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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

*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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maize, rice and wheat (García de Salamone, 2012a; García de Salamone and Döbereiner, 1996; García de Salamone et al., 2006, 2010).

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.

### 2.5. Statistical analyses

Absorbance values from CLPP analysis were used to calculate the Shannon's diversity (H') index.

The H index was calculated as $H = -\sum p_i (\ln p_i)$ where $p_i$ is the ratio of the activity on each substrate to the sum of activities on all substrates, according to Gómez et al. (2004). CLPP data were analyzed using discriminant analysis. Data from aerial biomass, grain yield, H' index and microbial determinations were analyzed by Kruskall-Wallis's test or ANOVA and Tukey's test for mean comparisons at $P \leq 0.05$, as appropriate. The software INFOSTAT/Professional 1.1 (Di Rienzo et al., 2011) was used.

## 3. Results

No interactions between nitrogen fertilization doses and PGPR inoculation treatments were observed, however some variables showed differences for main factors. PGPR inoculation and nitrogen fertilization modified grain yield of maize under field conditions. Both doses of nitrogen fertilization (90 and 180 kg urea $ha^{-1}$) increased grain yield by 26 and 38%, respectively, compared to the control without fertilization (Table 1). Fertilizer-use efficiency was greater in plots fertilized with 90 kg urea $ha^{-1}$ (25 kg grain per each kg of fertilizer) than plots fertilized with 180 kg urea $ha^{-1}$ (19 kg grain per each kg of fertilizer). Besides, maize plants fertilized with 90 kg $ha^{-1}$ showed higher root biomass than control plants at V5 stage (Table 1). However, no differences on total length of root density were observed due to nitrogen fertilization at this ontogenetic stage, with an average of 0.76 cm $cm^{-3}$. Root biomass at R3 stage could not be determined because several soil samples were taken with a shovel instead of the soil core which broke up during the R3 sampling (Table 1). Regarding to inoculation effect, PGPR inoculation response was different depending on inoculant type and the agronomic parameter being evaluated. Inoculation increased grain yield compared to control plants. Plants inoculated with 40 M + 42 M inoculant showed the greatest response on grain yield. In addition, plants inoculated with commercial inoculant showed less aerial biomass of maize at R3 stage than the others (Table 1). Furthermore, inoculation with 40 M strain significantly ($P \leq 0.05$) increased total length of root density from maize plants (1.08 cm $cm^{-3}$) compared to control plants (0.69 cm $cm^{-3}$) at R3 stage.

Nitrogen fertilization modified the MPN of cellulolytic, nitrifying and MNF microorganisms at V5 stage (Table 2). The highest MPN of cellulolytic and MNF microorganisms were observed in the rhizosphere of maize plants fertilized with 90 kg urea $ha^{-1}$. The highest MPN of nitrifying microorganisms were observed in the rhizosphere of maize plants without nitrogen fertilization. Interestingly, no differences were observed at R3 due to different nitrogen fertilizer doses. Inoculation only modified the MPN of MNF microorganisms at R3 stage. Maize plants inoculated with 40 M + 42 M showed higher MPN of MNF microorganisms in their rhizosphere than those inoculated with 40 M. Plant ontogeny modified only the MPN of cellulolytic microorganisms. They were higher in V5 than R3 stage (Table 2).

