Effects of inoculation on growth promotion and biological nitrogen fixation in maize (Zea mays L.) under greenhouse and field conditions

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The application of PGPB to agriculture is increasing as it offers an alternative to the use of chemical fertilizers, pesticides and other supplements. Maize (Zea mays L.) is one of the world’s principal cereal crops, grown for grain, silage and biofuel. Nitrogen fertilizer constitutes one of the major costs in maize production, and PGPB poses a potential alternative for achieving more sustainable production. In this study, we present the results of maize inoculation experiments with PGPB under greenhouse and field conditions. We found significant differences in shoot and root dry weight among cultivars and inoculation treatments in the greenhouse. The inoculation of Maizon with Rhanella spp. EMA83 increased shoot dry weight (22%) relative to the non-inoculated control. Similarly, the maize cultivar PAU871 increased in shoot biomass when inoculated with Pantoea agglomerans EMA82 (33%) and Rhanella spp EMA83 (50%) relative to the non-inoculated treatment. There was a negative correlation between % 15N a.e. and shoot dry weight (r=0.6, P<0.05, n=75). In the field, maize grain yield (kg ha⁻¹) increased among treatments with Rhanella spp. onto seed (15%) and soil (12%) and Azospirillum spp.in seed (15%) at 0 N fertilization rates. At maximum fertilization rates an increase was found only for maize inoculated with Rhanella spp. in soil (16%) relative to the non-inoculated control. Relative increases in 1000-grain dry weight (g) and yield (kg ha⁻¹) were also observed with inoculation treatments, regardless of N fertilization rate, respectively. In conclusion, Rhanella spp. EMA83 is a promising PGPB for growth enhancement of maize. This strain may potentially fix nitrogen, produce IAA and solubilize P. However, it is equally important to study the mechanisms by which this putative endophyte promotes growth as well as its potential for enhancing sustainable crop production.

Keywords: maize (Zea mays L.), putative endophytic bacteria, ¹⁵N isotope dilution technique

INTRODUCTION

An exponential increase in the world’s population will necessitate increased crop production. World population is expected to grow by over a third or 2.3 billion people between 2009 and 2050. Demand for cereals, is projected to reach some 3 billion tones by 2050 (FAO 2009). This rise in crop production has been a result of the indiscriminate use of chemical fertilizers (N, P, K) in combination with advancements in technology. Maize (Zea mays L.) is one of the world’s most important cereals and is grown for grain, silage and biofuel. To obtain an average yield of 7-9 t ha⁻¹, applications of 110-140 kg ha⁻¹ N, 20-50 kg ha⁻¹ P₂O₅, and 30-80 kg ha⁻¹ K₂O are generally recommended. Unfortunately, less than 50% of applied N fertilizer is used by plants (Halvorson et al., 2002). This poor use efficiency of nitrogen contributes to the nitrate contamination of soil and ground water supply, leading to health hazards and environmental degradation. Moreover, the use of chemicals, especially nitrogen – the most frequent limiting factor of intensive agriculture production – is extremely costly for many farmers.

Plant growth promoting bacteria (PGPB) comprise a group of microorganisms that colonize the root surface and internal plant tissues and pose many benefits to host...
plants (Kloepper et al. 1989). PGPB can stimulate plant growth by means of several processes, including biological nitrogen fixation (BNF); increases in nitrate reductase activity when growing as plant endophytes (Huergo et al., 2008); synthesis of hormones such as auxins, cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989), ethylene (Strzelezyk et al., 1994), and a variety of other molecules (Pering et al., 2007); solubilization of phosphate (Rodriguez et al., 2004); and biological control of pathogens (Correa et al., 2008). Overall, combinations of these mechanisms likely benefit plant growth (Dobbelaere et al., 2003). The use PGPB inoculants offer a sustainable alternative to chemical fertilizers and pesticides (Di Cello et al., 1997, Kennedy et al., 2004). A variety of PGPB have been found to colonize the roots and aerial parts of cereal crops and other grasses, including maize. PGPB found in association with maize include Azospirillum (Christansen-Weniger and Vanderleyden 1994); Klebsiella (Chelius and Tripplett 2000a), Dong et al., (2001); Pantoea, Herbaspirillum and Bacillus (Chelius and Tripplett 2000b), Palus et al, (1996); Rhizobium etli (Gutiérrez-Zamora and Martinez Romero 2001) and Burkholderia (Di Cello et al., 1997), Perin et al, (2006) all or some of which may supply plants with fixed N2.

