

Characterization of diazotrophic phosphate solubilizing bacteria as growth promoters of maize plants

Caracterización de bacterias diazotróficas solubilizadoras de fosfato como promotoras de crecimiento en plantas de maíz

*Mónica del Pilar López-Ortega**, *Paola Jimena Criollo-Campos**, *Ruth Milena Gómez-Vargas**, *Mauricio Camelo-Rusinque**, *Germán Estrada-Bonilla***, *María Fernanda Garrido-Rubiano**, *Ruth Bonilla-Buitrago**

Abstract

Phosphorus is limiting for growth of maize plants, and because of that use of fertilizers like rock phosphate has been proposed. However, direct use of rock phosphate is not recommended because of its low availability, so it is necessary to improve it. In this study, a group of diazotrophic bacteria were evaluated as phosphate-solubilizing bacteria, for their production of indolic compounds and for their effects on growth of maize plants. Strains of the genera *Azospirillum*, *Azotobacter*, *Rhizobium* and *Klebsiella*, were quantitatively evaluated for solubilization of $\text{Ca}_3(\text{PO}_4)_2$ and Rock Phosphate as a single source of phosphorous in SRS culture media. Additionally, the phosphatase enzyme activity was quantified at pH 5.0, 7.0 and 8.0 using p-nitrophenyl phosphate, and production of indolic compounds was determined by colorimetric quantification. The effect of inoculation of bacteria on maize was determined in a completely randomized greenhouse experiment where root and shoot dry weights and phosphorus content were assessed. Results showed that strain C50 produced $107.2 \text{ mg} \cdot \text{L}^{-1}$ of available-P after 12 days of fermentation, and AC10 strain had the highest phosphatase activity at pH 8 with 12.7 mg of p-nitrophenol $\text{mL} \cdot \text{h}^{-1}$. All strains synthesized indolic compounds, and strain AV5 strain produced the most at $63.03 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$. These diazotrophic bacteria increased plant biomass up to 39 % and accumulation of phosphorus by 10%. Hence, use of diazotrophic phosphate-solubilizing bacteria may represent an alternative technology for fertilization systems in maize plants.

Key words: phosphorus, indolic compounds, PGPR, maize.

Resumen

El fósforo es limitante para el crecimiento de plantas de maíz y debido a eso se ha propuesto el uso de fertilizantes como la roca fosfórica. Sin embargo, el uso directo de roca fosfórica no es recomendado por su baja solubilidad, por lo que es necesario mejorarlo. En este estudio, un grupo de bacterias diazotróficas fueron evaluadas como bacterias solubilizadoras de fosfato, productoras de compuestos indólicos y sus efectos sobre el crecimiento de plantas de maíz. Cepas de los géneros *Azospirillum*, *Azotobacter*, *Rhizobium* y *Klebsiella* fueron evaluadas cuantitativamente en la solubilización de $\text{Ca}_3(\text{PO}_4)_2$ y roca fosfórica como única fuente de fósforo en medio de cultivo SRS. Adicionalmente, la actividad de la enzima fosfatasa fue cuantificada a pH 5.0, 7.0 y 8.0 usando p-nitrofenil fosfato y, la producción de compuestos indólicos fue determinada por cuantificación colorimétrica. El efecto de la inoculación de las bacterias sobre plantas de maíz fue determinado en un experimento en invernadero con un diseño completamente al azar donde los pesos secos de raíz y hojas y el contenido de fósforo fueron evaluados. Los resultados mostraron que la cepa C50 produjo $107.2 \text{ mg} \cdot \text{L}^{-1}$ de fósforo disponible después

* Corporación Colombiana de Investigación Agropecuaria, Km 14 vía Mosquera, Cundinamarca-Colombia. Centro de Biotecnología y Bioindustria, Laboratorio Microbiología de Suelos. rbonilla@corpoica.org.co, mplopezo@unal.edu.co, modeplo_19@hotmail.com.

** Universidade de Sao Paulo, Escola Superior de Agricultura "Luiz de Queiroz" USP-ESALQ. Avenida Pádua Dias, 11 - Piracicaba/SP - CEP 13418-900. Sao Paulo-Brasil.

de 12 días de fermentación y que la cepa AC10 tuvo la más alta actividad fosfatasa a pH 8 con 12.7 mg de p-nitrofenol mL⁻¹.h⁻¹. Todas las cepas sintetizaron compuestos indólicos y la cepa AV5 produjo la más alta cantidad con 63.03 µg .mL⁻¹. Estas bacterias diazotróficas incrementaron la biomasa de las plantas por encima del 39 % y de la acumulación de fósforo por el 10 %. Aquí, el uso de bacterias diazotróficas solubilizadoras de fosfato puede representar una alternativa tecnológica para los sistemas de fertilización en plantas de maíz.

Palabras clave: fósforo, compuestos indólicos, PGPR, maíz.