Fig. 1.a shows the discriminant analysis of the CLPP of bacterial rhizosphere communities at both phenological stages. Axis 1 and Axis 2 explained the 72% of the total variation. Microbial communities are clustered on the Axis 1 mainly by arginine, oxalic acid and tween 20 and on the Axis 2 mainly by glutamine and putrescine. Discriminant analysis of the CLPP of bacterial rhizosphere communities at V5 stage is shown in Fig. 1.b. Axis 1 and Axis 2 explained the 57% of the total variation. Microbial communities are clustered on the Axis 1 mainly by malic acid, histidine and glutamine and on the Axis 2 mainly by oxalic acid, dextrose and lactic acid. Discriminant analysis of the CLPP of bacterial rhizosphere communities at R3 stage is shown in Fig. 1.c. Axis 1 and Axis 2 explained the 86% of the total variation. Microbial communities are clustered on the Axis 1 mainly by putrescine, dextrose, glycine and cellobiose and on the Axis 2 mainly by glycerin and proline. It is interestingly to note that the discriminant analysis of physiological profiles performed at R3 stage explained 29% less of total variance than the discriminant analysis performed at V5 stage (Fig. 1.b and 1.c). Discriminant analyses showed that plant ontogeny modified rhizosphere microbial communities physiological profiles more than inoculation and fertilization treatments (Fig. 1.a). According to this, functional diversity at R3 (2.99 of H' index) was significantly higher than the functional diversity at V5 stage (2.88 of H' index). Treatments did not modify the physiological profiles of rhizosphere microbial communities of maize plants at V5 (Fig. 1.b) but differences between treatments were observed at R3 stage (Fig. 1.c). However, no differences between treatments were observed in the H' index of microbial communities from maize rhizosphere at each ontogenetic stage.

## 4. Discussion

Interestingly, neither crop variables -grain yield, aerial and root biomass- nor microbial determinations showed interactions between nitrogen fertilization dose and PGPR inoculation treatments. Nevertheless, some variables showed differences for main factors. Some of the differences due to inoculation treatments could be explained by the use of different doses and concentration for commercial and experimental inoculants. However, it is important to point out that maize plants inoculated with the former showed similar grain yield than maize plants inoculated with the mono-strain experimental 40 M and 42 M inoculants (Table 1). This demonstrates that, in this work, differences in doses and concentrations are not relevant to explain differences in grain yield and, in consequence, other inoculation

**Table 1**
Agronomic response of maize to nitrogen fertilization and PGPR inoculation at three different crop stages[†].

| | Grain yield at PM[‡] (kg $ha^{-1}$) | Root biomass at V5[‡] | Root biomass at R3[§] | Aerial biomass at V5[‡] | Aerial biomass at R3[¶] |
|---|---|---|---|---|---|
| Dose of nitrogen fertilization (kg urea $ha^{-1}$) | | | | | |
| 0 | 8700 ± 414a | 213 ± 125a | nd | 2284 ± 940a | 9316 ± 1740a |
| 90 | 10,941 ± 324b | 337 ± 212b | nd | 1940 ± 454a | 9713 ± 1324a |
| 180 | 12,048 ± 513c | 268 ± 106ab | nd | 2043 ± 347a | 9146 ± 1681a |
| Inoculation treatments | | | | | |
| Control | 10,051 ± 1438a | 241 ± 176a | nd | 2028 ± 509a | 10,191 ± 1795b |
| Commercial | 10,588 ± 1379b | 261 ± 153a | nd | 1991 ± 600a | 7769 ± 1326 a |
| 40 M | 10,540 ± 1452b | 275 ± 169a | nd | 2040 ± 475a | 9568 ± 1280b |
| 42 M | 10,603 ± 1482b | 322 ± 206a | nd | 2038 ± 465a | 9473 ± 998b |
| 40 M + 42 M | 11,033 ± 1549c | 265 ± 104a | nd | 2349 ± 1038a | 9957 ± 1389b |

[†] V5, R3 and physiological maturity (PM) are three phenological stages as described by Ritchie and Hanway (1982).
[‡] Values ± standard deviation, with in a column for each main factor, followed by the same letter are not significantly different with Tukey's test at $P \leq 0.05$.
[§] nd: not determined.
[¶] Values ± standard deviation, with in a column for each main factor, followed by the same letter are not significantly different with Kruskall-Wallis' at $P \leq 0.05$.