Inoculation of maize with such bacteria has been shown to enhance crop yields (Jacoud et al., 1998), Riggs et al., (2001), Sharma and Johri, (2003). One study suggested that some maize cultivars may fix up to 60% of their N after inoculation with appropriate strains of Azospirillum (Garcia de Salmone et al., 1996). Under field conditions, selected strains of Azospirillum brasilense increased grain yields of maize by 24-30%, relative to non-inoculated controls, with low N-fertilizer starter (24 kg N ha\(^{-1}\)) at sowing (Hungría et al., 2010).

The use of microbial preparations for the enhancement of plant production is becoming a more widely accepted practice for both economic and ecological reasons. A key factor to the success of inoculation practices is the choice of appropriate bacterial strains. Although no specificity between plant species and bacterial strains has been demonstrated, some affinity exists between bacteria and plant species (Penot et al., 1992) or cultivars (Wani et al., 1985). An effect of maize genotype on plant interaction with bacteria has been also demonstrated (Garcia de Salamone et al., 1996). Despite the widespread occurrence of bacterial endophytes among non-leguminous plants, there is limited knowledge regarding the mechanism involved in endophyte-host interactions and their effect on plant growth. The most useful method for examining N\(_2\) fixation in the field and greenhouse experiments is the \(^{15}\)N isotope dilution technique (James 2000).

In this study we present results of maize inoculation experiments with various strains of PGPB under greenhouse and field conditions. To understand the effect of biological nitrogen fixation on plant growth, we used the \(^{15}\)N isotope dilution technique in the greenhouse experiment.

**MATERIALS AND METHODS**

**Greenhouse experiment**

The influence of four bacterial strains on plant growth and bacterial nitrogen fixation (BNF) was explored among three commercial maize cultivars, Maizom, PAU871 and NK940. The experiment was carried out in a greenhouse at the University with the objective of selecting a strain for further field experiment. Seeds of each cultivar were obtained from local private companies in Uruguay. PAU871 and NK940 (GMO-Bt) are hybrids and Maizom is a Uruguayan cultivar.

**\(^{15}\)N isotope dilution method**

The \(^{15}\)N isotope dilution method was used to estimate BNF among maize cultivars inoculated and non-inoculated by bacteria. This method involves the testing of a N\(_2\)-fixing plant and a suitable non-N\(_2\)-fixing control, both growths in a substrate with homogeneous \(^{15}\)N enrichment. It is important that the \(^{15}\)N enrichment in the soil changes slowly over time, that the fixing and reference plants have similar N uptake patterns, and that the roots of both plants explore the same volume of soil (Boddey and Döbereiner, 1995). Hardarson and Danso, (1990); IAEA, (2001). As such, given a sole supply of N by the soil and \(^{15}\)N-labeled fertilizer, a fixing plant and a non-fixing reference plant will contain the same ratio of \(^{15}\)N/\(^{14}\)N, as they are taking up N of the same \(^{15}\)N/\(^{14}\)N composition, but not necessarily the same quality of N. In both plants, the \(^{15}\)N/\(^{14}\)N ratio within plant tissues is lowered by the N absorbed from the unlabelled N source. Thus, the extent to which the \(^{15}\)N/\(^{14}\)N ratio decreases in the fixing crop, relative to the non-fixing plant, determines BNF. To evaluate nitrogen fixation, a solution containing \(^{15}\)N-labeled ammonium sulfate (5 mg N kg\(^{-1}\)soil) of 10% atom excess was added as a tracer. In this experiment non-inoculated treatments of maize plants that incorporated the highest levels of N from tracers and had the highest \(^{15}\)N atom excess (a.e.) were used as a control or reference for the calculation of \%Ndfa, assuming that these plants were not fixing N or had negligible nitrogen fixation.

