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Towards sustainable maize production: Glyphosate detoxification by *Azospirillum* sp. and *Pseudomonas* sp.

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ABSTRACT

To contribute to more sustainable crop production, this study evaluated the capacity of *Azospirillum* sp. and *Pseudomonas* sp. to degrade glyphosate residues both *in vitro* and *in vivo* in maize plants (*Zea mays* L.) at different growth stages. *In vitro*, both bacteria tolerated glyphosate and were capable of using it as a carbon source. In bioassays, inoculation with both bacteria improved germination and root emergence, primary root growth, root hair development and coleoptile growth in seeds previously treated with the herbicide. Foliar inoculation with *Azospirillum* sp. and *Pseudomonas* sp. in glyphosate-treated plants improved root and shoot biomass and increased foliar area, photosynthetic pigments and phytohormone content as well, thus increasing maize yield in the field while concomitantly decreasing herbicide accumulation in leaves and grains. The bacterial capacity to degrade glyphosate *in vivo* at different growth stages in maize plants growing in the field is a novel and promising biotechnological technique to minimize the persistence of xenobiotic compounds in the environment. This finding adds to the already known importance of the application of bacterial inoculants to crops to enhance plant growth, development and yield.

1. Introduction

Rhizospheric bacteria have a beneficial effect on plant growth (Döbereiner, 1992; Baldani et al., 1997; Mantelin and Touraine, 2004; Cohen et al., 2014). Due to this effect, these bacteria have been widely used as an alternative to reduce the use of pesticides in pursuit of more sustainable agriculture (Aguirre-Medina, 2008; Olalde-Portugal and Serratos, 2008).

Azospirillum and *Pseudomonas* are the most studied genera of Plant Growth Promoting Rhizobacteria (PGPR), due to their capacity to significantly enhance the growth, development, and yield of numerous vegetable species of agricultural interest (Okon and Labandera-González, 1994; Jaime et al., 1999; Bashan et al., 2004; Haas and Défago, 2005; Cohen et al., 2009). This capacity may be explained by the production of phytohormones such as gibberellins (GAs), IAA and ABA (Bottini et al., 1989, 2004; Crozier et al., 1988; Cohen et al., 2008).

In addition to their capacity to promote growth, it has also been shown that *Azospirillum* and *Pseudomonas* are able to tolerate herbicides and degrade xenobiotics (Venkateswarlu and Sethunatan, 1984; Omar et al., 1992; Gimsing et al., 2004; White and Metcalf, 2004; Ratcliff et al., 2006; Funke et al., 2006; Bazot and Lebeau, 2008; Moneke et al., 2010).

Herbicide use for weed control is feasible and widespread and is an important factor in current agriculture (Wardle and Parkinson, 1990; Sannino and Gianfreda, 2001). Glyphosate (N-phosphonomethyl glycine), is one of the most widely used non-selective broad-spectrum herbicides in agriculture worldwide (EPA, 1994; Franz et al., 1997). Commercial glyphosate products typically consist of a concentrated formula of an isopropylamine salt of

Abbreviations: ABA, Abscisic Acid; IAA, Indole-3-Acetic Acid; CFU, colony-forming unit; OD, optical density; RH, Relative humidity; JA, Jasmonic Acid; PGPR, Plant Growth Promoting Rhizobacteria; RR, Roundup Ready®.

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glyphosate for the destruction of weeds in wheat, beans, sorghum, tomatoes, vine, sorghum, potatoes, among others (Eslava et al., 2007).

The use of transgenic varieties of maize tolerant to glyphosate (RR: Roundup Ready®) has contributed to the wide use of this compound (Williams et al., 2000). However, its excessive use could have potentially toxic effects in crop products, which justifies the increasing concern at all levels associated with food safety. It is important to evaluate the risk these varieties have on people's health when grown using certain chemical compounds (Dobbelaere et al., 2002; Roy et al., 2002; Kozdroj et al., 2004).

Little is known about the interaction of PGPR inoculation with the application of agricultural herbicides; therefore, the aim of this study was to investigate that interaction. Specifically, the objective was to evaluate the capacity of *Azospirillum* and *Pseudomonas* to degrade residual glyphosate both *in vitro* and *in vivo* in maize plants at different growth stages, with the aim of improving the quality of crops in a sustainable way.

