

PHOSPHATE SOLUBILIZING BACTERIA ISOLATED FROM *Pennisetum clandestinum* ASSOCIATE TO LIVESTOCK SYSTEMS IN THE ANDEAN AREA

BACTERIAS SOLUBILIZADORES DE FOSFATO AISLADAS DE *Pennisetum clandestinum* ASOCIADAS A SISTEMAS GANADEROS EN EL ÁREA ANDINA

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Rev. U.D.C.A Act. & Div. Cient. 17(1): 423-431, Julio-Diciembre, 2014

SUMMARY

The grass *Pennisetum clandestinum* is the base of the dairy systems in high mountain regions in Colombia. Due to its fertilization requirements it is vital to find sustainable alternatives for its management. In this study, four native strains of the genera *Azotobacter*, *Pseudomonas*, *Micrococcus* and *Bacillus*, isolated from *P. clandestinum* were used. Characterization of growth promotion ability *in vitro* such as phosphorus solubilization, production of indole compounds and siderophores synthesis was made for all strains, followed by a test under greenhouse conditions using 50% tricalcium phosphate plus each bacterium. After two months the bacteria's potential to increment height, radical length, leaf and root weight in *P. clandestinum* was measured. The results indicated that the four strains have the ability for plant growth promotion. The treatment with the strain *Azotobacter beijerinckii* KA206 + 50% tricalcium phosphate increased significantly ($p \leq 0.05$) the assessed agronomical variables. These results surpassed the treatment with a 50% phosphate fertilization in terms of plant performance regarding roots length and dry weight. The four strains exhibited results that were significantly different ($p \leq 0.05$). These preliminary results suggest the existence of a feasible alternative that could reduce the phosphate fertilization with sources of low solubilization for the dairy production in sustainable agriculture systems.

Key words: *Azotobacter* sp., *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., phosphorus solubilization.

RESUMEN

El pasto *Pennisetum clandestinum* es la base de los sistemas lecheros de las regiones de alta montaña en Colombia. Debido a sus requerimientos de fertilización es vital encontrar alternativas sustentables para su manejo. En este estudio se utilizaron cuatro cepas nativas de los géneros *Azotobacter*, *Pseudomonas*, *Micrococcus* y *Bacillus*, aisladas de *P. clandestinum*. Se caracterizaron las capacidades de promoción de crecimiento *in vitro*, las cuales fueron solubilización de fosfatos, producción de compuestos indólicos y síntesis de sideróforos; seguido de un ensayo bajo condiciones de invernadero utilizando una dosis de 50% de fosfato tricálcico más cada cepa bacteriana. Después de dos meses se midió el potencial de las bacterias para incrementar la altura de la planta, longitud radical, peso foliar y radicular en *P. clandestinum*. Los resultados indicaron que las cuatro cepas cuentan con capacidades de promoción *in vitro*. La cepa *Azotobacter beijerinckii* KA206 + 50% fosfato tricálcico incrementó significativamente ($p \leq 0.05$) las variables agronómicas evaluadas. Estos resultados sobrepasaron el tratamiento con 50% de la dosis de fertilización fosfatada en términos de longitud y peso seco de la raíz. Las cuatro cepas arrojaron resultados significativamente diferentes ($p \leq 0.05$).

Este resultado preliminar sugiere una alternativa factible que podría reducir la fertilización fosfatada con fuentes de baja solubilización en la producción lechera en sistemas de agricultura sustentable.

Palabras clave: *Azotobacter* sp., *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., solubilización de fósforo.

INTRODUCTION

The grass *Pennisetum clandestinum* originally from tropical latitudes of central Africa is widely distributed in the Andes regions, characterized of low temperatures and high latitudes (Ataroff & Naranjo, 2009). In Colombia, in high mountain areas (> 2000masl), *P. clandestinum* has been established as the base of the middle and high intensity dairy systems (Murgueitio, 2000; Carulla *et al.* 2004; Mila & Corredor, 2004).

This perennial species has been of interest among researchers due to its high growth rate and good root development, as well as, high nutritional properties as a forage species (Jeffery, 1971). It has been pointed out that the protein content is between the 14 and 25%, 30 and 35 days after cutting. Besides, *P. clandestinum* has been reported as highly resistant to droughts, flooding and salinity conditions (Muscolo *et al.* 2003; 2013).