**Bacterial strains**

The bacterial strains *Pseudomonas fluorescens* EMA38, *Rhanelia* spp. EMA83, *Herbaspirillum frisingense* EMA117 and *Pantoea agglomerans* EMA82, were previously isolated from different maize cultivars and
characterized by Montañez et al., (2009) for several growth promoting abilities. All bacteria strains were nifH positive, produced indole acetic acid (IAA) and solubilized P, and *Pseudomonas fluorescens* EMA38 produced siderophores. Bacterial strains were grown in TY broth (Josey et al., 1979), for 48 h at 30°C and concentration was individually adjusted to $10^8$ cells ml$^{-1}$. Cell number was verified by the plate count dilution method. A volume of 2.5 ml of each individual strain was inoculated onto each seed, at the seedling stage and ten days after planting.

**Maize cultivars and growth conditions**

Seeds of Maizon, NK940 and PAU871 were surface sterilized with 4% sodium hypochlorite for 10 min, then washed five times with sterile distilled water and subjected to sterility checks on TY medium to ensure sterilization efficiency. The seeds were pre-germinated on agar water plates at 30°C. Two seedlings were transferred into each 3kg pot filled with non-sterile soil. Pots were previously washed with 20% sodium hypochlorite. Soil used in this experiment was a sandy soil: pH (H$_2$O) 5.0; organic matter, 1.3%; 0.065% total N; extractable P (Bray I), 10 ppm; exchangeable K, 0.29 Meq/100 g; exchangeable Ca, 0.6 Meq/100 g; exchangeable Mg, 0.4 Meq/100 g and exchangeable Na, 0.06 Meq/100 g (Black et al., 1965). Sterilized sand stones were spread on top of each pot to prevent the growth of airborne bacteria or fungi and reduce evaporation.

The greenhouse temperature ranged from 25 to 28 °C. Plants were watered on demand from the bottom plate with tap water and one half N free Fähraeus’s (1957) nutrient solution. Pots were placed at randomly selected locations in the greenhouse and rotated each week. Each inoculation treatment was replicated five times. Plants were harvested after 90 days, upon which root and shoots were excised and placed in paper bags in a drying oven at 70°C for 72 h. The dried shoots were ground to a fine powder, and total N in these samples were determined by the Kjeldahl method (Bremmer and Mulvaney, 1982). The analysis for $^{15}$N excess in plant tissue was determined by mass spectrometry (IAEA-Vienna).

**Field experiment**

On the basis of previous greenhouse studies, *Rhanella* spp. EMA83, was selected for a field trial at Ombues de Lavalle, Colonia, Uruguay (33° 54’S, 57° 46’W). Commercial inoculant containing *Azospirillum* spp. 39, was also included as an inoculation treatment in the field trial. The growing season in this location was from October 2008 to March 2009. The climate of the region is temperate with a mean monthly rainfall of 92 mm and a mean annual temperature of 17.4 °C.

Maize NK900 is the most commonly used maize cultivar in this agriculturally intensive region. Maize seeds of NK900, treated with fungicide Fludioxonil and Metalaxyl-M, were used in the field trial. Plant density was 80,000 plants ha$^{-1}$. The soil was a Brunosol Sub éutrico with a pH (H$_2$O) of 6.2, 42% clay, and had been previously used for sorghum crop. Chemical characteristics of the soil at sowing were 4.1% organic matter, 33 ppm N-NO$_3$ and 18 ppm extractable P Bray I.

Two inoculation treatments with *Azospirillum* spp. 39 and *Rhanella* spp. EMA83 were performed at sowing: 1) onto the seed and 2) in the soil. The dose for seed inoculation was 300 ml ha$^{-1}$ while the dose for soil inoculation was 600 ml ha$^{-1}$ of the inoculant formulations containing 1.5 x $10^2$ ufc ml$^{-1}$ of each microorganism.

Soil N at the 6 leaf stage (V6) was 60 kg ha$^{-1}$, which was considered sufficient for physiological growth. Nonetheless, application of urea (46% N) at that stage was performed at rates of 70 and 120 kg ha$^{-1}$. Weeds were controlled at pre-emergence with 2 l ha$^{-1}$ (500 g l$^{-1}$) of Atrazine and Acetochlor (90%) at a dose of 1 l ha$^{-1}$. Average total precipitation required during the growth period was 600 mm and total precipitation received was 258 mm.