2. Materials and methods

2.1. In vitro assays

To evaluate the capacity of *Azospirillum* sp. and *Pseudomonas* sp. (strains of which were provided by the agronomist A. Perticari, IMIZA-INTA-Castelar, Argentina) to tolerate and degrade glyphosate, preinocules were cultured overnight in LB medium (Luria Bertani DIBICO S.A. de C.V. Mexico, D.F.) to a final density of 1×10^8 CFU ml^{-1}. Then, these cultures were used to inoculate 1000 μL flasks containing 25 ml of NFb medium (Döbereiner, 1989) or modified NFb medium with a carbon source (malic acid, 5 g L^{-1}). To both conditions, 250 μL of either commercial herbicide (48% AGM Glyphosate AGM Glifoweed, Agriquemical Supplies S.A., Argentina) or pure glyphosate active ingredient (Sigma–Aldrich Co. LLC, USA) was added from a solution of 1.3 ppm. The treatments were: a) *Azospirillum;* b) Herbicide + *Azospirillum;* c) Glyphosate + *Azospirillum;* d) *Pseudomonas;* e) Herbicide + *Pseudomonas;* f) Glyphosate + *Pseudomonas.*

The medium was then incubated at 30 °C for 192 h on a shaker at 120 rpm. Viability was determined at 96 h by measuring the number of colony-forming units (CFU ml^{-1}), and optical density (OD_{590nm}) measurements were taken every 4 h in a ThermoSpectronic Helios spectrometer (Artisan Technology Group ® 101 E. Mercury Drive Champaign, IL 61822) to monitor growth throughout the biomass growth phase.

2.2. Germination assays

Experiments in Petri dishes were performed with 8 seeds each of the maize (*Zea mays* L.) DK 670 MGRR. Ten repetitions were carried out for each treatment. Fifteen (15) ml distilled water or solution of commercial herbicide was added with glyphosate as an active ingredient (0.25 L $(100\ L)^{-1}$). The herbicide dose used was determined in previous assays by means of a dilution curve, starting from a pattern solution at different concentrations. The maximum concentration corresponded to the required amount for the effect of the herbicide to be observed and for germination to be uninhibited. The Petri dishes were incubated at approximately 29 °C. The treatments were: a) Herbicide + Seeds without inoculation; b) Herbicide + Seeds inoculated with *Azospirillum* sp.; c) Herbicide + Seed inoculated with *Pseudomonas* sp.

After 48 h, the germination percentage was evaluated according to the International Seed Testing Association Plant Evaluation Manual (ISTA, 2003); after 96 h, the coleoptile (Sixto et al., 1997) and radicle growth (Beckie et al., 1990) were observed.

2.3. Plants assays under controlled conditions

Experiments were performed in 6 replicates in 300 cm^3 pots filled with soil/vermiculite, with one maize plant DK 670 MGRR per pot. Hoagland universal solution was used for irrigation. Leaves of plants were sprayed with PGPR inoculant in the V3 and V5 stages of growth (Zadocks et al., 1974), the amount of solution of both inoculants being 1 ml/plant with a concentration of 10^7 CFU ml^{-1}. The herbicide was applied by spraying at the V2–V3 stages. The concentration used was 2.5 L $(100\ L)^{-1}$ (the dose commonly used in the field). The treatments were: a) Foliar application of Herbicide; b) Foliar application of Herbicide + Foliar inoculation with *Azospirillum* sp.; c) Foliar application of Herbicide + Foliar inoculation with *Pseudomonas* sp.

Pots were incubated in a growth chamber (16 h light at 28° C/8 h darkness at 20 °C, 80% RH). After the treatments and up to 30 days post sowing, the samples were collected to evaluate the following variables:

The 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 (Montgomery, 1911).

Aboveground and belowground biomass were determined on a dry weight (DW) basis by placing sample aliquots for 7 days at 65 °C in a fan-ventilated oven. For pigment measurement, 50 mg fresh weight of flag leaf was homogenized in a mortar with 10 ml of 80% acetone. The homogenate was loaded in Eppendorf tubes and incubated for 1 h at 4 °C in the dark to extract the pigment; then, the homogenate was centrifuged twice for 5 min at 5000 rpm (radius: 15 cm). Aliquots were taken from the supernatant, and chlorophyll *a* and *b* levels were measured by spectrophotometry at 650 and 665 nm, respectively. Five millimeters of 1 M NaOH and 15 ml of diethyl ether were added to the total volume. Carotene content was assessed from the ether fraction by spectrophotometry at 450 nm (modified from Mackinney (1938)).