**Table 2**
Most probable number (MPN) of cellulolytic, nitrifying and microaerophilic $N_2$ fixing (MNF) microorganisms in the rhizosphere of maize plants under different treatments at two different crop stages[†].

| | V5 stage | | | R3 stage | | |
|---|---|---|---|---|---|---|
| | Cellulolytic[‡] (Log MPN $g^{-1}$ dry root) | Nitrifying[‡] | MNF[‡] | Cellulolytic[‡] | Nitrifying[‡] | MNF[‡] |
| Dose of nitrogen fertilization (kg urea $ha^{-1}$) | | | | | | |
| 0 | 6.56 ± 0.33a | 6.21 ± 0.72b | 6.69 ± 0.73ab | 6.21 ± 0.61a | 6.00 ± 0.67a | 7.10 ± 0.89a |
| 90 | 7.34 ± 0.45b | 5.88 ± 0.87ab | 6.96 ± 0.69b | 6.29 ± 0.29a | 5.81 ± 0.44a | 6.88 ± 0.59a |
| 180 | 6.92 ± 0.50a | 5.45 ± 0.89a | 6.37 ± 0.55a | 6.40 ± 0.41a | 5.62 ± 0.44a | 6.88 ± 1.00a |
| Inoculation treatments | | | | | | |
| Control | 7.08 ± 0.38a | 6.16 ± 1.06a | 6.53 ± 0.61a | 6.40 ± 0.51a | 5.60 ± 0.48a | 6.87 ± 0.80ab |
| Commercial | 6.91 ± 0.39a | 5.94 ± 0.92a | 6.79 ± 0.72a | 6.35 ± 0.65a | 5.75 ± 0.56a | 6.76 ± 0.60ab |
| 40 M | 6.96 ± 0.64a | 5.68 ± 0.68a | 6.80 ± 0.71a | 6.13 ± 0.31a | 5.84 ± 0.45a | 6.46 ± 0.58a |
| 42 M | 6.71 ± 0.41a | 5.81 ± 0.77a | 6.63 ± 0.62a | 6.28 ± 0.42a | 5.86 ± 0.71a | 7.09 ± 0.94ab |
| 40 M + 42 M | 7.02 ± 0.78a | 5.64 ± 0.97a | 6.62 ± 0.89a | 6.34 ± 0.35a | 5.99 ± 0.49a | 7.59 ± 0.88b |
| Average values[§] | 6.94 ± 0.53b | 5.85 ± 0.87a | 6.67 ± 0.69a | 6.30 ± 0.45a | 5.81 ± 0.54a | 6.95 ± 0.83a |

[†] V5 and R3 are two phenological stages as described by Ritchie and Hanway (1982).
[‡] Values ± standard deviation, with in a column for each main factor, followed by the same letter are not significantly different with Tukey's test at $P \leq 0.05$.
[§] Values ± standard deviation, for each microbial group, followed by different letters show significant differences between crop stages with Tukey's test at $P \leq 0.05$.

responses.

It has previously been demonstrated increases in maize grain yield due to *A. brasilense* inoculation in the order of 9% (Díaz Zorita and Fernández Canigia, 2008), 18% (Rodríguez-Cáceres et al., 2008), or more than 70% (García de Salamone, 2012b). Besides, co-inoculation of two *A. brasilense* strains increased wheat grain yield in the order of 30%, while individual inoculation of these strains increased grain yield in the order of 18% (Hungria et al., 2010). To this regard, a mixture of *Azospirillum* strains showed better and differential performance than individual strains when several maize genotypes were inoculated under field experimental conditions (García de Salamone et al., 1996). According to this, in this work, inoculation with 40 M or 42 M strains of *A. brasilense* increased maize grain yield in the order of 5%, while co-inoculation of both strains increased maize grain yield in the order of 11%. Interestingly, it is necessary to have in mind that the grain yield obtained with an addition of 90 kg urea $ha^{-1}$ has a cost six times higher than the grain yield obtained with the use of PGPR bioinsumes. This economic benefit for the farmers is in accordance with the paradigm of conservation agriculture to reduce the amount of chemical fertilizer inputs.