P. clandestinum thrives well in fertile soils with high levels of nitrogen. It is very sensitive to the lack of magnesium, iron, potassium and other minerals. Regarding phosphorus, although this grass is very efficient in the use of this element, high available soil concentrations are required for the initial establishment of pasture (Marais, 2001).

Considering the above, under unsuitable soil nutritional conditions and soil degradation process, there is low protein content in the tropical pastures. Due to this lack, the pastures respond positively to chemical fertilization (Marais, 2001; Barton *et al.* 2009), consisting in 200kg ha⁻¹year⁻¹ and 137kg ha⁻¹year⁻¹ of P₂O₅ for a yield of 8 tha⁻¹year⁻¹. However, due to high economic and ecological costs of this fertilization system it is necessary to develop biotechnological alternatives that allow a good performance and increase in the quality of these pastures.

Environmentally friendly technologies have considered the implement of plant growth promoting rhizobacteria (PGPR), which inhabit the soil and are isolated from the rhizosphere. The effects of these microorganisms in plant development are evident, including beneficial influence on seed germination, seedling emergence and plant growth (Sánchez *et al.* 2014). The use of these microorganisms has been extensively reported due its positive effect on the development of plant species of the genus *Pennisetum* (Hameeda *et al.* 2006; De

Morais *et al.* 2012). Among the direct effects of PGPR on plants are the production of plant growth promoters, siderophores synthesis and mineral solubilization (Asgar *et al.* 2002; Hameeda *et al.* 2006). These effects may increase the nutrient availability for the plant or facilitate its colonization by other beneficial microorganisms (Bashan *et al.* 2012).

Some PGPR make phosphorus available from insoluble compounds that would normally not be available for plant absorption (Yadav *et al.* 2014). Thus, primary mechanisms for phosphorus solubilization are proton excretion, organic acids production and phosphatases biosynthesis, the latter when phosphorus sources are organic. Regarding organic acids, these may chelate cations as iron or aluminum which are present in soil and can form complexes with P, releasing P to be assimilated by plants. In addition, these acids can block absorption sites on soil particles enabling it to be available for plants. This mechanism has been previously associated with effects on growth promotion in diverse vegetable species. In this way, inoculation of plants with microorganisms capable to solubilize phosphorus, might improve health and plant development or even increase crop yield (Behera *et al.* 2014).

In addition to phosphorus solubilization, indolic compounds and siderophores production by microorganisms has been reported for having positive effects in plant growth promotion. Indolic compounds stimulate root system formation, development and growth (Tsavkelova *et al.* 2007), and siderophores production is recognized by its effect on increasing iron availability for plants, due to chelation activity in soil. These mechanisms are related to growth and plant development variable increases such as length, height, biomass production and seed germination percentages (Sharma & Johri, 2003).

The aim of this study was to characterize different bacterial genera as plant growth rhizobacteria promoters of *P. clandestinum* plants for the development of possible biofertilizers, allowing to take properly advantage from Colombian soils' biodiversity in the Andean zone and to reduce cost in fertilization.

MATERIALS AND METHODS

Experiment location and bacteria strains: The experiment was developed at the research center "Tibaitatá - Corporación Colombiana de Investigación Agropecuaria (CORPOICA)", located in Mosquera way km 14 at coordinates 4.71 °N, 74.23 °O and 2.291 m above the sea level.

Four bacteria isolates were assessed in this study: *Bacillus* sp. K24, *Micrococcus lectus* K32, *Pseudomonas* sp. K35, and *Azotobacter beijerinckii* KA206. These microorganisms belong to the Soil Microbiology Laboratory Collection from the

Center for Biotechnology and Bioindustry. The strains were previously isolated from *P. clandestinum* rizosphere in Mosquera, Cundinamarca.