Phytohormone analysis was performed on 200 mg samples of leaf tissue collected in liquid nitrogen. After collection, the samples were lyophilized and kept at −20 °C. The samples were ground to powder with a mortar and pestle and weighed (100–200 mg per sample). The extraction was performed with 5 ml of deionized water with pH adjusted to 2.8 at 4 °C. After centrifugation (15 min, maximum speed), the supernatant was collected, and the pellet was then re-suspended and re-extracted with 2 ml of fresh buffer (pH: 2.8) to be re-centrifuged as before. 50 ng aliquots of each of deuterated JA, ABA and IAA (provided by Olchelmn Ltd, Czech Republic) were added as internal standards. The extracts were transferred to 50 ml tubes and mixed with ethyl acetate. Then, the organic phase was extracted and evaporated at 37 °C in a Speed-Vac. Dried extracts were dissolved in 50 μL methanol (100%), and placed in vials. For liquid chromatography, analyses were performed using an Alliance 2695 (Separation Module, Waters, USA) quaternary pump equipped with an auto-sampler. A Restek C18 (Restek, USA) column (2.1 × 100 mm, 5 μm) was used at 28 °C with an injected volume of 10 μL. The binary solvent system used for elution gradient consisted of 0.2% acetic acid in H_2O (solvent B) and MeOH (solvent A) at a constant flow-rate of 200 μL min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied [t (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration. MS/MS experiments were performed on a Micromass Quatro UltimaTM ^{PT} double quadrupole mass spectrometer (Micromass, Manchester City, UK). All analyses were performed using a turbo ion spray source in negative electrospray ionization mode (*ESI*) with the following settings for phytohormones: capillary voltage −3250 V, energy cone 35 V, RF Lens1 (20), RF Lens2 (0.3), source temp. 100 °C, desolvation temp. 350 °C, gas cone 100 L h^{-1}, gas desolvation 701 L h^{-1}, collision cell potential of 15 V

and multiplier (650). MS/MS parameters were optimized in infusion experiments using individual standard solutions of each hormone. MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the double quadrupole mass spectrometer, and mass was analyzed using the second analyzer of the instrument. In negative mode, the spectrum for each hormone gave deprotonated molecule [M−H]. Quantitation was performed by injection of samples in multiple reaction monitoring (MRM) modes because many compounds could present the same nominal molecular mass. The combination of parent mass and unique fragment ions was used to selectively monitor hormones. MRM acquisition was performed by monitoring the 209/59 and 215/59 transitions for JA and ($^2H_6$)-JA; 263/153 and 269/159 for ABA and ($^2H_6$)-ABA; and 174/130 and 179/135 for IAA and ($^2H_5$)-IAA, respectively, with a dwell time of 2000 ms for each transition. Data were acquired and analyzed using MassLynxTM 4.1 and QuanLynxTM 4.1 (Micromass, Manchester, UK) software. For quantification, values were obtained from a calibration curve previously constructed using known amounts of each hormone and its pure standard (Sigma, St. Louis, MO, USA)/deuterated internal standard ratio (del Mar Soto et al., 2010).

2.4. Field experiments

The maize hybrid was the same used for assays 2.2 and 2.3, DK 670 MGRR. Experiments were conducted during the 2011–13 crop seasons in the experimental field of the Universidad Nacional de Río Cuarto campus, Río Cuarto, Córdoba, Argentina (33° 07′ S, 64° 14′ W), located at 432 m.a.s.l., with monsoon-type precipitation in the area. The region's soil is a typical Hapludoll. An experimental unit (a parcel) was comprised of 6 rows, each 16 m long. Blocks were separated by 0.7 m unplanted distances. Each treatment had 6 repetitions in a randomized complete block. A necessary number of rows around and between the assays were used to avoid border effects. The maize was sown on a suitable date for that area (Andrade and Cirilo, 2000) to its optimal density of 8 plants m^{-2} (Echarte et al., 2000). Weed, pests and disease control were performed in a timely manner. The field was fertilized according to the recommendations from soil analysis and common practices of the area to avoid possible nutritional inferences. The treatments were the same as the ones described for the plant assays under controlled conditions: a) Foliar application of Herbicide; b) Foliar application of Herbicide + Foliar inoculation with *Azospirillum* sp.; c) Foliar application of Herbicide + Foliar inoculation with *Pseudomonas* sp.

In this experimental field, both PGPR bacterial suspensions were prepared at 1L (100 L)$^{-1}$ with biomass of 1 × 10^8 CFU ml^{-1} and were applied at the 6-leaf vegetative growth stage (V6) and repeated prior to the flowering reproductive stage (R1), according to the Zadocks scale (Zadocks et al., 1974). Glyphosate was applied by spraying 48% isopropylamine salt of glyphosate at the V3 and V6 stages. The dose usually used per field hectare was 2.5 L 100 L^{-1} in both stages. Grain yield and yield components per area unit as well as the residual content of herbicide in leaves and grains were determined at physiological maturity. For this purpose, the procedure used was the same as the ones used for the phytohormone analysis previously described, although a greater amount of vegetal tissue content was used for analysis (1 g per leaf sample, 5 g per grain sample). Calibrations were made using the pure and the commercial formulation (Roundup®). The identification of glyphosate residue was based on the following transitions: 170/88 for pure glyphosate and 229/184 for glyphosate salt (isopropylammonium or N-phosphonomethylglycine ammonium salt).