Field plots consisted of 4 rows of 81 m long spaced at 0.70 m apart and arranged in a split-plot design with either inoculation or non-inoculation as the main-plot treatments and N-fertilization rates (0, 70 and 120 kg ha$^{-1}$) as the sub-plot treatments. The total area of each plot was 25.2 m$^2$. This design provided for 3 replicates and 5 treatments. Each of the main and sub-plot treatments and replicates were randomly distributed to minimize experimental error from uncontrolled factors typically accompanying field experimentation. Treatments with and without N-fertilizer were (1) non-inoculated control, (2) *Azospirillum* spp. 39 onto seed, (3) *Azospirillum* spp. 39 in soil, (4) *Rhanella* spp. EMA83 onto seed, (5) *Rhanella* spp. EMA83 in soil.

**Evaluation of agronomic parameters**

Aerial biomass was determined at both V6 (6 leaves, 51 days after sowing) and F (flowering, 65 days after sowing) stages and the dry weight of a thousand grains was evaluated at the F stage. At each sampling time (V6 and F) 10 randomly selected plants from each split-plot were removed, excluding edge plants. Samples were dried at 70°C and weighed after a week of constant weight. Total N content in plant tissue was obtained with the Kjeldahl method (1956).

**Statistical analysis**

Data from each experiment were analyzed by ANOVA
Table 1. Effect of bacterial inoculation on dry weight and atom % $^{15}$N excess and nitrogen uptake in three maize cultivars

<table>
<thead>
<tr>
<th>Maize Cultivar</th>
<th>Treatment</th>
<th>Dry weight g plant $^{-1}$</th>
<th>Atom excess (%) $^{15}$N</th>
<th>N accumulation (g plant$^{-1}$)</th>
<th>N concentration (mg g$^{-1}$ dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>shoot</td>
<td>root</td>
<td>shoot</td>
<td></td>
</tr>
<tr>
<td>Maizon</td>
<td>Non-inoc</td>
<td>18.50 ab</td>
<td>7.72 ab</td>
<td>0.02 b</td>
<td>0.13 a-c</td>
</tr>
<tr>
<td></td>
<td>Pantoea agglomerans EMA82</td>
<td>15.87 a</td>
<td>6.99 a</td>
<td>0.23 a</td>
<td>0.10 a</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas florescens EMA38</td>
<td>20.44 a-c</td>
<td>14.56 b</td>
<td>0.26 ab</td>
<td>0.14 bc</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas and Herbaspirillum EMA117</td>
<td>23.48 bc</td>
<td>12.31 ab</td>
<td>0.22 ab</td>
<td>0.12 ab</td>
</tr>
<tr>
<td></td>
<td>Rhanella spp EMA83</td>
<td>24.73 c</td>
<td>14.44 b</td>
<td>0.19 a</td>
<td>0.16 c</td>
</tr>
<tr>
<td></td>
<td>CV%</td>
<td>20.61</td>
<td>46.8</td>
<td>5.9</td>
<td>22.5</td>
</tr>
<tr>
<td>PAU871</td>
<td>Non-inoc</td>
<td>16.47 a</td>
<td>5.42 c</td>
<td>0.27 ab</td>
<td>0.09 a</td>
</tr>
<tr>
<td></td>
<td>Pantoea agglomerans EMA82</td>
<td>24.52 bc</td>
<td>7.81 bc</td>
<td>0.19 b</td>
<td>0.13 ab</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas florescens EMA38</td>
<td>19.53 ab</td>
<td>8.79 a-c</td>
<td>0.32 a</td>
<td>0.11 ab</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas and Herbaspirillum EMA117</td>
<td>22.66 a-c</td>
<td>9.37 ab</td>
<td>0.19 b</td>
<td>0.14 b</td>
</tr>
<tr>
<td></td>
<td>Rhanella spp EMA83</td>
<td>26.97 c</td>
<td>11.55 a</td>
<td>0.18b</td>
<td>0.13 ab</td>
</tr>
<tr>
<td></td>
<td>CV%</td>
<td>23.06</td>
<td>31.48</td>
<td>6.19</td>
<td>28.45</td>
</tr>
<tr>
<td>NK940</td>
<td>Non-inoc</td>
<td>22.35 ab</td>
<td>6.83 ab</td>
<td>0.16 b</td>
<td>0.13 a</td>
</tr>
<tr>
<td></td>
<td>Pantoea agglomerans EMA82</td>
<td>17.78 a</td>
<td>5.29 b</td>
<td>0.28 a</td>
<td>0.11 a</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas florescens EMA38</td>
<td>19.33 ab</td>
<td>8.41 a</td>
<td>0.24 ab</td>
<td>0.14 a</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas and Herbaspirillum EMA117</td>
<td>21.16 ab</td>
<td>8.75 a</td>
<td>0.25 b</td>
<td>0.12 a</td>
</tr>
<tr>
<td></td>
<td>Rhanella spp EMA83</td>
<td>23.78 b</td>
<td>7.21 ab</td>
<td>0.22 ab</td>
<td>0.13 a</td>
</tr>
<tr>
<td></td>
<td>CV%</td>
<td>21.20</td>
<td>29.74</td>
<td>6.69</td>
<td>26.74</td>
</tr>
</tbody>
</table>