Recibido: diciembre 27 de 2012

Aprobado: noviembre 12 de 2013

Introduction

Even though the phosphorus (P) content in soils is generally high, its availability for plants is often limited (Anwar and Jalaluddin, 1999), and therefore, P is considered the second most limiting element for crops, after nitrogen (Arcand and Schneider, 2006). Rock phosphate represents the greatest reservoir of P in nature; however, even the highest quality rock phosphate has low solubility and cannot always be recommended for direct use in crops. Traditional techniques to increase the quantity of soluble P in rock phosphate are not cost-effective, and therefore, new techniques are needed (Vanlauwea *et al.*, 2000). Richardson *et al.* (2009) suggested improve the efficiency of phosphorus fertilizers by using a specific inoculant capable of improving availability of P in soil, or assimilation of this element by the roots. The phosphate solubilizer bacteria can increase the P availability in soils through different mechanisms as the organic acid excretion, the phosphatase enzymes activity or the synthesis of chelating agents (Rodríguez and Fraga, 1999). Although immediate effects of phosphate rock used in maize crops are frequently not seen (Vanlauwea *et al.*, 2000), it has been reported that bacterial strains of the genera *Serratia* and *Pseudomonas* capable of solubilizing rock phosphate can promote plant growth of maize by improving the uptake of P by plants when they are fertilized with phosphate rock as source of P (Hameeda *et al.*, 2008). Microorganisms belonging to the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Azotobacter* and *Azospirillum*, among others, frequently have the ability to solubilize phosphorus (Rodríguez and Fraga, 1999). This microorganisms are capable to synthesize plant growth regulators as indolic compounds (Patten and Glick, 2002; Cassán *et al.*, 2009), defined as a group of organic substances with an important role in cell division, elongation, differentiation, development and growth of roots, tropism regulation and adult plant structure (Woodward and Bartel, 2005). The main indolic compound produced by bacteria is indol acetic acid (IAA), an active biological form of the auxins, which stimulates radical system growth (Dobbelaere

et al., 2003; Vessey, 2003) and increases the uptake of the nutrients by the plant. The aim of this study was to present the role of diazotrophic bacteria of the genera *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, *Rhizobium* and *Klebsiella* as phosphate solubilizing bacteria and indolic compounds producers, as well as to determine in a preliminary way the effect of the inoculation of maize plants with these bacteria and the assimilation of phosphorus under greenhouse conditions.

Materials and methods

Strains. Nine bacterial strains were used: *Azospirillum brasilense* (C16 and SP7), *Azospirillum lipoferum* C15, *Azotobacter chroococcum* AC1 and AC10, *Azotobacter vinelandii* AV5, *Bradyrhizobium japonicum* USDA110, *Rhizobium* sp. C50 and *Klebsiella variicola* BRCG3. The strains were supplied by CORPOICA.

Phosphorus solubilization assay. Determination of released P by the evaluated strains was carried out in SRS culture broth (Sundara and Sinha, 1963; Nautiyal, 1999) and SRS broth supplemented with rock phosphate (RP) named SRS-RP, in all cases all the assays were realized in triplicate. Inocula were produced in sterile solution of NaCl (0.85 % w/v) at OD₆₀₀ = 0.500 (approximately 10⁸ cells .mL⁻¹). One mL of each inoculum was inoculated in 24 mL of the culture broth SRS and SRS-RP in flasks (capacity of 125 mL). These broths were incubated at 30 ± 2 °C, 150 rpm (Labline Hertz 3525). At the end of this period, the soluble P was quantified using the phosphomolybdenum blue method using an absorbance of 712 nm (Murphy and Riley, 1962; Watanabe and Olsen, 1965). The final pH was evaluated by a potentiometer (Consort C861). Rock Phosphate. The rock phosphate was from a deposit of the Pesca municipality (Boyacá - Colombia) with the following chemical composition: 30 % P₂O₅, 40 % Ca, 12 % Si, 0.1 % Mg, 40 ppm Mn, 30 ppm Cu, 10 ppm Mo, 300 ppm Zn and 3 % of moisture.

Determination of phosphatase enzymes. Quantification of phosphatase enzyme activity was estimated at

pH 5.0, 7.0 and 8.0. The inoculum was produced in SRS culture broth without P, and it was inoculated at 1 % of the effective volume (EV) and incubated in agitation at 120 rpm, 30 ± 2 °C for 48 - 96 h according to the growth kinetics of each strain during her stationary phase (data not shown). In order to evaluate the activity of phosphatase enzymes it was used p-nitrophenyl phosphate as substrate in all cases; to quantify the acid phosphatases it was used an acid buffer (sodium acetate 0.5 M and MgCl₂ 0.1 M) and for alkaline phosphatases it was used an alkaline buffer (Tris-Cl 0.5 M and MgCl₂ 0.1 M) (Patel *et al.*, 2010). The reaction was incubated at 35 °C (ShellLab-WGM) for 1 hour and then NaOH 20 mM was added to stop the reaction. The released concentration of p-nitrophenol was measured by spectrophotometry at 450 nm (Tabatabai and Bremner, 1969).

Production of indolic compounds. Cultures were grown at 30 ± 2 °C in K-lactate culture medium supplemented at 1% with L-tryptophane (10 %) for 48 to 96 h (based on the growth kinetics of each strain) and were centrifuged at 10000 rpm for 10 minutes. Indolic compounds were determined in the supernatant by Salkowsky's method at 540 nm (Carreño *et al.*, 2000; Glickmann and Dessaux, 1995).