2.5. Statistical analysis

The results were analyzed using the Info-Stat statistical analysis software package (professional version 1.1, infostat@agro.uncor.edu). An ANOVA was performed, and Fisher's LSD test (α = 5%) was used to compare the treatments.

3. Results

3.1. Tolerance and degradation of glyphosate in vitro

In vitro, both bacteria (*Azospirillum* and *Pseudomonas*) tolerated the glyphosate and were able to use it as a carbon source. Bacterial growth in response to 250, 500 and 1000 μL doses of herbicide solution was examined. An increase in glyphosate concentration led to a concomitant decrease in the growth of the isolates compared with the control which contained no glyphosate (data not shown); for this reason, doses of 250 μL were used for all further experiments in this section.

In the bacterial growth experiments with carbon sources added, *Azospirillum* showed a longer lag phase than *Pseudomonas*. Both bacteria had lower growth in the presence of pure glyphosate (9.4 × 10^5 and 1.1 × 10^5 CFU ml^{-1} for *Azospirillum* and *Pseudomonas*, respectively); in the presence of the commercial herbicide preparation, both bacteria showed a growth profile similar to the bacteria grown in the absence of herbicide (1.7 × 10^6 and 1.4 × 10^6 CFU ml^{-1}, respectively). This culture reached a clearer maximum of growth at 96 h of incubation. If this time is compared with the one obtained in the experiment without carbon source (Fig.1a), it can be observed

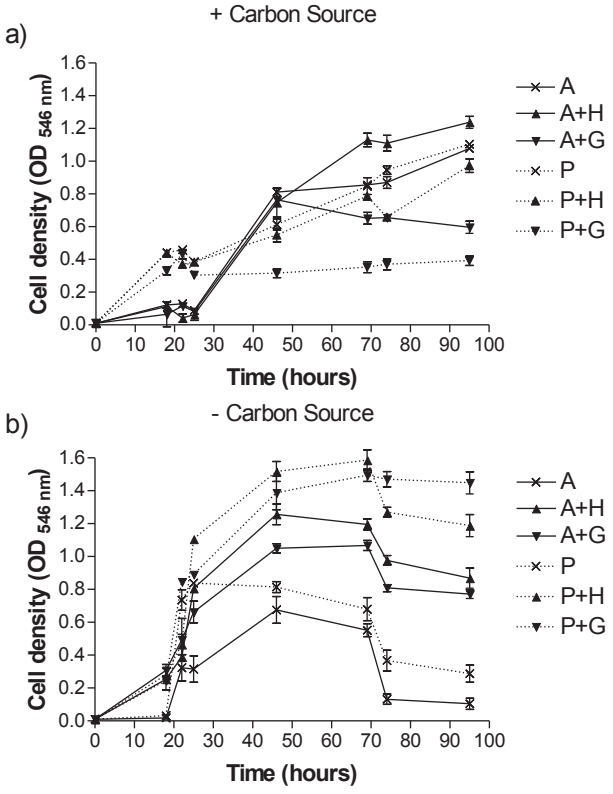

Fig. 1. Biomass of *Azospirillum* and *Pseudomonas sp*. at different incubation times, in the presence of commercial herbicide or pure active principle, glyphosate; with carbon source (a) and without incorporated carbon source (b). Treatments: A: *Azospirillum;* A + H: *Azospirillum* with commercial herbicide; A + G: *Azospirillum* with pure glyphosate; P: *Pseudomonas*; P + H: *Pseudomonas sp*. with commercial herbicide; P + G: *Pseudomonas* with pure glyphosate.

that the bacteria had not yet entered the death phase due to greater nutrient availability.

In the *in vitro* experiments without carbon source, the biomass growth of both bacteria was affected (Fig.1b). Final titers were two orders of magnitude lower than in the experiments with carbon source added (on the order of 1.8×10^4 CFU ml^{-1}). The short lag phase, coupled with the rapid growth of the two bacteria, showed the effective use of glyphosate and glyphosate salt because, before the 24 h mark, the incubations were in exponential phase. *Pseudomonas* and *Azospirillum* attained maximum growth at 48 h of incubation, while both bacteria with glyphosate or commercial herbicide achieved maximum growth at 72 h of incubation. Both bacteria showed higher growth with the addition of commercial glyphosate than with pure glyphosate. In the presence of both chemical products, the stationary phase was longer and the beginning of the death phase was delayed. The culture of both bacteria without carbon source and with commercial or pure glyphosate also showed increased total growth (the values of CFU ml^{-1} were two and three orders of magnitude higher than *Azospirillum* and *Pseudomonas* alone, respectively).