Regarding to commercial inoculant, which is formulated with *A. brasilense* and *P. fluorescens*, it showed inoculation response in the order of 5% similar than the response of 40 M and 42 M inoculation treatments. In addition, maize plants inoculated with the commercial inoculant showed less aerial biomass at R3 stage than the other inoculation treatments (Table 1). These results could be explained by phosphorous fertilization to the entire experimental plot at sowing, which could be affect commercial inoculation response due to phosphate solubilization is one of the plant growth promotion mechanisms of *P. fluorescens* (Antoun and Prévost, 2006). Also, the *A. brasilense* strains included in the commercial inoculant -Az39- have not been isolated from corn and it showed to be very phenotypically (Di Salvo et al., 2014) and genotypically (Jijón-Moreno et al., 2015) different to the 40 M and 42 M strains. As it was reported, these two strains have shown to produce higher levels of indole-acetic acid than Az39 strain (Jijón-Moreno et al., 2015).

Maize inoculation with 40 M and 42 M strains increased grain yield with respect to control plants although no differences in aerial biomass were observed. It could be explained by other factors, such as end-of-cycle diseases affecting reproductive structures but not aerial biomass. In this work, the maize plants showed a high incidence of common corn smut at R3 stage. This disease is caused by *Ustilago maydis* which produce galls on corn ears and decreases in grain yield (Windauer et al., 2004). Although no field measurements were considered in order to evaluate common corn smut incidence, it could be interesting to evaluate, in the future, if *A. brasilense* inoculation could reduce the incidence of this plant disease. This idea is based on the fact that 40 M and 42 M *A. brasilense* strains, among other PGPR mechanisms, have shown the ability to produce siderophores under *in vitro* conditions (Di Salvo et al., 2014) which could help to control this phytopathogenic fungus.

Probably because it is an arduous method, it is unusual to find field experiments in which total length of root density was determined. Thus, it is more frequent the determination of root biomass. However, total length of root density considers different thicknesses of the root, unlike the root biomass, which did not consider the fine roots. This work makes a valuable contribution to the knowledge of the root systems because is one of the few in which values of total length of root density were established for maize crops under productive field environments. In this work, nitrogen fertilization modified root biomass while *A. brasilense* inoculation modified total length of root density. These results demonstrate that both variables are complementary to evaluate the effect of these two agronomic practices on maize production.

It has been demonstrated that *A. brasilense* inoculation increases the quantity or length of both hair roots and adventitious roots (Okon and Vanderleyden, 1997). In this work, this effect was only observed in the total length of root density of maize plants inoculated with the 40 M strain. Also, total length of root density did not show differences between ontogenetic stages. Regarding to this, the lack of differences in this variable does not mean that plants did not have differences in their soil exploration patterns, considering that total length of root density were measured in a specific volume of soil from the top layer of 20 cm of the soil profile.

Although, cultivable soil microbiota constitutes a small fraction of the total diversity, they are considered as the most active microorganisms in soils. Thus, cultivable-dependent techniques are useful to demonstrate human impact on soil bacterial communities (Chessa et al., 2016). In this work, PGPR inoculation did not modify the MPN of cellulolytic and nitrifying at both ontogenetic stage but modified the MPN of MNF bacteria at R3 stage. At this stage, plants inoculated with 40 M + 42 M showed higher MPN of MNF than plants inoculated with 40 M strain. This result suggests a competitive advantage of the combined inoculant over the single-strain inoculant formulations. Besides, the MPN of MNF in the rhizosphere of control plants was similar than the MPN of this microbial group in the rhizosphere of inoculated plants. Although some authors reported PGPR inoculation effect on MPN of MNF bacteria in the rhizosphere of maize (Abril et al., 2006; Cappelletti et al., 2004; Casaretto and Labandera, 2008) and rice (García de