Characterization of plant growth promotion mechanisms *in vitro*: Indolic compounds production was estimated using the colorimetric test described by Glickmann & Dessaux (1995). K-lactato medium supplemented with tryptophan was used for this (Carreño-López *et al.* 2000). Microorganism incubation was carried out for 72h at 150 rpm in complete darkness in the culture medium indicated. Sarkowsky reagent was used for indole production determination (12 g L⁻¹ FeCl₃ in 7.9 M H₂SO₄) with a 1:1 relation between the reagent and the supernatant from microorganism culture. The reaction lasted for 30 min in darkness. Indolic compounds concentration was analyzed by spectrophotometry at 540nm.

Quantitative determination of tricalcium phosphate solubilization in liquid medium was accomplished using Pikovskaya broth (g L⁻¹): Glucose 10; (NH₄)₂SO₄ 0.5; MgSO₄ 0.1; KCl 0.2; yeast extract 0.05; Ca₃(PO₄)₂ 2.5. Microorganisms were incubated for 5 days at 30 ± 2°C and 150rpm (Pikovskaya, 1948). The supernatant from each culture and the control treatments were used for phosphorus content measuring (Fiske & Subbarow, 1925).

For siderophores synthesis determination, it was made a bacterial suspension from each strain in study in NaCl 0.85% at an OD₆₀₀ = 0.500. Aliquots of 10 µL from the suspension were streaked by triplicate on petri dishes with CAS medium (g L⁻¹): Chrome azurol S(CAS), 0.06; FeIII solution (1mM FeCl₃.6H₂O), 10mL; 1mM HCL, 10mL; haxadeciltrimetilammoniumbromide (HDTMA), 0.07; Agar, 37.00; glycerol, 10mL; piperazine-N-N-bis [2 sulfuric acid ethanol](Pipes), 3.24. pH 6.8; at a one micro droplet proportion per dish, then, were incubated for 48 h at 30 ± 2°C. Positive outputs were distinguished by blue to yellow color turning surrounding the bacterial growth (Schwyn & Neilands, 1987).

Test under greenhouse conditions: Phosphorus source: Ca₃(PO₄)₂ Tricalcium phosphate (TF), low solubilization in water and not available to the plant.

In this evaluation, a complete randomized design was used, with seven treatments: T0. Control; T1. 100% TF, T2. 50% TF, T3. 50% TF + K24, T4. 50% TF + K32, T5. 50% TF + K35 y T6. 50% TF + KA206, and five repetitions for each treatment. Chemical fertilization was based on soil analysis (12.23% OM; 2.7 P (mg kg⁻¹); 0.72K; 1.20 Mg; 2.32Ca (Cmol kg⁻¹); pH=5.5), using as phosphorus source tricalcium phosphate. The assessed variables were: plant height (cm), root length (cm), shoots dry weight (g) and root dry weight (g). Each treatment was inoculated with 5mL of cellular suspension at 1x10⁸ UFC ml⁻¹ concentration in Luria Bertani medium (g L⁻¹): Tryptone 10, yeast extract 5, NaCl 10. The experiment was kept under greenhouse conditions for two months.

Statistical analysis: Data were statistically evaluated using an ANOVA and Tukey`s HSD Test with 95% confidence level.

RESULTS AND DISCUSSION

Characterization *in vitro* of plant growth promotion features: Results indicated that all strains have the ability to solubilize the P source evaluated. *Bacillus* sp. K24 (31.37 mg L⁻¹) and *Azotobacter beijerinckii* KA206 (30.17 mg L⁻¹) strains displayed the best results with statistical significant differences among other treatments (p ≤ 0.05). In relation to indolic compounds production there were a similar behavior within all assessed microorganisms where *Micrococcus lectus* K32 (10.72 µg mL⁻¹) and *A. beijerinckii* KA206 (9.43 µg mL⁻¹) strains exhibited higher values. However, regarding to siderophores synthesis *M. lectus* K32 strain was the only one lacking the activity (Table 1).

Table 1. Characterization of *in vitro* growth promotion features of the four bacteria strains evaluated.