3.2. Growth, development and yield of maize

Before the evaluation of maize seed germination subjected to glyphosate, the effects of different concentrations of glyphosate were analyzed (2.5, 1.0, 0.5, 0.25 and 0.005 L $(100\ L)^{-1}$). The glyphosate concentration of 0.25 L $(100\ L)^{-1}$ was used in the later assays of germination because, even though there was a lower percentage of germination in relation to the control one, it was the most concentrated solution that affected the root growth to a lesser extent and allowed for better coleoptile development. The concentration normally used in the field (2.5 L $(100\ L)^{-1}$) could not be used because direct contact of the seed with that solution inhibited germination and growth.

Inoculation with either bacterium in the presence of herbicide significantly improved germination rates and root emergence. This effect was largest in the treatment with *Pseudomonas* sp. (Fig.2a). Treatment with either PGPR resulted in longer primary roots (Fig.2b), as well as better radical hair and coleoptile development.

The plants inoculated with *Azospirillum* sp. and *Pseudomonas* sp. showed a greater foliar area and greater aboveground and belowground biomass than uninoculated plants (Figs. 3 and 4). The foliar inoculation increased the total content of both chlorophylls and carotenes (Fig.5). When glyphosate was applied, the foliar application of either bacterium significantly increased the analyzed phytohormone content in comparison with the uninoculated plants. The IAA and ABA levels were highest in those plants treated with *Azospirillum* (Table 1).

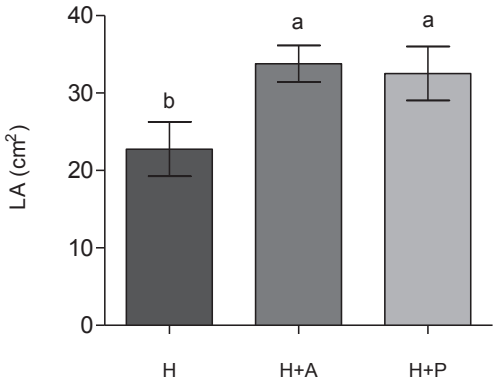

Fig. 3. Average leaf area (LA) of maize seedlings grown in plastic pots during 30 days. Treatments: H: Application of herbicide; H + A: herbicide application and inoculation with *Azospirillum sp.* at leaf level; H + P: herbicide application and inoculation with *Pseudomonas sp.* at leaf level. Different letters show significant differences at $p < 0.05$ with the Fisher alpha Test.

The results from maize crops in the field experiment showed a significant decrease in the accumulation of herbicide in salt form in leaves that received PGPRs in comparison with uninoculated control leaves, two and three times lower in the treatments with *Azospirillum* sp. and *Pseudomonas* sp., respectively (Table 2). These bacterial applications also had the capacity to mitigate herbicide accumulation in grains (Fig.6). When residual herbicide contents in maize grains were determined, it was clearly observed that the control treatment contained a significant amount of active glyphosate and a lower proportion of salt. Inoculated plants showed lower accumulation in grains of glyphosate in both forms (glyphosate salt and glyphosate).

Additionally, a yield increase in the crops treated with *Azospirillum* and *Pseudomonas* was observed, 11.1% and 47.5%, respectively. The uninoculated control averaged 1021.5 kg grain/ha and the PGPR-treated plants averaged 1135.0 and 1502.5 kg grain/ha, respectively.

4. Discussion

Studies on the effect of herbicides on bacterial growth *in vitro* have found reductions in population counts when glyphosate was added to culture media (Quinn et al., 1988; Santos and Flores, 1995; Kryzsko-Lupicka and Orlik, 1997). The toxicity of artificial media is

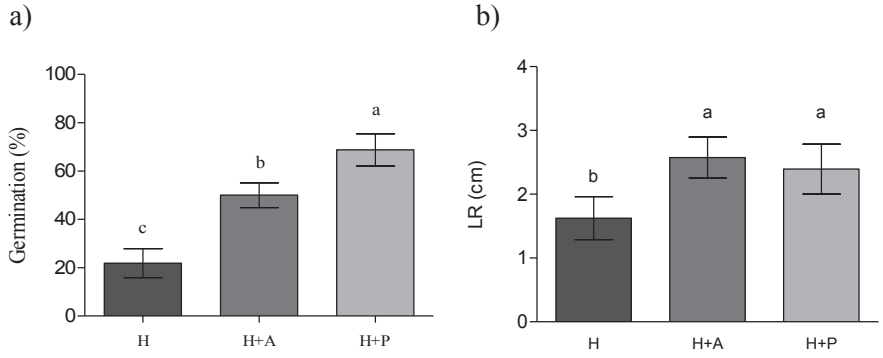

Fig. 2. Germination percentage (a) and root length (b) of maize seeds incubated in Petri dish to 96 h. Treatments: H: Application of herbicide; H + A: herbicide application and inoculation with *Azospirillum sp.*; H + P: herbicide application and inoculation with *Pseudomonas sp.* Different letters show significant differences at $p < 0.05$ with the Fisher alpha Test.