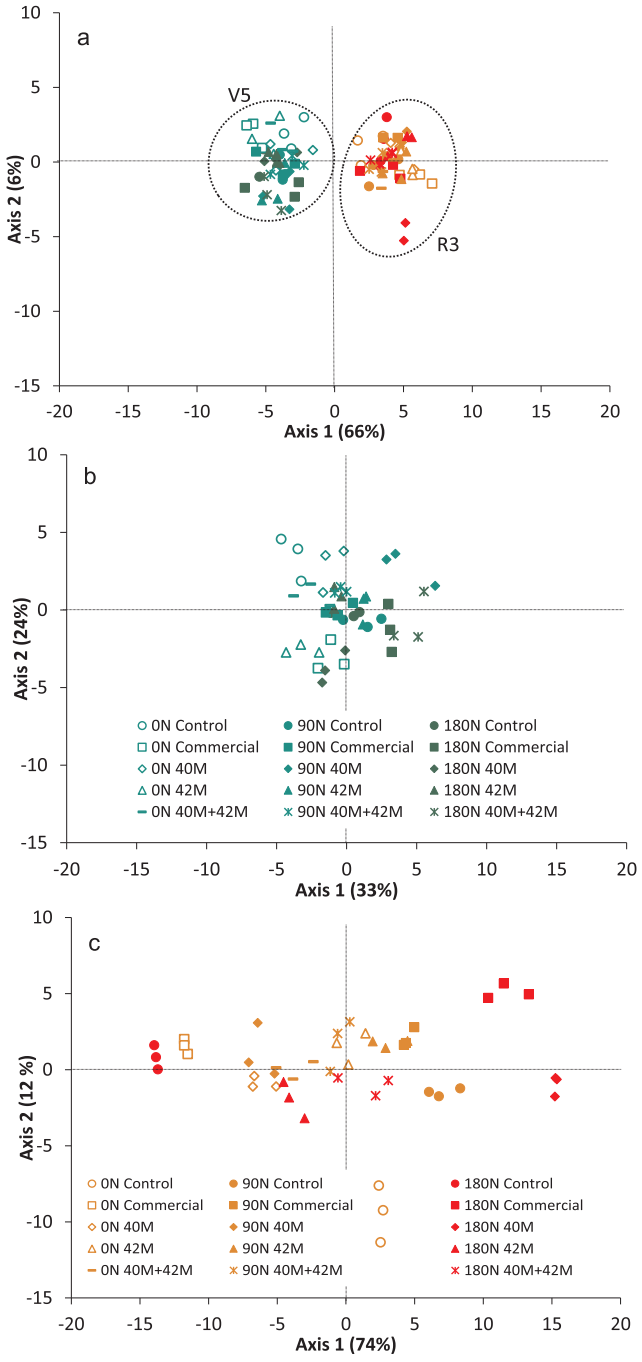


**Fig. 1.** Discriminant analysis of the physiological profiles of microbial communities from rhizosphere of maize under different levels of nitrogen fertilization and PGPR inoculations at two different ontogenetic stages (a), at V5 stage (b) and R3 stage (c). Data used for the analysis corresponded to 72 h absorbance values. Total explained variance by each axis is in parenthesis.

Salamone et al., 2010; Pedraza et al., 2009), other authors demonstrated that not all field experiments show these differences (Abril et al., 2006; Naiman et al., 2009). Our results to this respect could be explained because in NFb medium can grow both native *A. brasilense* strains different than inoculated strains and other bacterial species which can fix $N_2$ different than *A. brasilense* (Di Salvo et al., 2014).

Nitrogen fertilization modified the MPN of cellulolytic, nitrifying and MNF microorganisms at V5 stage but no differences were observed at R3 stage. The incorporation of nitrogen fertilizer in V4 stage could promote growth of soil microorganisms, which are generally under nutritional deficiency (Madigan et al., 2000). Nitrogen fertilization can promote degradation of organic matter (Treseder, 2008), a phenomenon known as "positive priming effect" (Kuzyakov et al., 2000). This could increase MPN of cellulolytic microorganisms at V5 stage, immediately following nitrogen fertilization. The activity of these mostly heterotrophic microorganisms provides carbon compounds of lower molecular weight for other heterotrophic microorganisms, such as $N_2$ fixing bacteria. This generates more heterotrophic activity (Kumar and Goh, 1999), which would result in a greater amount of microbial biomass and therefore increased nitrogen immobilization, primarily as ammonia. Nitrogen immobilization causes a reduction in soil ammonium availability for nitrifying microorganisms. When nitrogen fertilizer was applied, soil ammonium availability increases. Thus, heterotrophic activity increases and the MPN of nitrifying microorganisms could decrease, because they are mainly chemoautotrophs. This could be the result of a lower competitive ability of this functional group to immobilize this nutrient with respect to the heterotrophic microbial community (Verhagen and Laanbroek, 1991). Moreover, nitrogen fertilization with 180 kg urea $ha^{-1}$ reduced the MPN of cellulolytic, nitrifying microorganisms, probably due to toxic effect of ammonium (Bollmann and Laanbroek, 2001; Koops et al., 2006). Even though it has been demonstrated that bacteria have protection mechanisms to ammonium toxic effect (Müller et al., 2006), only three bacterial genera were evaluated.