Bacterial strain	Indolic compounds	Soluble phosphorus (ppm)	Index of synthesis of siderophores (mm)
K24	6.69 ± 0.23 ^b	31.37 ± 0.24 ^a	2.33 ± 0.07 ^b
K32	10.72 ± 1.13 ^a	18.20 ± 0.83 ^b	0.00 ± 0.00 ^c
K35	4.52 ± 0.37 ^c	29.80 ± 1.50 ^a	2.52 ± 0.08 ^a
KA206	9.43 ± 0.51 ^a	30.17 ± 1.19 ^a	2.25 ± 0.03 ^b

Means with no common letters within each variable differ statistically according to Tukey`s test p ≤ 0.05 (n = 3)

Bacillus and *Pseudomonas* genera have been previously reported as microorganisms that have the three plant growth promotion mechanisms evaluated *in vitro* in this essay (Ramani, 2011; Gupta *et al.* 2013). The same results related to the ability for total indole production and siderophores synthesis were reported by Sánchez *et al.* (2012). Babana *et al.* (2013) reported for *Pseudomonas* sp. BR2 strain values of 90 mg L⁻¹ for phosphorus solubilization after seven days of incubation as well as indole production and siderophores synthesis. This latter mechanisms are well known for chelating ferric ion in rhizosphere, which inhibits growth of pathogenic microorganisms which affinity for iron is low. Likewise it allows to increase the element availability to plants when it is found in the form Fe⁺³, improving plant nutritional balance and therefore plant growth (Glick & Bashan, 1997).

With regards to the genus *Azotobacter* other authors have found that it does not have the ability to solubilize high phosphorus concentrations (Husen, 2003), Kumar & Nair (1999) reported solubilization values between 0.18 and 0.19 mg L⁻¹, results that are below to those ones resulting in this study for the strain *A. beijerinckii* KA206. Respecting to indole production, it has been found that several species belonging to the genus *Azotobacter* have the capability to produce different concentrations of these compounds, ranging between 11 y 12.2 µg m L⁻¹ (Fiorelli *et al.* 1996; Ravikumar *et al.* 2004), values close to those displayed by KA206. Similarly, siderophores production has been found in previous studies of this genus (Duhme-Klair, 2003).

For the genus *Microccus*, it has been found as well the capacity to produce indole compounds, solubilize different inorganic phosphorus sources and synthesize siderophores (Dastager *et al.* 2010). Nevertheless, the last activity was absent in *M. lectus* K32 strain in this research.

Test under greenhouse conditions

Height and root length of *P. clandestinum*: Results revealed that inoculation with selected strains positively influenced plants height. Inoculation with *A. beijerinckii* KA206 ($p \leq 0.05$) increased plant growth three times in regards to control, whereas that 100% fertilization treatment did not display differences.

In the same way, root length was increased by bacterial inoculation. Differences were observed with *A. beijerinckii* KA206 and *M. lectus* K32 compared to the control. However, no differences were evident with 100% fertilization treatment. In relation to 50% fertilization dose treatment, it was evident that treatments with the same fertilization dose plus bacterial inoculation displayed higher results. With *A. beijerinckii* KA206 strains, there were statistically significant ($p \leq 0.05$) differences, revealing increases of 80.6% (Figure 1).

For plant species of the genus *Pennisetum*, it has been previously reported that some *Pseudomonas* sp. bacterial strains are capable of improving height and root length. These features are closely related to plant growth promoting mechanisms in this study, such as phosphorus solubilization and siderophores production (Gupta *et al.* 2013), which is consistent with results shown by *Pseudomonas* sp. K35. Siderophores production is linked to iron use by plants, an essential element for photosynthesis and other biological processes. Thus, siderophores allow oxidized iron plant-availability, improving plant nutrition (Taiz & Zeiger, 2010). Therefore, both *Pseudomonas* sp. and *Bacillus* sp. have been distinguished by producing positive significant effects on these variables with up to 30% increases in plant height and 16% in root length (Sánchez *et al.* 2012).

Other studies, have linked *Pseudomonas* sp. own capability to promote plant growth with efficient rhizosphere colonization leading to a significant increase in plant height and root length, which is mainly related to the strain's competence to solubilize phosphorus (Katiyar & Goel, 2003). This is due to limitation of available P to plants that can generate various nutritional deficiencies, so the increase in the element availability due to P solubilization contributes to plant nutrition and development (Rodríguez & Fraga, 1999).