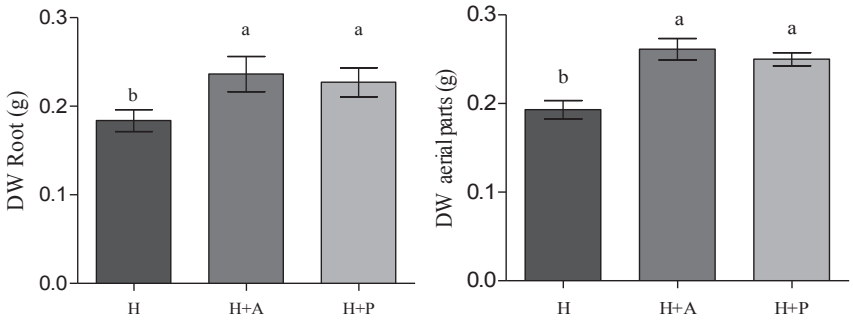

Fig. 4. Dry weight (DW) of roots and aerial parts, of maize seedling grown in plastic pots during 30 days. Treatments H: Application of herbicide; H + A: herbicide application and inoculation with *Azospirillum sp.* at leaf level; H + P: herbicide application and inoculation with *Pseudomonas sp.* at leaf level. Different letters show significant differences at $p < 0.05$ with the Fisher alpha Test.

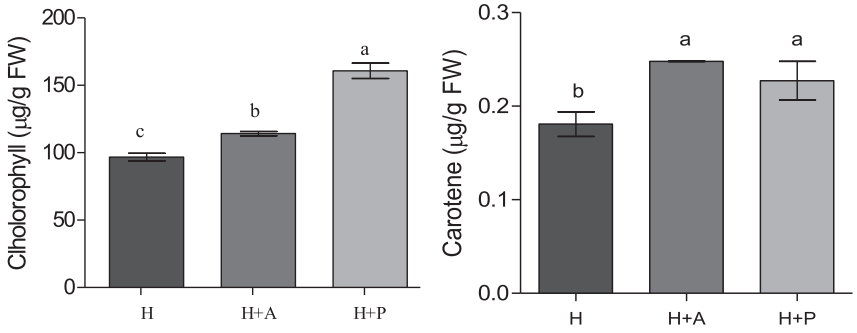

Fig. 5. Average of total Chlorophyll and Carotene content per gram of fresh weight of maize seedlings grown in plastic pots during 30 days. Treatments: H: Application of herbicide; H + A: herbicide application and inoculation with *Azospirillum sp.* at leaf level; H + P: herbicide application and inoculation with *Pseudomonas sp.* at leaf level. Different letters show significant differences at $p < 0.05$ with the Fisher alpha Test.

Table 1
Phytohormone content per gram fresh weight of maize seedlings grown in plastic pots during 30 days. Treatments: H: Application of herbicide; H + A: herbicide application and inoculation with *Azospirillum sp.* at leaf level; H + P: herbicide application and inoculation with *Pseudomonas sp.* at leaf level. Different letters show significant differences at $p < 0.05$ with the Fisher alpha Test. AIA: indole acetic acid. ABA: abscisic acid, JA: jasmonic acid.

Phytohormone (ng/FW)	H	H + A	H + P
AIA	31172c	194999.5a	100854.9b
ABA	1846.5c	5333.5a	2873.5b
JA	52b	111.5a	137.5a

Table 2
Glyphosate Residual content (micrograms of glyphosate per grams fresh weight). Treatments: H: Application of herbicide; H + A: herbicide application and inoculation with *Azospirillum sp.* at leaf level; H + P: herbicide application and inoculation with *Pseudomonas sp.* at leaf level. Different letters in the same row indicate a statistically significant difference ($p < 0.05$).

