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Plant growth-promoting rhizobacteria inoculation and nitrogen fertilization increase maize (*Zea mays* L.) grain yield and modified rhizosphere microbial communities

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) were used as inoculants of cereal crops to improve their growth and grain yield. The crops responses to inoculation are complex because are defined by plant-microorganisms interactions, many of them still unknown. Thus, it is necessary to improve the knowledge about the microbial ecology of the rhizosphere of crops under different agricultural practices. The aim of this study was to evaluate the effects of certain PGPR inoculants and nitrogen fertilization on maize (*Zea mays* L.) production and some associated microbial communities under field conditions in order to increase the knowledge about microbial ecology to improve crop response to PGPR inoculation. A field experiment of maize was performed to evaluate five PGPR inoculation treatments -including commercial and experimental inoculants of *Azospirillum brasilense* or *Pseudomonas fluorescens*- and three levels of nitrogen fertilization. Particular microbial groups belonging to the carbon and nitrogen soil cycles were analyzed. Nitrogen fertilization and PGPR inoculation increased maize grain yield. Inoculation only modified the number of microaerophilic nitrogen fixing (MNF) microorganisms at the reproductive stage of the crop, while fertilization modified the amount of cellulolytic, nitrifying and MNF microorganisms, only in the vegetative stage of maize. In addition, it was observed that both inoculation and fertilization modified the physiology of the rhizosphere microbial communities in the reproductive stage. Physiological changes observed in different ontogenetic stages of the crop had higher impact than both agricultural practices. All the results demonstrate that changes in the relationships between plant and microorganisms are due to different management decisions. This work gives a better understanding of maize-rhizosphere microbial ecology which can be used to improve PGPR inoculation response in order to obtain a sustainable agricultural production.

1. Introduction

Crop yield increases are based on plant breeding which includes the application of high doses of chemical fertilizers that can generate negative environmental impact to the ecosystem (Tilman et al., 2002). For that reason, it is important to find and improve agricultural practices in order to increase and maintain high production levels in a more sustainable way (Altieri and Nicholls, 2000). Regarding to this, inoculation with plant growth-promoting rhizobacteria (PGPR) is an economical and ecological alternative to increase crop yields (García de Salamone, 2011; Verma et al., 2010) and improve fertilizer-use efficiency (Hayat et al., 2012).

Cereal crops, such as maize (*Zea mays* L.), can associate with many species of beneficial bacteria, usually called as PGPR (Barea, 2004). Some of these PGPR are *Azospirillum brasilense* and *Pseudomonas fluorescens*, which have shown capabilities related to biological N_2 fixation (Franche et al., 2009; García de Salamone, 2012a) and improvement for nutrient absorption (Dobbelaere et al., 2001; Hayat et al., 2012). In association with the rhizosphere of crop plants, PGPR produce direct and indirect beneficial effects on plant growth (Cassán and Díaz-Zorita, 2016; Pliego et al., 2011; Verma et al., 2010). In this regard, some strains of these PGPR promote grain yield and aerial biomass growth of maize, rice and wheat (García de Salamone, 2012a; García de Salamone and Döbereiner, 1996; García de Salamone et al., 2006, 2010).

Abbreviations: PGPR, plant growth-promoting rhizobacteria; MNF, microaerophilic nitrogen fixing microorganisms; CFU, colony-forming units; DAS, days after sowing; MPN, most probable number; CLPP, community-level physiological profiles; H' index, Shannon's diversity index

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The rhizosphere is a small volume of soil surrounding plant roots, which is under its direct influence (Morgan et al., 2005). It is a highly dynamic and diverse microenvironment (Hisinger et al., 2009; Pliego et al., 2011). Many of the processes that occur in the rhizosphere (Hisinger et al., 2009) and microbial communities responsible for them (Kent and Triplett, 2002) are still unknown. Because of that, it is necessary to improve the knowledge about the microbial ecology of the rhizosphere (García de Salamone, 2012b; Minz and Ofek, 2011). Besides, the inclusion of maize in the crop sequences guarantees the addition of great amount of crop residues. This is essential to keep soil quality as the conservation agriculture guidelines state. Thus, the aim of this study was to evaluate the effects of certain PGPR inoculants and nitrogen fertilization on maize production and some associated microbial communities under field conditions.

2. Materials and methods

2.1. Field site and climate conditions

Field experiment was performed in Pehuajó, province of Buenos Aires, Argentina (35°30′9″S and 61°54′24″W). This region has warm and humid weather with an average temperature of 15.4 °C and average annual rainfall of 1000 mm (SMN, 2017). The maize crop was conducted under rain-fed conditions. The soil was a Norumbega silty loam (Entic Hapludoll) (GeoInta, 2013) and chemical characteristics of the upper soil layer (20 cm) before sowing were: pH 5.8 (1:2.5 soil:water), electrical conductivity 0.92 dS m^{-1}, 3.2% of total organic matter, 0.19% of organic nitrogen, 8.54 ppm available phosphorous, determined according to Donagema et al. (2011).