Inoculation tests under greenhouse conditions. The assay was carried out in the Mosquera municipality (Cundinamarca - Colombia) 4.71 °N, 74.23 °W and 2291 meters high, to determine the effect of the strains over the development of maize seedlings. The variety 135 of maize used was 136 ICA 503-7, obtained from Vegetal Germoplasm Bank of Corpoica. This assay was carried out under gnotobiotic conditions. Seeds were surface sterilized with sodium hypochlorite (2%) and alcohol (70%). Ten-day-old seedlings were 137 cultured individually in 1 kg plastic bags with a mixture of vermiculite: sand (2:1) as substrate. Inoculation of each strain was made at transplanting with 5 mL of bacterial suspension in SRS broth at 10^8 CFU .mL⁻¹, concentrations according with the established treatments. The treatments used were T1: Chemical control as Hoagland's solution without phosphorus and supplemented with phosphate rock; T2: *A. vinelandii* AV5; T3: *A. chroococcum* AC1; T4: *A. chroococcum* AC10; T5: *B. japonicum* USDA110; T6: *Rhizobium* sp., C50; T7: *Klebsiella variicola* BRCG3. The inoculated treatments were fertilized using Hoagland's solution without phosphorus and supplemented with rock phosphate dosage per bag was 0.1 g. The seedlings were randomized with 10 repetitions per treatment under semi-controlled conditions in greenhouse with a maximum temperature of 33.14 °C and a minimum

Table 1. Phosphorus solubilization in SRS broth with tricalcium phosphate and rock phosphate.

Strain	Solubilization of tricalcium phosphate in broth		Solubilization of rock phosphate in broth	
	Available P (mg .L ⁻¹)	Final pH	Available P (mg .L ⁻¹)	Final pH
<i>A. brasilense</i> SP7	19.01±0.13 ^s	6.42±0.05	9.71±0.53 ^{de}	6.92±0.05
<i>A. lipoferum</i> C15	15.12±0.33 ^h	5.11±0.11	3.22±0.29 ^f	5.91±0.18
<i>A. brasilense</i> C16	12.12±0.6 ⁱ	5.97±0.13	4.74±1.6 ^{ef}	6.27±0.23
<i>A. vinelandii</i> AV5	54.01±0.28 ^e	6.15±0.28	12.37±0.97 ^d	5.26±0.09
<i>A. chroococcum</i> AC1	41.30±0.18 ^f	4.91±0.07	12.95±0.43 ^d	4.48±0.05
<i>A. chroococcum</i> AC10	93.72±0.18 ^b	4.39±0.02	67.01±4.3 ^a	5.25±0.24
<i>B. japonicum</i> USDA110	88.74±0.15 ^c	5.29±0.17	39.21±3.8 ^c	4.19±0.03
<i>Rhizobium</i> sp. C50	107.23±0.07 ^a	4.02±0.09	62.78±2.4 ^a	4.26±0.05
<i>Klebsiella variicola</i> BRCG3	84.01±0.07 ^d	6.96±0.02	54.70±1 ^b	6.72±0.27

Initial pH: 7.2. In the same column values with the same letter have no significant statistical differences at a confidence level of 95%.

temperature of 12.85 °C during the assay. Plants were watered once every three days. Agronomic variables (shoot and root length and dry weigh) were evaluated 20 days after inoculation. Shoot P content was determined for each treatment (Bray and Kurtz, 1945).

Statistical analysis. Data were analyzed by SPSS version 17.0. One-way analyses of variance (ANOVA) and comparison among treatments were done by Tukey's HSD. All analyses were performed at the $P = 0.05$ level.

Results and discussion

Phosphorus solubilization. The results of phosphorus solubilization capacity of the diazotrophic bacteria evaluated are shown in table 1. All the strains solubilized tricalcium phosphate and phosphate rock in broth.

In the presence of tricalcium phosphate, the concentration of soluble P was between 12.12 and 107.23 mg .L⁻¹ after 12 days of fermentation. Strain C50 (*Rhizobium* sp.) produced the highest concentration with a final pH value of 4.02. In contrast, in SRS broth supplemented with phosphate rock, the concentration of soluble P varied between 3.22 and 62.78 mg .L⁻¹. Strain AC10 had the greatest solubilization of P with a final pH value of 5.25. The phosphorus solubilization in culture media supplemented with tricalcium phosphate and phosphate rock was accompanied by decreases in the initial pH (7.2) due to the activity of the different bacteria, ranged between 4.02 and 6.92. In a generally way, a negative correlation was observed between the available P and the pH values, thus the greatest solubilization by the evaluated bacteria was presented at lower pH values.

The potential of genus *Rhizobium* as a phosphate solubilizing bacterium has been previously described using sources as hydroxyapatite, FePO₄, AlPO₄ and Ca₃(PO₄)₂ (Sridevi and Mallaiah, 2009). They used tricalcium phosphate as source of P and reported solubilization levels between 156 and 620 mg .L⁻¹ of P₂O₅, meanwhile in the present study it was shown a solubilization of 107.23 mg .L⁻¹. *B. japonicum*, has been reported as a non-phosphate solubilizing bacteria (Fernandez *et al.*, 2005). However, our results showed that the strain USDA110 was able to solubilize a great quantity of P using the two sources of phosphates.

None of the strains of the genus *Azospirillum* was able to solubilize high concentrations of P in comparison with the other evaluated strains. Ramachandran *et al.*, (2007) and Vikram *et al.*, (2007) reported similar results for several species of *Azospirillum*. Nevertheless Rodríguez *et al.*, (2004) reported that in presence of

fructose and glucose, strains of *A. brasilense* and *A. lipoferum* produced gluconic acid that is involved in phosphate solubilization process.

The solubilization capacity of the two sources of P by the strain BRCG3 (*Klebsiella variicola*) exceeded 50 mg .L⁻¹, at evaluated conditions, confirming that the genus is able to solubilize insoluble sources of P. Ahmad *et al.*, (2008) reported the genus *Klebsiella* as organic acid producer from the glucose metabolism to solubilize phosphates present in the soil solution, Ahemad and Saghir (2011) reported levels of solubilization up to 294 mg .L⁻¹ using as source of P tricalcium phosphate.