Importance of phosphorus in this context lies in its impact on forage quality and hence in the high demand of chemical fertilizers use (Marais, 2001). This is due to phosphorus is an essential macronutrient to plant development, involved in structural stability and energetic efficiency in plants. Therefore, increases in the element availability to plants will be reflected in its development (Taiz & Zeiger, 2010).

In other grasses such as wheat, it has been pointed out the increases in plant growth caused by strains of the genus *Azotobacter*, which is also due to phosphates solubilization activity and plant growth promoting substances production such as indole compounds. These mechanisms were observed in *A. beijerinckii* KA206 strain in this study (Kumar *et al.* 2001). It has been noted that indole production stimulates cell elongation and apical plant growth, thus this mechanism is closely involved in plant height and root length risings (Taiz & Zeiger, 2010).

Likewise, the genus *Microccus* increases up to 100 and 39.2% root length and plant height respectively, compared to control treatment (Dastager *et al.* 2010). In this study, increases caused by *M. lectus* K32 strain were statistically significant ($p \leq 0.05$) rising in a 128% in respect to 50% fertilization dose treatment (Figure 1).

Shoot and root dry weight of *P. clandestinum*: Results showed that inoculation with microorganisms influenced

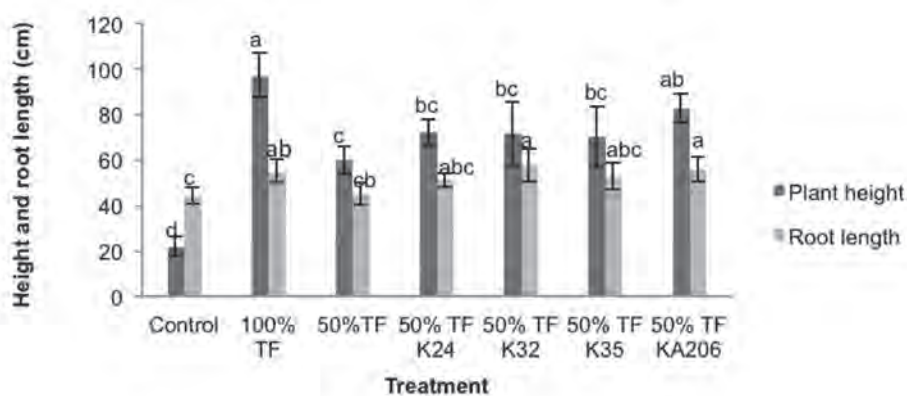


Figure 1. Height (cm) (dark grey bars) and root length (light gray bars) (cm) of *P. clandestinum*. Error bars represent standard deviation. Letters represent significant differences ($p \leq 0.05$).

shoot weight positively with tenfold higher values using *A. beijerinckii* KA206 and *Bacillus* sp. K24 strains compared to the control treatment ($p \leq 0.05$); whereas no significant differences were found among 100% fertilization treatment. It was observed that root dry weight from inoculated plants with *A. beijerinckii* KA206, *M. lectus* K32 and *Bacillus* sp. K24 strains, exhibited a significant increase ($p \leq 0.05$), in relation to control, 100% and 50% fertilization treatments. Results obtained with *A. beijerinckii* KA206 strain stand out because of its best performance in root dry weight variable (Figure 2).

In relation to the increase in plant dry weight, it should be pointed out that the rise in values of root biomass has significant effects on plants ability to absorb nutrients from the soil as it represents a greater exploration on soil (Antoun & Prevost, 2006). In this sense, several studies have demon-

strated that auxin type 3-indolacetic acid (IAA) has a notable impact on root development of plants (Lambrecht *et al.* 2000; Tsavkelova *et al.* 2006; Ashrafuzzaman *et al.* 2009). Taking this into account, it is important to emphasize that the four strains had the capacity to produce indolic compounds, which may be related to the improvement of plant development, compared to the control and fertilized treatments (Figure 2).

