completely even at higher glyphosate concentration (Shahid and Khan, 2018).

On the other hand, in the trials referring to the use of atrazine as the sole source of nitrogen, it should be noted that no results have been published with bacteria of the genus *Azospirillum* and its behavior towards atrazine. Furthermore, bacteria of this genus have not been isolated in studies of isolation of microorganisms from soils exposed to this herbicide. However, it is interesting to highlight the survival capacity of both strains when exposed to this agrochemical, as long as they could survive nutritionally at the expense of other essential sources, thus constituting a new contribution in relation to this bacterial genus.

Plant growth promoting bacteria stand out for their potential as biofertilizers, constituting an agroecological and sustainable alternative. Because of this, it is necessary to design experiments on the long-term effect of pesticides on microbial communities and their eco-toxicological effects; since it is widely known that pesticides and their use can cause serious hazards to soil environment and human health (Meena *et al.* 2020). This has become critical because most harmful effects are caused by the application doses that exceed the recommended rates. In this sense, based on the novel results of this study, in our work group (Cardozo *et al.*, in preparation) effects of the bioinoculation of *A. brasilense* Az39 in field crop maize with different combinations and doses of herbicides are evaluated. This is done in order to increase production and to improve sustainable management practices. The education of farmers, distributors, industry, policymakers, and other stakeholders in the discriminate use of pesticides is critical to reducing the adverse effects they have on humans and the environment.

Materials and methods

Microorganisms and in vitro experimental design

The bacterial strains Az39 and Sp7 were provided by the agronomist A. Perticari, IMIZA-INTA-Castelar, Argentina and Dr. Y. Okon, Fac. Agriculture, Food and Environment of the Hebrew University of Jerusalem. The survival capacity of both strains was determined regarding the presence of glyphosate (monoammonium salt or N-phosphonomethyl-glycine dimethylamine, Panzer Gold, Dow Agrosciences) and atrazine (2-chloro-4-ethylamine-6-isopropylamine, Gesaprim 90 WC, Syngenta), adding them to the culture medium from the beginning of their growth. The recommended culture medium for each strain was NFb (Dobereuier *et al.*, 1976) for Az39, Fructose (Reem *et al.*, 2015) for Sp7 and NFb-Congo Red (Rodríguez Cáceres, 1982) for both. The doses of glyphosate and atrazine were 0.5g L^{-1} and 0.04g L^{-1}, corresponding to 0.2 and 0.4% of the dose used in the field, selected according to previous trials in the work group (Travaglia *et al.*, 2015). The ability to use herbicides as the sole source of carbon or nitrogen was also evaluated

(Moneke *et al.*, 2010; Martínez-Nieto *et al.*, 2012; Pinelli *et al.*, 2012). For this, each culture medium was prepared without the addition of malic acid (Az39) or fructose (Sp7) as a carbon source and without ammonium chloride as a source of nitrogen. These were replaced by glyphosate and atrazine using the doses mentioned above. The pH was adjusted to 6.8 - 7.0 before sterilization (1 atm, 121 °C, 20 min). The treatments corresponding to the Sp7 strain were: Sp7: control without herbicides; A7: Fructose medium + atrazine; G7: Fructose medium + glyphosate; W/o $ClNH_4$ + A7: Fructose medium without addition of $ClNH_4$ as a source of N + atrazine; W/o C + G7: Fructose medium without addition of malic acid as a source of C + glyphosate. While those corresponding to the Az39 strain were: Az39: control without herbicides; A39: NFb medium + atrazine; G39: NFb medium + glyphosate; W/o $ClNH_4$ + A39: NFb medium without addition of $ClNH_4$ as a source of N + atrazine; W/o C + G39: NFb medium without addition of malic acid as a source of C + glyphosate.

The growth was monitored by 70 h incubation (28 ° C and 140 rpm) measuring its optical density (OD_{600}nm) and its purity through the Gram stain technique. Fresh mobility and viability was determined by measuring the number of colony-forming units (CFU mL^{-1}) in NFb-Congo Red Medium, with visualization of characteristic colonies. Data obtained the *in vitro* assays were from three repetitions (n=3) and the values are recorded as the mean standard error of each group of results. The comparisons between the different treatments were carried out using ANOVA.

Plant materials and experimental design

The modern maize hybrid DK7210 VT3pro was used, which is the recommended for the maize area of Argentina. The plants were incubated in a growth chamber under controlled conditions (16 h light / 8 h darkness, 25 ° C, 80% relative humidity). The treatments consisted of applications by irrigation of glyphosate and atrazine (1 mL L^{-1} and 1g L^{-1}, corresponding to 4 and 5% of the doses used in the field, respectively) in two phenological stages: V2 and V4 (Ritchie and Hanway, 1982) with and without the foliar inoculations of the different strains of *A. brasilense*. The treatments were: C) distilled water applications; G) applications of glyphosate; A) applications of atrazine; 7) foliar applications of Sp7; 39) foliar applications of Az39; G7) applications of glyphosate and Sp7; G39) applications of glyphosate and Az39; A7) applications of atrazine and Sp7; A39) applications of atrazine and Az39.

In V6 (about 25 days post sowing), the following morpho-physiological variables were determined. Aboveground and belowground biomass was determined on a dry weight (DW) by incubation in an oven at 60 ° C, until reaching a constant weight. Foliar area was determined by multiplying the total length by the maximum width of each leaf. The result was multiplied by the particular correction factor for each crop (Ahmad *et al.*, 2015). Chlorophyll *a* measurement was determined by extraction in 80% ethanol according to the method of Vernon and Mac Kinney (modified by Mac Kinney, 1938). Damage of cell membranes was evaluated as the relative conductivity rate (RCR) according to Prásil and Zámecník (1998). For this, 0.1 g of fresh material was weighed and placed in distilled water at 4 ° C for 24 hours. After that time, measurements of electrical conductivity were taken in the solution at 25 ° C. Data were obtained in the plant materials from three repetitions (n=8) and the

values were given as the mean standard error of each group of results.

Statistical analysis

The comparisons between the different treatments were carried out using ANOVA. Fisher's LSD test was used to establish the significance of differences between groups and factorial analysis to shed light on the effect of each strain-herbicide interaction. The significance level in all cases was set at $p < 0.05$ when compared between different results. The results were analyzed using the GraphPad Prism statistical analysis software package (professional version 5.0.3.0 GraphPad Prism).

Conclusions

The strain *Azospirillum brasilense* Az39 survives to the presence of both commercial herbicides and uses glyphosate as the sole source of carbon. The strain of *Azospirillum brasilense* Sp7 only survives in the presence of atrazine. Both strains are capable of ameliorating the damaging effects of herbicides at the root level in maize plants. Most of the negative effects of both herbicides on these plants are attenuated only with foliar inoculation of the native strain. Therefore, the native strain of *A. brasilense* Az39 has better adaptive characteristics against herbicides widely used in maize. This highlights the importance of the use of native microorganisms for effective inoculations in crops.

Acknowledgments

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