	H	H + A	H + P
Glyphosate Residual (µg/g FW)	6195.5a	3040.5b	2033.0c

expected to be based on the mode of action of glyphosate (inability of the organism to synthesize the needed aromatic amino acids). Unlike the response in artificial media, no toxicity was found when glyphosate was added in laboratory bioassays (Busse et al., 2001). In the results of this experiment, both bacteria showed the capacity to tolerate the presence of glyphosate and showed effective use of glyphosate as a carbon source *in vitro*. In the presence of either chemical product, the stationary phase was longer and the onset of the death phase was delayed, an effect present in both bacteria. This outcome shows that both bacteria were capable of using and metabolizing glyphosate and its salt as nutrition sources and corroborates an earlier report (Masciarelli et al., 2013), which found that the content of these chemical compounds was lower in spent culture fluid. The same dose–response profile was obtained for commercial herbicide as for pure glyphosate (500 and 1000 µL), both showing higher growth at lower doses, which is in agreement with other studies (Grant et al., 2002; Jilani and Altaf, 2006; Malik et al., 2009; Murugesan et al., 2010). These findings demonstrate that PGPRs can degrade the herbicide and its active compound *in vitro*, and PGPRs would thus mitigate residual herbicide accumulation in grains.

Studies using ^{14}C-glyphosate radiolabeling have suggested that *Pseudomonas* sp. strain LBr degrades glyphosate via aminomethylphosphonic acid (AMPA) and sarcosine (Jacob et al., 1988). In

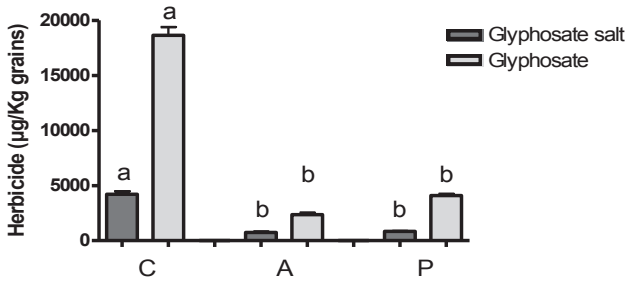

Fig. 6. Glyphosate (light bars) and glyphosate salt (dark bars) content in micrograms per kilograms of grains. Treatments: C: Control; A: *Azospirillum.sp.* P: *Pseudomonas* sp. Different letters in the same bars indicate a statistically significant difference ($p < 0.05$).

another study, members of five bacterial genera capable of using glyphosate as a sole carbon source were isolated; of these genera, the *Pseudomonas* spp were dominant, and many of them are being used as inoculants (Martínez-Nieto et al., 2012). Herbicide tolerance has also been demonstrated in *Azospirillum* (Gadkari, 1988; Omar et al., 1992). Both bacteria used in this study showed appreciable growth in the culture medium containing glyphosate and glyphosate formulations. The differences observed in the growths of the isolates in the medium are indicative of the differences between the organisms in tolerating the glyphosate, and suggest further biodegradation studies based on their short lag phase and rapid utilization of glyphosate. There have been several reports of the ability of different PGPR genera to effectively use glyphosate by naturally synthesizing appropriate enzymes or as a result of genetic mutation (Shinabarger et al., 1984; Kishore and Jacob, 1987; Jacob et al., 1988; White and Metcalf, 2004; Funke et al., 2006; Bazot and Lebeau, 2008; Moneke et al., 2010).

Moreover, the biostimulant action of PGPR on the maize root system upon inoculation (Hadar and Okon, 1987; Fulchieri and Frioni, 1994; Bellone et al., 1999) is already well known. In this study, longer roots and an increase in the growth of radical hairs were the main effects, and were the main activities responsible for greater nutrient uptake by plants (Burdman et al., 2000). Both bacteria were able to enhance the growth and early development of maize in the presence of herbicide, which is symplastically transported towards the meristems of the growing plant. Just as it was shown in the seedling experiments, inoculation with *Azospirillum* and *Pseudomona* sp. promoted root development in the early growth stages of maize, according to Santillana (2001), and would be able to alleviate the effects caused by the glyphosate in the commercial herbicide during germination. These advantages reflect the important role played by the aforementioned PGPRs in the degradation of toxic residues, as was observed in a previous study on RR soybeans treated with glyphosate (Krishna et al., 2010). Several studies on this topic worldwide have found that both glyphosate and other organophosphonate compounds are degraded by an assortment of different soil microorganisms, mainly by several species of *Pseudomonas* spp. (White and Metcalf, 2004; Funke et al., 2006; Bazot and Lebeau, 2008; Moneke et al., 2010).