2.2. Sowing and crop management

The maize hybrid was AX886 MG with glyphosate resistance, an excellent behavior to leaf rust and genetic resistance to *Diatraea saccharalis* (Nidera™, Buenos Aires, Argentina). Soybean was the preceding crop. Maize sowing occurred on 30 September 2010, which is an early date during the typical sowing period for the location which was decided based on environmental conditions. The sowing density was adjusted to 80,000 seeds ha^{-1} with a row distance of 70 cm. At sowing, the entire experimental plot was fertilized with 20 kg ha^{-1} of phosphorous as monoammonium phosphate. Crop management was under no-tillage system.

2.3. Experiment design and treatments

The experiment had a completely randomized block design with a factorial arrangement of three levels of nitrogen fertilization (0, 90 and 180 kg urea ha^{-1}) and five inoculation levels (control without inoculation and four inoculation treatments with different inoculants). Three blocks were applied perpendicularly to the topographic slope which was less than 0.5%. Forty-five plots were considered in the experiment and the dimensions of each plot were 20 m by 3.5 m. Seed inoculation was carried out on the day of sowing and nitrogen fertilization was performed at V4 stage (Ritchie and Hanway, 1982). Inoculation treatments were carried out on the sowing day by mixing each inoculant with the maize seeds and let them dry under shadow for 1 h before sowing. One of the inoculants used in the experiment was a commercial liquid formulation of both *A. brasilense* and *P. fluorescens* (Rhizoflo Premium Maíz™, Laboratorios CKC™, Argentina). The dose per each kg of seeds was 5 ml of commercial inoculant containing 10^9 CFU ml^{-1} as indicated by manufacturer's instruction. Besides, other three experimental inoculants of *A. brasilense* which were formulated with the strains 40 M (GenBank accession number HM002661), 42 M (GenBank accession number HM002662) and 40 M + 42 M, were used. Both strains were previously isolated from maize rhizosphere (García de Salamone and Döbereiner, 1996), identified (García de Salamone et al., 2010) and vastly characterized (Di Salvo et al., 2014; García de Salamone 2012a,b). Experimental inoculants were a liquid formulation of NFb medium with 1 g L^{-1} of ammonium chloride (García de Salamone et al., 2010). In order to formulate the 40 M + 42 M inoculant, both strains were cultured separately and their mixture 1:1 was prepared 24 h before sowing of the maize seeds. The dose per each kg of seeds was 10 ml of the 40 M, 42 M and 40 M + 42 M inoculants containing 10^{10} CFU ml^{-1}.

2.4. Sampling and determinations

Rhizosphere soil, roots and aerial parts of the maize plants were sampled at V5 stage (Ritchie and Hanway, 1982) (62 days after sowing or DAS) and R3 stage (Ritchie and Hanway, 1982) (132 DAS). After physiological maturity (225 DAS), grain yield was determined. At the first two phenological stages, aerial parts were sampled by cutting the plants growing in a line of 0.5 m. (García de Salamone et al., 2012). Every line was randomly selected in each plot as representative of the canopy, avoiding the border effects. In order to determine aerial biomass, sampled maize plants, excluding their reproductive structures, were dried to constant weight at 55 °C.

At V5 and R3 stages, samples of rhizosphere soil and roots were taken with a soil core on the seeding line at a depth of 0–20 cm in duplicates. Roots were manually separated from soil. One of the root samples were used to determine root biomass by drying the roots to constant weight at 55 °C. Before drying, roots were used to determine the total length of root density by the line intersection method. This method consists in putting randomly each stained root sample onto a rectangular grid and counting the number of intersections between every root and the straight lines of the grid. The total length of root density was estimated by the formula $R = \frac{\pi \times A \times N}{2 \times H}$, where R is the total length of root density, A is the area of the rectangular grid, N is the number of intersections between the root and the straight lines, and H is the total length of the straight lines, according to Newman (1966).

The other root samples were used to perform soil suspensions in aqueous solution of NaCl (9 g L^{-1}). Ten-fold dilutions were prepared for each sample. Dilutions were used to analyze the most probable number (MPN) of microaerophilic N_2 fixing (MNF) bacteria, using N-free NFb semisolid medium (Döbereiner, 1998). Also, MPN of cellulolytic and nitrifying microorganisms were determined using 96-well microplates with different culture media. Each well of the microplate was inoculated with 50 µl of soil sample dilution and 200 µl of specific culture media for cellulolytic or nitrifying microorganisms (Alef, 1998). According to the MPN technique, four dilutions of each soil sample were inoculated in triplicates. Besides, control wells without sample inoculation were included. Positive wells and the characteristic numbers were determined by comparison with the control wells (Man, 1983). Microplates were incubated at 28 °C for 15 days. After incubation, MPN of cellulolytic microorganisms was determined by a colorimetric scale according to filter paper degradation, as sole carbon source in culture medium. MPN of nitrifying microorganisms was determined by the quantification of nitrite and nitrate concentration, using Quantofix™ dipsticks (Macherey-Nagel™, GmbH & Co. KG, Germany). Ten-fold dilutions of rhizosphere soil samples were also used to evaluate functional diversity of rhizosphere microbial communities by community-level physiological profiles (CLPP). Thus, 50 µl of 10^{-4} dilutions were inoculated in microplates with 23 sole carbon sources and incubated at 30 °C for 96 h, according to Di Salvo and García de Salamone (2012). Absorbance values were taken every 24 h with a microplate reader Multiskan EX™ (Labsystems, Vantaa, Finland) at 590 nm. Absorbance values from 72 h of incubation were used to perform further analyzes described below.