Both strains *A. chroococcum* AC1 and *A. vinelandii* AV5 released similar amount of P, however, the values were significantly lower in comparison with the ones obtained from *Rhizobium* sp. C50, meanwhile the strain *A. chroococcum* AC10 showed similar quantities of soluble P in the culture medium in comparison with this strain. Several studies have reported that the genus *Azotobacter* does not present high levels of solubilization. In that way, Kumar and Narula (1999) found values of P solubilization between 0.18 and 0.19 mg .L⁻¹ for native strains of *Azotobacter chroococcum* using rock phosphate as source of P. Similarly, Husen (2003) reported that *A. vinelandii* Mac 259, was not able to solubilize tricalcium phosphate in Pikovskaya culture medium. Those results do not coincide with our results reported here especially with *A. chroococcum* AC10, which presented one of the greatest activities.

Phosphatase enzymes determination. All tested strains produced phosphatases at the pH values tested (figure 1). Strains SP7, C15, C16, USDA110 and BRCG3 had the lowest enzymatic activity at the three pH values. In contrast, AC1, AC10 and AV5 (genus *Azotobacter*) and C50 showed the highest values of enzymatic activity at the pH values evaluated. Strain AC10 showed the greatest activity at pH 8 with 12.70 mg of p-nitrophenol mL⁻¹ .h⁻¹, at pH 7.0 AV5 demonstrated the greatest enzymatic activity with 8.77 mg of p-nitrophenol mL⁻¹ .h⁻¹. At pH 5.0 AC1 presented an enzymatic activity of 9.01 mg of p-nitrophenol mL⁻¹ .h⁻¹. Overall, the results show that the strains of genus *Azotobacter* have the greatest phosphatase activity in the different pH evaluated in comparison with the other strains.

Characterization of the bacteria was complemented with the phosphatase activity measurement, which has not been studied in the genera of the present study at great length before. Other authors have reported productions of phosphatases from 2.62 µg mL⁻¹ .h⁻¹ released p-nitrophenol by *Pantoea ananatis* to 70.98

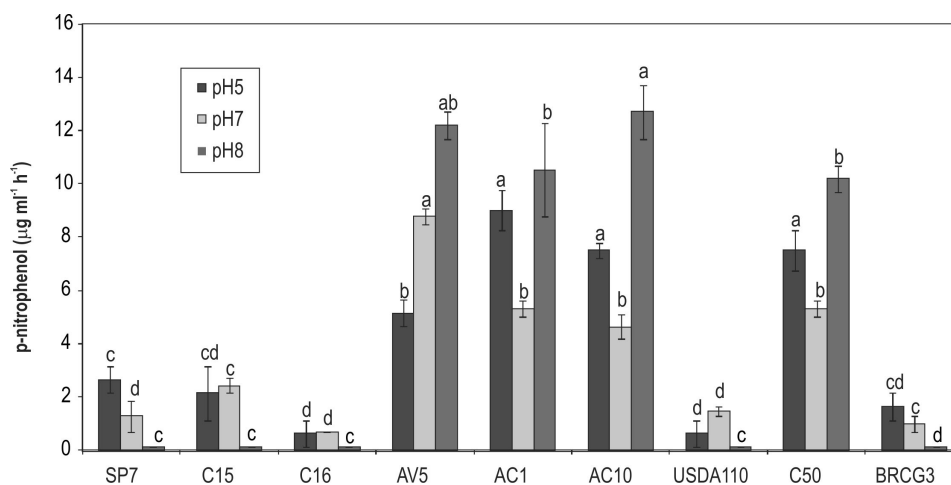


Figure 1. Phosphatase enzymatic activity of the evaluated strains at different pH. For each pH value bars with the same letter are not significantly different at a confidence level of 95%.

released p-nitrophenol ($\mu\text{g mL}^{-1} \cdot \text{h}^{-1}$) by *Burkholderia cepacia*, either because of acid or alkaline phosphatase activity (Oliveira *et al.*, 2009), thus the obtained results from this study are among the mineralization rates reported by other bacteria genera.

The evaluated strains in the present study, were isolated from different soils, crops and climatic conditions therefore it is likely that several factors, as temperature, pH and redox potential, could affect the phosphatase enzyme expression (Šarapatka, 2003). Additionally, the enzyme activity was evaluated during the stationary phase of each strain. In this phase the organic

P associated to death cell, could act as an inducer of phosphatase synthesis (Jagadish *et al.*, 2001). All of the tested strains expressed phosphatase enzyme at all three tested pH levels. However, according with Šarapatka (2003) acid phosphatases are more common than alkaline phosphatases in soil microorganisms, which may account for the large production of phosphatases in acid environments such as in tropical soils. In contrast, in this study the greatest expression of phosphatases occurred under alkaline conditions.

Indolic compounds production. All of the tested strains synthesized indolic compounds from tryptophan as

