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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