Regarding to MPN of MNF bacteria, differences among inoculation treatments were observed at V5 stage but no differences were observed at R3 stage, according to Roesch et al. (2006). It is known that the nitrogen availability in soils inhibits biological $N_2$ fixation (Cocking, 2003), but does not inhibit growth of heterotrophic MNF microorganisms or crop response to PGPR inoculation (Bashan and Levanony, 1990). Thus, in this work, differences in the MPN of MNF bacteria in the rhizosphere of maize plants were observed between both nitrogen fertilization doses. Besides, decreases in the MPN of MNF bacteria caused by 180 kg urea $ha^{-1}$ could be explained by less competitive ability of this functional group than other heterotrophic microorganisms under high nitrogen availability conditions. Some authors have reported that high nitrogen availability for plant nutrition increases secretion of carbon compounds in the root exudates. They promote the proliferation of heterotrophic microorganisms which can negatively affect the MNF microorganisms by competition (Dobbelaere et al., 2002). As some authors demonstrated, urea application at sowing in combination with *A. brasilense* inoculation reduced bacteria survival (Puente et al., 2008). For that reason, in this work, urea fertilizer was applied at V4 stage. At this regard, there are important aspects to consider when selected agricultural practices associated with crop management are applied.

Interestingly, only the MPN of cellulolytic microorganisms showed differences between both ontogenetic stages of the maize crop. This microbial group could be more sensitive than the nitrifying and MNF microorganisms to the changes in the rhizospheric environment across crop development. During the first stages of maize crop, residues of the preceding crop on soil surface are less degraded than remaining residues at the end of this summer crop. These residues are the substrate for cellulolytic microorganisms, which can degrade them under favorable environmental conditions by mostly mesophilic microorganisms. As the crop grows, its root exudates are the source of low molecular weight carbon compounds (Aulakh et al., 2001; Badri and Vivanco, 2009). These carbon compounds allow other heterotrophic microorganisms to grow, which compete for carbon substrates with cellulolytic microorganisms in the rhizosphere. This could explain differences in the MPN of cellulolytic microorganisms between ontogenetic stages.

Crop residues degradation causes nutrient immobilization in microbial biomass, such as nitrogen immobilization. In addition, nitrate uptake in maize plant is higher during vegetative and flowering stages than during grain-filling stage. Thus, soil nitrate concentration is lower at the first stages of maize crop in comparison with the latest stages due to both microbial immobilization and plant assimilation (Paul and Clark, 1996). For this reason, it would be expected that the MPN of

nitrifying microorganisms at V5 stage was lower than the MPN of this functional group at R3 stage, due to competition for ammonia with heterotrophic microorganisms, which are involved in nutrient mineralization. However, no differences were observed, probably due to the effect of nitrogen fertilization, which has been applied at V4 stage and modified MPN of this functional group at V5 stage, as it was discussed before. Also, no differences between ontogenetic stages were observed in the MPN of MNF, contrary to what was previously reported (Cappelletti et al., 2004; Garcia de Salamone et al., 2010; Reis et al., 2000). Despite the fact that this functional group did not show differences in its MPN by counting with NFb culture medium, it is important to note that the genetic structure of microorganisms with biological $N_2$ fixation ability could probably show differences, according to Soares et al. (2006).