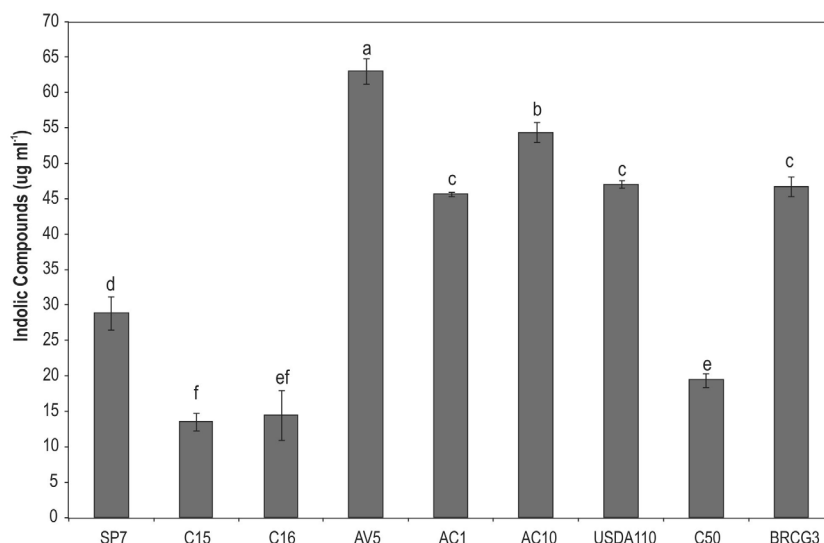


Figure 2. Total indolic compounds production by the evaluated bacteria. Bars with the same letter are not significantly different at a confidence level of 95%.

the precursor (figure 2). Strain AV5 had the greatest production of indolic compound with an average of 63.03 $\mu\text{g} \cdot \text{mL}^{-1}$, followed by AC10 with 54.41 $\mu\text{g} \cdot \text{mL}^{-1}$.

Previous studies have reported that the genus *Rhizobium* synthesizes indol acetic acid from several tryptophan isomers (DL and L) (Perrine *et al.*, 2004) and reaches production levels up to 90.6 $\mu\text{g} \cdot \text{mL}^{-1}$ with tryptophan at 1% (Datta and Basu, 1997) and 267.5 $\mu\text{g} \cdot \text{mL}^{-1}$ with tryptophane at 4 % (De and Basu, 1996). In the present study, the production of indolic compounds with tryptophane at 0.1 % by *Rhizobium* sp. C50, was lower than the reported in literature, however, this could be related with the concentration of tryptophane in the culture medium (Datta and Basu, 1997).

For genus *Azotobacter* it has been reported several species that can produce different quantities of IAA. *A. chroococcum* produced 12.2 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA, *A. beijerinckii* produced 12 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA, *A. vinelandii* produced between 11 $\mu\text{g} \cdot \text{mL}^{-1}$ and 49.07 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA (Fiorelli *et al.*, 1996; Ravikumar *et al.*, 2004). These results are overcome by the results obtained in this study with the different species of *Azotobacter* evaluated. Similarly, the production of indolic compounds by the strain *B. japonicum* USDA110 were superior than those found in literature where are reported productions of 11.8 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA (Badawi *et al.*, 2011) and 6.62 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA in absence of tryptophan (Cassán *et al.*, 2009), in this way in the present study it was evaluated the production of indolic compounds in presence of tryptophan as precursor and it could stimulate the production.

The genus *Klebsiella* has been reported as indolic compound producer before. Ahemad and Saghir (2011)

found a production of 42 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA, this result can be compared with the levels of production obtained from the strain BRCG3, under the established conditions of this work.

It is known that in the genus *Azospirillum* the primary pathway for production of IAA is the indol-3-pyruvic acid pathway (Patten and Glick, 1996; Malhotra and Srivastava, 2008). This pathway is dependent on tryptophan (Malhotra and Srivastava, 2008). Although tryptophan was used in the current study as a precursor, the concentration of indolic compounds produced by the strains of the genus *Azospirillum* was lower than the concentration produced by the other strains with the exception of the strain SP7. Also it is known that *Azospirillum* synthesizes these compounds in the absence of tryptophan, as reported by Cassán *et al.* (2009) who reported that *Azospirillum brasilense* produced 13.16 $\mu\text{g} \cdot \text{mL}^{-1}$ of IAA, which is consistent with our results.

Inoculation test under greenhouse conditions. Maize plants inoculated with strains AC1, AC10, USDA110 and BRCG3, had the highest production of biomass compared to the chemical control with Hoagland's solution supplemented with rock phosphate (HS*RP) (table 2). Inoculation with strain BRCG3 increased root dry weight by 39 % greater than the control HS+RP, with a mean of 1.01 g, followed by treatment with strain AC10 with 29 % of increase and 0.87 g (table 2). The strains AC1 and AC10 increased shoot dry weight in approximately 33 % in comparison with the control (table 2).

The plants inoculated with strains AC1 and BRCG had the greatest concentration of phosphorus in shoot with

Table 2. Shoot and root dry weight and phosphorus concentration in shoot of maize plants under greenhouse conditions. For each variable, data with the same letter are not significantly different at a confidence level of 95%.

Treatments	Shoot Dry weight (g)	Root Dry weight shoot (g)	P total shoot (mg P)
Non inoculated	0.35±0.04 ^d	0.62±0.07 ^{de}	1.15±0.11 ^{bc}
AV5	0.38±0.04 ^{cd}	0.7±0.04 ^{cd}	0.99±0.10 ^{cd}
AC1	0.52±0.05 ^a	0.76±0.08 ^c	1.42±0.12 ^a
AC10	0.52±0.04 ^a	0.87±0.06 ^b	1.32±0.07 ^{ab}
USDA110	0.44±0.04 ^{bc}	0.8±0.04 ^{bc}	1.17±0.12 ^b
C50	0.34±0.04 ^d	0.54±0.04 ^e	0.97±0.11 ^d
BRCG3	0.47±0.05 ^{ab}	1.01±0.09 ^a	1.35±0.13 ^a

1.4 mg and 1.33 mg respectively with statistically significant differences ($P \leq 0.05$) in comparison with the control. However, inoculation with strains AC10 and USDA110 did not increase P in shoots.