*Values represent the mean of seven replicates. Values in the same column for each cultivar followed by the same letter are not significantly different (LSD test, P<0.05).

RESULTS

Greenhouse experiment

Significant (P<0.05) differences in shoot and root dry weights were found among cultivars and treatments Table (1). The inoculation of Maizon with Rhanella spp. EMA83 significantly increased shoot dry weight (22%) compared to the non-inoculated control. PAU871 plants also showed a significant increase in shoot weight when inoculated with Pantoea agglomerans EMA82 (33%) and Rhanella spp. EMA83 (50%) compared with the non-inoculated treatment. PAU871 plants also showed a significant (P<0.05) increase in root dry weight in comparison to non-inoculated controls in when inoculated with Herbaspirillum frisingense EMA117 (42%) and Rhanella spp. EMA83 (53%). Regardless, the maize cultivar with Rhanella spp. increased significantly (P<0.05) in shoot dry weight compared to non-inoculated control (n=75, CV=21.7%). The cultivar NK940 showed no response to inoculation treatments.

The %$^{15}$N a.e. values ranged from 0.16% (NK940 non-inoculated) to 0.32% (PAU871 with Pseudomonas florescens EMA38). Maize cultivars with lower %$^{15}$N a.e. for each inoculated treatment (P<0.05) were: Maizon inoculated with Rhanella spp. and PAU871 inoculated with Rhanella spp., Pseudomonas florescens and Herbaspirillum frisingense Table (1).

According to this technique, the input of unlabelled nitrogen derived from BNF was calculated from the proportional dilution of the plant $^{15}$N enrichment derivate from the $^{15}$N-labeled fertilizer, relative to the non-fixing plant. In this experiment, non-inoculated treatments were not suitable as a reference treatment for calculating BNF.

Either N shoot accumulation or N concentration varied among treatments and cultivars, but differences were non-significant Table (1). In general, an increase in shoot dry weight was observed among inoculated plants with
higher N content (g plant⁻¹; r=0.5, P<0.05, n=75). Meanwhile, a negative correlation was observed between shoot dry weight and % ¹⁵N a.e. (r=0.6, P<0.05, n=75).

**Field experiment**

Significant increases (P<0.05) in shoot dry weight were observed among plants inoculated with *Azospirillum* spp. in soil at V6 (22.6%), at F without N application (23.7%) and with maximum N fertilization (15.7%) relative to non-inoculated controls. At stage V6, N accumulation (mg plant⁻¹) was significantly higher (P<0.05) when no N was applied to the soil of plants inoculated with *Rhanella* spp. on seeds and *Azospirillum* spp. on seeds and in soil. At stage F, a significant increase in shoot dry weight was observed among plants inoculated with *Azospirillum* spp. in soil at N fertilization rates of 0 and 120 kg ha⁻¹. An identical response was observed for plant N (mg g⁻¹) concentration Table 2.

In comparison with non-inoculated controls without N fertilization, the dry weight of 1000 grains increased significantly (P<0.05) among plants inoculated with *Rhanella* spp. (8%) in soil and *Azospirillum* spp. (5%) in seed, at 70 N fertilization rate, with both *Rhanella* spp. (8%) and *Azospirillum* spp. (8%) in seeds, and with *Azospirillum* spp. in seed at 120N rate (9%) and *Rhanella* spp. in soil (8%) Table 3.