Analysis of functional diversity of rhizosphere microbial communities by CLPP is an estimation of the potential catabolism of cultivable microorganisms from environmental samples. This methodology was widely used to analyze both soil and rhizosphere microbial communities (Di Salvo and García de Salamone, 2012). Some authors have demonstrated that the physiology of rhizosphere microbial communities is modified by inoculation (Conn and Franco, 2004; García de Salamone et al., 2010, 2012; Naiman et al., 2009), mainly at early stages of crop development (Minz and Ofek, 2011). In this work, microbial communities physiological profiles of maize rhizosphere were modified by the interaction between inoculation and fertilization treatments, only at R3 stage of the crop (Fig. 1.c). These results demonstrate that the sampling moment determination, according to crop development, is essential in order to show differences between agricultural practices and make conclusions which could have an impact on the crop management.

As the crop grows, root exudates have different quantity and quality of organic compounds (Aulakh et al., 2001; Kamilova et al., 2006). The composition of root exudates can change microbial communities of the rhizosphere (Kristin and Miranda, 2013; Houlden et al., 2008). According to this, other authors showed that plant ontogeny modified the physiology of microbial communities (Baudoin et al., 2002; Houlden et al., 2008; Kristin and Miranda, 2013). In this work, ontogenetic stages of the maize plants had stronger effect on the physiological profiles and the functional diversity of rhizosphere microbial communities than the agriculture practices of PGPR inoculation and nitrogen fertilization (Fig. 1). These results are relevant information to take into account before making decisions about the crop management.

## 5. Conclusions

Crop response to *A. brasilense* inoculation is determined by interactions between the inoculated bacterial strain, plant genotype (Abril et al., 2006; García de Salamone, 2012a) and environmental conditions (Rani and Goel, 2012), such as nutrient availability (Dobbelaere et al., 2001) and native microbial community (Aeron et al., 2011). The latter constitutes an ecological competition for the applied inoculant (Cummings, 2009). Accordingly it was expect, nitrogen fertilization increased maize grain yield. However, no interaction between fertilization and PGPR inoculation were observed. Maize grain yield increased by the inoculation with the 40 M and 42 M strains of *A. brasilense* individually or in combination. This inoculation response was even higher than the crop response to the commercial inoculant with both *A. brasilense* and *P. fluorescens*. This work demonstrates the potential of 40 M and 42 M strains to be used as bioinsumes for maize production. Besides, this work is one of the few to measure the variable total length of root density in a crop at field conditions. This variable showed to be useful to complement the information obtained with the variable root biomass, usually determined in many researches. Both variables together could be used to evaluate more exhaustively the effect of agricultural practices, such as chemical fertilization and PGPR inoculation, or the changes in root morphology during the crop cycle.

Nitrogen fertilization modified the MPN of cellulolytic, nitrifying and MNF microorganisms, only at vegetative stage of maize. The different levels of nitrogen fertilization cause different effects on these microbial communities at this ontogenetic stage. Besides, only some PGPR strains modified the MPN of MNF in the maize rhizosphere at reproductive stage of the crop, while the other evaluated microbial communities were not affected at any ontogenetic stage of the crop. Finally, PGPR inoculation and nitrogen fertilization modified the physiology of rhizosphere microbial communities in maize rhizosphere mainly at the reproductive stage. However, this work showed that plant ontogeny modified the physiological profiles of rhizosphere microbial communities more than inoculation and fertilization treatments.

This work shows, for the first time, the effects of both agricultural practices and crop development on maize rhizosphere microbial communities. The results regarding to the effects of inoculation with *A. brasilense* and *P. fluorescens* and nitrogen fertilization on certain microbial communities are a contribution to the knowledge of maize rhizosphere ecology at field conditions. They can be used to improve crop response to these PGPR inoculations in interaction with chemical fertilization and environmental risk characterization of both agronomic practices for a more sustainable agricultural production.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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