The results of the growth promotion tests are similar to the results obtained by Hameeda *et al.* (2008) where it was observed under greenhouse conditions a significant increase of the dry weight of maize plants due to inoculation with foreign strains that showed the ability to solubilize phosphates and other mechanisms to promote plant growth. This shows that isolations from other crops, or even from out of rhizosphere, can promote plant growth. In the present study it was evaluated strains that were not isolated from maize, which showed an important effect on maize growth promotion.

It was observed a major increment in maize root dry weight in comparison to shoot dry weight. Some plant species spent a great portion of their total dry matter in roots growth when are farmed in P deficiency (Hill *et al.*, 2006). Liu *et al.*, (2004) showed that in two different genotypes of maize, roots respond first than shoot in P deficiency through the production of lateral roots and radicular hairs because of the plant need to cover a greatest area for searching this element. In the present study, the P was supplied as non-available phosphate rock. The inoculation with phosphate solubilizing bacteria could have a positive effect over the acquisition of P by the plant and over plant development.

The growth-promoting effect was evident by *A. chroococcum* strains AC1 and AC10, which are capable to solubilize phosphate and produce indolic compounds. It has been reported the effect of strains of *A. chroococcum*, phosphate solubilizing and phyto-hormone producer bacteria, over plant growth parameters on several wheat varieties. It has been found that phosphate solubilization and indolic compounds production have a positive influence in plant height (19 %), crop yield (14 %) and root biomass (12 %), with reports were the lower doses of fertilization are matched or overcome (Kundu and Gaur, 1980; Kumar *et al.*, 2001).

In the study by Kumar and Narula (1999), increased plant growth was attributed to the production of plant growth promoting substances and to the phosphate solubilizing activity of *A. chroococcum*. This bacterial species can improve the availability of P in soil, in that way; according to Chabot *et al.*, (1998) phosphate solubilization is an effective mechanism in plant growth promotion.

B. japonicum USDA110 also showed important results in maize growth promotion. Previously it has been de-

monstrated that strains of *Bradyrhizobium*, under greenhouse conditions, improved germination of up to 8 % and stimulated shoot weight by 35 % and root weight by 32 % (Cassán *et al.*, 2009). Other authors report increases in soy up to 31% (Molla *et al.*, 2001) beans up to 64 % (Gupta *et al.*, 1998) and peanuts up to 37 % (Badawi *et al.*, 2011). In the present work, the strain USDA110 increased shoot dry weight in maize plant up to 20 % and root dry weight up to 23 %.

The results of assay control were higher than those of treatment inoculated with *Rhizobium* sp. C50. This contrast with the reports of strains of *R. leguminosarum*, that after 20 days (Chabot *et al.*, 1998) and *Rhizobium etli*, that after 40 days (Gutiérrez and Martínez, 2001), have the ability to significantly increase dry weight compared with controls (Antoun *et al.*, 1998).

With *Klebsiella*, Farzana *et al.*, (2009) found an increase of roots dry weight and volume in potato plants compared with the non-inoculated control, and concluded that this effect may be the result of bacterial indolic compounds production. These results agree with the ones of the present study where it is shown that the strain that belongs to this genus present the higher values in root dry weight (1.1 g) regarding the non-inoculated control (0.61 g).

It is well known that root tissues are extremely sensitive to changes in concentrations of indolic compounds, like IAA, and also, that the root development could be affected by production of IAA by plant growth-promoting rhizobacteria (Tanimoto, 2005). The synthesis of indolic compounds by the evaluated microorganisms could stimulate the root system by the development of lateral roots and apical divisions of the meristem that conduces to the roots growth (Patten and Glick, 1996; Vessey, 2003; Dobbelaere *et al.*, 2003), and also to the increase of the plant access to soil's nutrients, allowing a greatest production of vegetal biomass (Patten and Glick, 1996; Vessey 2003; Barazani and Friedman, 1999). The evaluated strains in the present study showed an *in vitro* ability to synthesize indolic compounds using tryptophan as precursor and they could use this amino acid found in root exudates free in rhizosphere (Dakora and Philips, 2002; Malhotra and Srivastava, 2009; Barea *et al.*, 1976).

The ability of bacterial genus to increase the P supply to the plant using rock phosphate has been well documented, Yu *et al.*, (2012) found that in walnut trees, *Pseudomonas chlororaphis* and *Arthrobacter pascens* with the ability to solubilize P under *in vitro* conditions, increases the shoot and root dry weights with significant differences up to 22 % when were compared

with the non-inoculated control and phosphate rock added. In addition, the authors found an increase in the concentration of P up to 21 % compared with the control. According with Kumar *et al.*, (2001) can be suggested that the inoculation with bacteria capable to solubilize phosphates, in this case phosphate rock, increases the availability of this element in substrate, and as consequence, increases its acquisition by the plant. The description above coincides with the results of the present study where was shown that the use of phosphate rock as source of P in maize crops with the inoculation of bacterial strains can increase up to 10 % the uptake of this element in comparison with the non-inoculated control supplied with phosphate rock.

Conclusion

In the present study the results allow to affirm in a preliminary way that the evaluated diazotrophic bacteria can increase the maize plant biomass in shoot and root, and also, the accumulation of P in plant. This represents a possible alternative for the maize phosphate fertilization system in our country with sources of low solubilization like as rock phosphate.

Acknowledgment

The authors thank the Agricultural Ministry for the financing of the research, the Microbiology laboratory of CORPOICA for the development of this work and Dr. Joseph Kloepper for his contribution to the review of the manuscript.