The number of grains per cob ranged from 333 (non-inoculated without N) to 397 (with *Azospirillum* spp. in soil at 120 kg N ha⁻¹). There were no significant differences in grain count per cob between inoculated and non-inoculated treatments for each N fertilization rate. There were significant differences (P<0.05) in the grain yields of all inoculation treatments compared to non-inoculated controls when N was not applied. Only one treatment, *Rhanella* spp. in soil at 120 kg N ha⁻¹, showed significant differences compared to the non-inoculated treatment. There were significant differences (P<0.05 in grain yield (kg ha⁻¹) at 0 N fertilization rate among plants inoculated with *Rhanella* spp. onto seed (15%) and soil (12%) and *Azospirillum* spp. onto seed (15%). At maximum fertilization rates, significant differences were only found among plants inoculated with *Rhanella* spp. in the soil (16%), compared to non-inoculated controls (Table 3). Overall, significant increases were observed in 1000 grain yield (g) and yield (kg ha⁻¹) among inoculation treatments in relation to the control, regardless of N fertilization rate (P<0.05, n=135, CV 7.14% and CV=14.08%).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogen fertilization (kg ha⁻¹)</th>
<th>Plant Shot stage</th>
<th>Dry weight (g)</th>
<th>N Accumulated (mg pl⁻¹)</th>
<th>N Concentrated (mg g⁻¹ dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-inoculated</td>
<td>0</td>
<td>V6</td>
<td>59.33 a</td>
<td>13 a</td>
<td>781 a</td>
</tr>
<tr>
<td><em>Rhanella</em> spp in Soil</td>
<td>0</td>
<td>V6</td>
<td>63.33 a</td>
<td>15.33 a</td>
<td>972 ab</td>
</tr>
<tr>
<td><em>Rhanella</em> spp in Seed</td>
<td>0</td>
<td>V6</td>
<td>68.33 a</td>
<td>16.67 b</td>
<td>1146 b</td>
</tr>
<tr>
<td><em>Azospirillum</em> spp in Seed</td>
<td>0</td>
<td>V6</td>
<td>70.33 a</td>
<td>16.67 b</td>
<td>1179 b</td>
</tr>
<tr>
<td><em>Azospirillum</em> spp in Soil</td>
<td>0</td>
<td>V6</td>
<td>82.00 b</td>
<td>19.67 c</td>
<td>1617 c</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td></td>
<td>9.29</td>
<td>8.40</td>
<td>17.07</td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>70</td>
<td>F</td>
<td>137 a</td>
<td>21 a</td>
<td>2880 a</td>
</tr>
<tr>
<td><em>Rhanella</em> spp in Soil</td>
<td>70</td>
<td>F</td>
<td>154 a</td>
<td>23.67 a</td>
<td>3654 a</td>
</tr>
<tr>
<td><em>Rhanella</em> spp in Seed</td>
<td>70</td>
<td>F</td>
<td>154.67 a</td>
<td>23.67 a</td>
<td>3735 a</td>
</tr>
<tr>
<td><em>Azospirillum</em> spp in Seed</td>
<td>70</td>
<td>F</td>
<td>153 a</td>
<td>23.67 a</td>
<td>3630 a</td>
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<tr>
<td><em>Azospirillum</em> spp in Soil</td>
<td>70</td>
<td>F</td>
<td>181.33 b</td>
<td>28.33 b</td>
<td>5144 b</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td></td>
<td>10.06</td>
<td>9.88</td>
<td>19.37</td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>120</td>
<td>F</td>
<td>148.33 a</td>
<td>22.33 a</td>
<td>3330 a</td>
</tr>
<tr>
<td><em>Rhanella</em> spp in Soil</td>
<td>120</td>
<td>F</td>
<td>148.33 a</td>
<td>22.33 a</td>
<td>3330 a</td>
</tr>
<tr>
<td><em>Rhanella</em> spp in Seed</td>
<td>120</td>
<td>F</td>
<td>159 a</td>
<td>25 a</td>
<td>4027 a</td>
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<tr>
<td><em>Azospirillum</em> spp in Seed</td>
<td>120</td>
<td>F</td>
<td>154.67 a</td>
<td>23.67 a</td>
<td>4152 ab</td>
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<tr>
<td><em>Azospirillum</em> spp in Soil</td>
<td>120</td>
<td>F</td>
<td>176.67 b</td>
<td>27.33 b</td>
<td>4837 b</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td></td>
<td>15.33 a</td>
<td>11.23</td>
<td>21.72</td>
</tr>
</tbody>
</table>