The beneficial effect of bacteria on several crops, especially cereals, has been well established (Okon and Labanderas-González, 1994). Therefore, the increases in biomass, the foliar area, the photosynthetic pigment content and the cell membrane integrity in the inoculated plants may all be related to better adaptation and lower oxidative damage (Randall et al., 1977). The adverse effects of glyphosate on photosynthesis and biomass production were evident in the first and second soybean crop generation (Cessnal and Cain, 1992; Saes et al., 2010). That result agrees with Davis et al. (1978), who stated that approximately 50% of commercial herbicides are photosynthesis inhibitors. This study found that inoculation with either bacterium seems to mitigate the damage caused in the pigment due to the mentioned herbicide. The PGPRs increased plant growth by several mechanisms, including the production of phytohormones. The application of either bacterium, *Azospirillum* or *Pseudomonas*, increased plant phytohormone contents in the presence of herbicide. The increase in the endogen content of jasmonic acid (JA) in inoculated plants in comparison with the non-inoculated ones indicates that there could be a better response signal before the herbicide presence because this phytohormone modulates the responses to stress and development (Creelman and Mullet, 1997; Farmer et al., 2003). Indole-3-acetic acid (IAA) and abscisic acid (ABA) contents were higher in the treatment with *Azospirillum*, which suggests that the presence of such bacterium raises the levels of these phytohormones. One of the explanations of *Azospirillum*'s stimulatory effects on plant growth involves the production of growth regulators (Bashan and Levanony, 1990; Okon and Labandera-González, 1994); several such regulators have been identified in the supernatants of these bacterial cultures (Perrig et al., 2007; Masciarelli et al., 2010). IAA produced by the bacteria can modify the phytohormone content of the plants, leading to their growth stimulation. For example, in previous studies, fluoridone (inhibitors of ABA synthesis) treatments diminished the growth of maize plants that had been well watered in a similar manner to drought, but inoculation with *Azospirillum lipoferum* completely reversed this effect. These results were correlated with ABA levels assessed by GC-EIMS (Cohen et al., 2009). The effect attributed to inoculations with commercial products based on *Pseudomonas* is mainly associated with antibiosis and phosphate solubility, but is less well associated with phytohormone production. There are only a few studies in which IAA is included, but they are limited to production *in vitro* (Kang et al., 2006). Thus, the current report of different phytohormone production following *Pseudomonas* inoculation is very interesting and novel.

More recently, Weyens et al. (2009) reviewed the benefits of using plant-associated endophytes in bioremediation and emphasized that, although successfully applied in several laboratory-scale experiments, the large-scale field application of this technology is limited. Thus, different experiments in the field were performed to improve crop quality in this study. Studies on the glyphosate levels in grains to be exported from main Argentine ports found 0.2–0.7 mg kg^{-1} in grains stored in silos (CONICET, 2009). In experimental RR soybean crops, glyphosate residues between 0.30 and 0.31 mg kg^{-1} were found, as well as similar concentrations of its metabolite AMPA. In conventional non-RR soybean grains, neither glyphosate nor this metabolite was found (Lorenzatti et al., 2004). There are studies in which the presence of glyphosate residues was shown after being applied to foods such as strawberry, blueberry, cereals, raspberries, lettuce, carrots and barley (Cessnal and Cain, 1992; Eslava et al., 2007). One of the most serious problems for plant, animal, and human health is the persistence of glyphosate residues, which increases their toxicity and risk (Dalvie et al., 2003; Holmes et al., 2008; Murugesan et al., 2010). The average lifetime in the soil may be 60 days, but residues are usually found the next year in the field (EPA, 1999). In this study, glyphosate residual content was decreased in grains from plants treated with either bacterium in comparison with plants that were not inoculated; this could be because these PGPR are able to metabolize residual glyphosate, using them as a carbon, nitrogen or phosphorus source. In the present *in vitro* studies, both bacteria tolerated the presence of glyphosate and were capable of using it. Thus, foliar application of PGPR-based inoculants could mitigate the accumulation of xenobiotic compounds in grains or as residues.

5. Conclusions

In regions where modern agriculture is practiced, the application of *Azospirillum* and *Pseudomonas* reduces fertilizer demand and decreases both production costs and problems derived from fertilizer use, mainly contamination, without decreasing yields. Thus, the application of biological compounds in maize crops will not only enhance crop growth, development and yield, but will also improve soil geochemical cycles by allowing a reduction in excessive chemical fertilization and minimizing the persistence of xenobiotic compounds widely used in current agricultural practices. This study of the bacterial capacity to degrade glyphosate, both *in vitro* and at different growth stages in maize plants, reports novel results mainly from its field studies. This research shows that novel detoxification capabilities and the ability of these isolates to utilize glyphosate effectively provide a means of removing this

compound from the environment.

Given that there is compatibility between them, many fertilizers and pesticides could be mixed in the same spray to save costs and simultaneously lessen their counterproductive effects on crops and the environment. Extensive environmental damage to the microbiota results from the excessive use of agrochemicals, and we propose the use of bacteria capable of degrading toxic synthetic organic compounds in combination with specific plants as a biological recomposition measure for the soil-plant ecosystem. This tool may offer an effective, economical and sustainable remediation technology for the twenty-first century.

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