References

- Ahemad, M., Saghir, M. 2011. Effects of insecticides on plant-growth-promoting activities of phosphate solubilizing rhizobacterium *Klebsiella* sp. strain PS19. *Pesticide Biochemistry and Physiology*. 100: 51-56.
- Ahmad, F., Ahmad, I., Khan, M. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research*. 163: 173-181.
- Antoun, H., Beauchamp, C., Goussard, N., Chabot, R., Lalonde, R. 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus* L.). *Plant and Soil*. 204: 57-67.
- Anwar, Q., Jalaluddin, M. 1999. Reactions of VMA and *Azospirillum* species on wheat growth. *Pakistan Journal of Biological Sciences. Agricultural*. 2 (1): 227-232.
- Arcand, M., Schneider, K. 2006. Plant and microbial-based mechanisms to improve the agronomic effectiveness of phosphate rock: a review. *Annals of the Brazilian Academy of Sciences*. 78: 791-807.
- Badawi, F., Biomy, A., Desoky, A. 2011. Peanut plant growth and yield as influenced by co-inoculation with *Bradyrhizobium* and some rhizo-microorganisms under sandy loam soil conditions. *Annals of Agricultural Science*. 56: 17-25.
- Barazani, O., Friedman, J. 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *Journal of Chemical Ecology*. 25: 2397-2406.
- Barea, J., Navaro, E., Montoya, E. 1976. Production of plant growth regulators by rhizosphere phosphate-solubilizing bacteria. *Journal of Applied Bacteriology*. 40: 129-134.
- Bray, B., Kurtz, L. 1945. Determination of total, organic, and available forms of phosphorus in soils. *Soil Science*. 59: 39-45.
- Carreño, R., Campos, N., Elmerich, C., Baca, B. 2000. Physiological evidence for differently regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in *Azospirillum brasilense*. *Molecular Genetics and Genomics*. 264: 521-30.
- Cassán, F., Perrig, D., Sgroy, V., Masciarelli, O., Penna, C., Luna, V. 2009. *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). *European Journal of Soil Biology*. 45: 28-35.
- Chabot, R., Beauchamp, C., Kloepper, J., Antoun, H. 1998. Effect of phosphorus on root colonization and growth promotion of maize by bioluminescent mutants of phosphate-solubilizing *Rhizobium leguminosarum* Biovar *phaseoli*. *Soil Biology and Biochemistry*. 30 (12): 1615-1618.
- Dakora, F., Philips, D. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil*. 245: 35-47.
- Datta, C., Basu, P. 1997. Growth behaviour and IAA production by a *Rhizobium* sp. isolated from root nodules of a leguminous medicinal herb, *Dolichos bijlorus* L., in culture. *Microbiological Research*. 152: 353-357.
- De, P., Basu, P. 1996. Growth behaviour and IAA production by a *Rhizobium* sp. isolated from root nodules of a leguminous medicinal herb, *Tephrosea purpurea* Pers., in culture. *Microbiological Research*. 151: 71-76.
- Dobbelaere, S., Vanderleyden, J., Okon, Y. 2003. Plant growth promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences*. 22: 107-149.
- Farzana, Y., Saad, S., Kamaruzaman, S. 2009. Growth and Storage Root Development of Sweetpotato Inoculated with Rhizobacteria Under Glasshouse Conditions. *Australian Journal of Basic and Applied Sciences*. 3 (2) : 1461-1466.
- Fernández, L.; Zalba, P.; Gómez, M.; Sagardoy, M. 2005. Bacterias solubilizadoras de fosfato inorgánico aisladas de suelos de la región Sojera. *Ciencia del suelo*. 23 (1) : 31-37
- Fiorelli, F., Pasetti, L., Galli, E. 1996. Fertility-Promoting Metabolites Produced by *Azotobacter vinelandii* Grown on Olive-Mill Wastewaters. *International Biodeterioration & Biodegradation*. 38(3-4) : 165-167.
- Glickman, E., Dessaux, Y. 1995. A Critical Examination of the Specificity of the Salkowsky Reagent for Indolic Compounds Produced by Phytopathogenic Bacteria. *Applied and Environmental Microbiology*. 61 (2) : 793-796.
- Gupta, A., Saxena, A., Gopal, M., Tilak, K. 1998. Effect of plant growth promoting rhizobacteria on competitive ability of introduced *Bradyrhizobium* sp. (*Vigna*) for nodulation. *Microbiological Research*. 153: 113-117.
- Gutiérrez, M., Martínez, E. 2001. Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). *Journal of Biotechnology*. 91: 117-126.