*Means in the same column followed by the same letter are not significantly different at the P<0.05 level according to LSD tests.
A net increase (10.5%) in grain yields (kg ha⁻¹) was observed for all maize inoculation treatments compared to non-inoculated controls, across N fertilization treatments. Considering the international price (2012) of one ton of maize grain, such increase could represent an estimative profit of 143 dollars per ton for this particular farmer under these experimental conditions.

**DISCUSSION**

The mechanisms of plant growth promotion by endophytic bacteria are still not fully understood (Bhattacharyya and Jha, 2012), Wang et al. (2006). Most endophytes with plant growth-enhancing properties produce phytohormones (Biowas et al., 2000), Yanni et al. (2001), Verma et al., (2001), iron-sequestering siderophores (Yanni et al., 2001), Verma et al. (2001), phosphate solubilizing enzymes (Verma et al., 2001), L-aminoacyclopropan-1-carboxylate (ACC) deaminase (Khalid et al., 2005) and BNF (Boddley and Döbereiner, 1995). In this study, the inoculated strains used in the greenhouse experiment were selected for several traits associated with plant growth promotion such as the presence of the nif H gene, production of IAA *in vitro* and P solubilization capacity (Montañez et al., 2009). These factors are known to enhance plant growth (Rodriguez and Fraga, 1999), and their combination may explain the observed increases in root and shoot growth of maize plants here. In our greenhouse study, maize cultivars differed significantly in their response to inoculation treatments. In general, inoculation with *Rhanella* spp. promoted a relative increase in shoot dry weight by 25% in Maizon and 39% in PAU871 compared to non-inoculated controls. In both cases, the growth effect was not reflected in N accumulation data.

Plant-bacteria associations can occur at different levels of interaction. As a result, the genetic characteristics of both the bacteria and host plant can define the level of inoculation response in a given association (Azvedo et al., 2005). When the same strain is applied as inoculant to different cultivars, significantly different responses can be obtained, which can be attributed to several mechanisms for plant growth promotion (Bashan et al., 2004). For example, when inoculated with *Rhanella* spp., both Maizon and PAU871 increased in shoot dry weight but NK940 showed no response. Alternatively, plants inoculated with *Herbaspirillum frisingense* showed no difference in shoot dry weight among cultivars but did show a decrease in %¹⁵N a.e. among Maizon and PAU871. Putative endophytic bacteria are also reported to promote plant growth without enhancing plant nitrogen content, implying that these bacteria can influence plant growth by means other than nitrogen fixation (Chi et al., 2005). Our results emphasize that the selection of the strain-host combination is crucial for enhancing the efficiency of the association under different conditions.

Biological nitrogen fixation by endophytic bacteria has largely been tested in leguminous plants and less so among grasses. It has been suggested that endophytic bacteria enhance their nitrogen fixation potential inside plant tissues due to reduced competition for nutrients and
protection with high levels of O₂ present in the rhizosphere. In this study, there was a negative correlation between %¹⁵N a.e. and shoot dry weight under greenhouse experimental conditions (r²=0.6). This relationship indicated that putative endophytic and rhizospheric bacteria may enhance plant growth by providing fixed nitrogen. These results indicate the need for future research on the interaction of endophytic diazotrophic bacteria and plant genotype in order to optimize BNF.