- Hameeda, B., Harini, G., Rupela, O., Wani, S., Reddy, G. 2008. Growth promotion of maize by phosphatesolubilizing bacteria isolated from composts and macrofauna. *Microbiological Research*. 163: 234-242.
- Hill, J., Simpson, R., Moore, A., Chapman, D. 2006. Morphology and response of roots of pasture species to phosphorus and nitrogen nutrition. *Plant and Soil*. 286: 7-19.
- Husen, E. 2003. Screening of soil bacteria for plant growth promotion activities *in vitro*. *Indonesian Agricultural Sciences*. 4 (1) : 27-31.
- Jagadish, C., Tarafdar, J.C., Yadav, R.S., Niwas, R. 2001. Relative efficiency of fungal intra- and extracellular phosphatases and phytase. *Journal of Plant Nutrition and Soil Science*. 165: 17-19.
- Kumar, V., Narula, N. 1999. Solubilization of inorganic phosphates and growth emergence of wheat as affected by *Azotobacter chroococcum* mutants. *Biology and Fertility of Soils*. 28: 301-305.
- Kumar, V., Behl, R., Narula, N. 2001. Establishment of phosphate-solubilizing strains of *Azotobacter chroococcum* in the rhizosphere and their effect on wheat cultivars under greenhouse conditions. *Research in Microbiology*. 156: 87-93.
- Kundu, B., Gaur, A. 1980. Establishment of nitrogen fixing and phosphate solubilizing bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop. *Plant and Soil*. 57: 223-230.
- Liu, Y., Mia, G., Chena, F., Zhang, J., Zhang, F. 2004. Rhizosphere effect and root growth of two maize (*Zea mays* L.) genotypes with contrasting P efficiency at low P availability. *Plant Science*. 167: 217-223.
- Malhotra, M., Srivastava, S. 2008. Organization of the ipdC region regulates IAA levels in different *Azospirillum brasilense* strains: Molecular and functional analysis of ipdC in strain SM. *Environmental Microbiology*. 10: 1365-1373.
- Malhotra, M., Srivastava, S. 2009. Stress-responsive indole-3-acetic acid biosynthesis by *Azospirillum brasilense* SM and its ability to modulate plant growth. *European Journal of Soil Biology*. 45: 73-80.
- Molla, A., Shamsuddin, Z., Halimi, M., Morziah, M., Puteh, A. (2001) Potential for enhancement of root growth and nodulation of soybean coinoculated with *Azospirillum* and *Bradyrhizobium* in laboratory systems. *Soil Biology and Biochemistry*. 33: 457-463.
- Murphy, J., Riley, I.P. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*. 27: 31-36.
- Nautiyal, C. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*. 170: 265-270.
- Oliveira, C., Alves, V., Marriel, I., Gomes, E., Scotti, M., Carneiro, N., Guimaraes, C., Schaffert, R., Sá, N. 2009. Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil Biology and Biochemistry*. 41 : 1782-1787.
- Patel, K., Singh, A., Nareshkumar, G., Archana, G. 2010. Organic-acid-producing, phytate-mineralizing rhizobacteria and their effect on growth of pigeon pea (*Cajanus cajan*). *Applied Soil Ecology*. 44 : 252-261.
- Patten, C., Glick, B. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian Journal of Microbiology*. 42 : 207-220.
- Patten, C., Glick, B. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology*. 68 : 3795-3801.
- Perrine, F., Rolfe, B., Hynes, M., Hocart, C. 2004. Gas chromatography-mass spectrometry analysis of indoleacetic acid and tryptophan following aqueous chloroformate derivatisation of *Rhizobium* exudates. *Plant Physiology and Biochemistry*. 42 : 723-729.
- Ramachandran, K., Srinivasan, V., Hamza, S., Anandaraj, M. 2007. Phosphate solubilizing bacteria isolated from the rhizosphere soil and its growth promotion on black pepper (*Piper nigrum* L.) cuttings. *Development in Plant and Soil Sciences*. 102 : 325-331.
- Ravikumar, S., Kathiresan, K., Ignatiammal, S.T.M., Babu Selvam, M., Shanthi, S. 2004. Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers. *Journal of Experimental Marine Biology and Ecology*. 312(1) : 5-17.
- Richardson, A., Barea, J., McNeill, A., Prigent-Combaret, C. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*. 321: 305-339.
- Rodríguez, H., Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*. 17: 319-339.
- Rodríguez, H., Gonzalez, T., Goire, I., Bashan, Y. 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. *Naturwissenschaften*. 91: 552-555.
- Šarapatka, B. 2003. Phosphatase activities (ACP, ALP) in agroecosystem soils. Uppsala, Sweden: Dissertation Acta Universitatis agriculturae Sueciae. p. 59
- Sridevi, M., Mallaiah, K. 2009. Phosphate solubilization by *Rhizobium* strains. *Indian Journal of Microbiology*. 49: 98-102.
- Sundara, R., Sinha, W. 1963. Phosphate dissolving microorganism in the soil and rhizosphere. *Indian Journal of Agricultural Sciences*. 33: 272-278.
- Tabatabai, M., Bremner, J. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry*. 1: 301-307.
- Tanimoto, E. 2005. Regulation of root growth by plant hormones-roles for auxin and gibberellin. *Critical Reviews in Plant Sciences*. 24: 249-265.
- Vanlauwea, B., Nwoke, O., Diels, J., Sanginga, N., Carsky, R., Deckers, J., Merckx, R. 2000. Utilization of Rock Phosphate by crops on a representative toposequence in the Northern Guinea savanna zone of Nigeria: response by *Mucuna pruriens*, *Lablab purpureus* and maize. *Soil Biology and Biochemistry*. 32: 2063-2077.
- Vessey, J. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil*. 255: 571-586.
- Vikram, A., Alagawadi, A., Krishnaraj, P., Kumar, K. 2007. Transconjugation studies in *Azospirillum* sp. negative to mineral phosphate solubilization. *World Journal of Microbiology & Biotechnology*. 23: 1333-1337.
- Watanabe, F., Olsen, S. 1965. Test of an ascorbic acid method for determining phosphorus in water and NaHCO₃ extracts from soil. *Soil Science Society of America Journal*. 29: 677-678.
- Woodward, A., Bartel, B. 2005. Auxin: regulation, action, and interaction. *Annals of Botany*. 95: 707-735.
- Yu, X., Liu, X., Zhu, T., Liu, G., Mao, C. 2012. Co-inoculation with phosphate-solubilizing and nitrogen-fixing bacteria on solubilization of Rock Phosphate and their effect on growth promotion and nutrient uptake by walnut. *European Journal of Soil Biology*. 50: 112-117.