While inoculation experiments reveal the effects of endophytes on maize plants, it is well-known that resident or indigenous endophytes are already present in seeds. Maize plant tissues harbor a large diversity of diazotrophic bacterial endophytes (Chelious and Tripplett, 2001), Montañez et al. (2009), Montañez et al. (2011). In this study the highest %¹⁵N a.e. values were not always maize cultivars with non-inoculated treatments as expected, meaning that control plants had intrinsic N fixation due to the presence of intrinsic diazotrophic bacteria. In addition, Montañez et al. (2009) showed that non-inoculated NK9400, PAU871 and Maizzon obtain significant nitrogen from BNF using the ¹⁵N isotope-dilution technique in a greenhouse experiment. Our greenhouse inoculation experiment showed that certain strains can improve this capacity while others can have a negative or inhibitory effect on the native endophytic community associated with maize plants. The presented %¹⁵N a.e. values provide additional evidence to the existing literature (IAEA, 2001), (Knowles and Blackburn, 1993) that the populations of diazotrophic bacteria associated with maize are able to obtain substantial contributions from BNF. Tissue culture has been used to eliminate or reduce endophyte communities (Leifert et al., 1994) but this strategy is not necessarily effective for all cultivars. In the case of maize, there should be a selection of lesser to higher N fixing cultivars and further studies to explore which cultivars respond to inoculation treatments.

Potential factors that may affect the performance of endophytes include soil nitrogen content, soil type and host plant stage and variety (Bhattacharyya and Jha, 2012). Trials with PGPB demonstrate increases in yields for rice (Sudha et al., 1999), barley (Sahin et al., 2004), wheat (Çakmakçı et al., 2007), maize (Pal, 1998), sugarcane (Sundara et al., 2002) and apples (Aslan et al., 2007). Both greenhouse and field results showed that the overall promotion of growth and nitrogen assimilation is not solely due to BNF by putative endophytes. Similar results were found by Riggs et al., (2001) for maize plants inoculated with *Herbaspirillum seropedicae* under greenhouse conditions as well as with *Azospirillum* with maize and other crop species under field conditions (Okon and Labandera Gonzalez 1994), Itzigshon et al. (2000), Steenhoudt and Venderleyden (2000). Nevertheless, diazotrophic bacteria other than *Azospirillum* can consistently increase maize yields in the field with N fertilization (Garcia de Salomone et al., 1996). This may indicate that other mechanisms for increasing yield, such as the production of bacterial hormones, may be a factor, but there is no evidence to support this hypothesis. Further research should explore the relative levels of plant hormones in inoculated vs. non-inoculated plants.

In our field experiment there was a 10.5% increase in grain yield in inoculated vs. non-inoculated plants The estimative profit obtained in field experiment with inoculated vs non inoculated maize plants could not only be attributed to BNF benefit. The net increase in grain yield in inoculated plants may be a consequence of a combination of factors. However could be possible to add another component of the profit regarding cost of N fertilization but the authors understand that the information is not enough for a statistical strong financial evaluation.

Finally, it has been suggested that free-living diazotrophs do not excrete N from their cells (Kleiner 1984). Rather, fixed nitrogen remains mainly in bacterial cells and is released to the host only at later stages of plant growth (Rao et al., 1998). This process is inefficient, and perhaps delayed, when compared with the active release of the immediate products of N₂ fixation by living bacteria, as occurs in legume nodules (Mylona et al., 1995). This may explain in part why we did not find a plant response to inoculation at earlier vegetative stages of growth (V6 and F) but did observe differences at later stages in grain yield and other yield components. Diazotrophic bacteria colonizing the plant tissue may release N after decay of the bacterial biomass.

**CONCLUSIONS**

*Rhanella* spp. EMA83 is a promising PGPB for growth enhancement of maize. This strain may potentially fix nitrogen, produce IAA and solubilize P. Nonetheless, it is equally important to study in detail the potential of this putative endophyte along with their mechanisms of action for sustainable crop production. The ¹⁵N isotope dilution technique is a useful method for screening nitrogen fixation in greenhouse conditions, and the natural abundance of ¹⁵N can be used in field experiments. PGPR may use multiple mechanisms to enhance plant growth, and experimental evidence suggests that plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously (Martinez-Viveros et al., 2010). In addition, there is a plant genotype-strain interaction that should be taken into account. PGPB strains have diverse applications in agriculture, success and commercialization which depend in part upon communication between scientists, farmers and industry. Further understanding of how PGPBs promote plant growth may lead to expanded exploitation of these biofertilizers in order to reduce the potentially
negative consequences associated with enhancement/intensification of food and fiber production.

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