In the genera *Azotobacter*, *Pseudomonas*, *Bacillus* and *Micrococcus*, the capacity to increase dry matter production is related to mechanisms such as phosphorus solubilization, indole production and siderophores synthesis, which has been previously reported (Kumar *et al.* 2001; Dastager *et al.* 2010). Therefore, inoculation with phosphate solubilizing microorganism improve dry weight on treated plants. Accord-

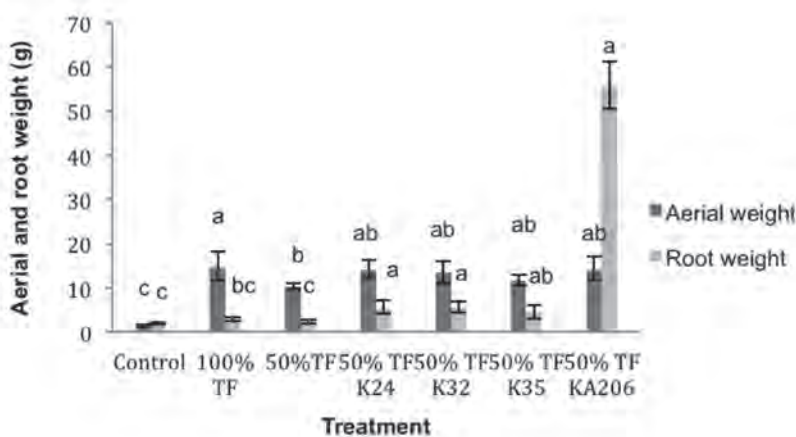


Figure 2. Aerial weight (g) (dark grey bars) and root weight (light grey bars) (g) for *P. clandestinum*. Error bars represent standard deviation. Letters represent significant differences ($p \leq 0.05$).

ing to Kumar *et al.* (2001), this fact may be associated with P increases on substratum, incrementing at the same time its absorption by plants. The latter has been previously reported for the genera *Azospirillum* and *Azotobacter*, which are capable of solubilizing phosphorus. Thus increasing capture of phosphorus by plants, when these are inoculated, is related with additions on dry matter production (López-Ortega *et al.* 2013). So, in accordance with Chabot *et al.* (1998), phosphates solubilization might be an effective mechanism for plant growth promotion. In that way, Madigan *et al.* (2004) and Fernández *et al.* (2005) have noted that since microbial metabolism is capable of releasing trapped phosphorus in insoluble compounds, it is important to take into account the use of microorganisms to increase phosphorus plant uptake.

In addition, it is important to emphasize that this test was carried out on soil which may contain some other sources of phosphate compounds not available for plants; however they could become available due to microorganisms activity. Besides, it should be outlined that the phosphate fertilization dose used in inoculated treatments was 50%. In this way, the results obtained with these treatments surpassed the treatment with the same fertilization dose and without inoculation and also in some cases the treatment with 100% fertilization with statistically significant differences ($p \leq 0.05$).

Nevertheless, for grasses, it has been previously reported that indole compounds, siderophores production and phosphorus solubilization is related to plant growth promotion, increasing values of variables associated with growth and plant development (Castanheira *et al.* 2014). Therefore, accomplished results can not only be attributed to phosphorus solubilization but also to the other mechanisms assessed in this assay and also to those not evaluated as biological nitrogen fixation, earlier and widely conferred for the genus *Azotobacter* (López-Ortega *et al.* 2013).

Conforming to the response displayed by *P. clandestinum* to inoculation with phosphate solubilizing bacteria, this alternative emerges as a way for using low solubility phosphorus sources plus microbial inoculants instead of using chemical phosphate fertilization. The preceding can make production systems sustainable over time within an organic system.

The presented results contribute to sustainability concept and its application in production systems. On the other hand, more research is needed to elucidate how these bacteria play a role in *P. clandestinum* growth. Thus, the strains used in this research ought to be evaluated in more experiments under greenhouse and field conditions to confirm its potential as plant growth promoters.

In conclusion, the use of this microorganism may lead to a substantial reduction in chemical fertilizers application and

therefore in production costs, transforming the livestock system in a sustainable one at different levels such as economic, environmental and social.

Acknowledgment: The authors would like to render thanks to the Colombian Ministry of Agriculture and Rural Development and to the Soils Microbiology Laboratory-Corpoica.

Conflict of interest: The manuscript was prepared and revised by all authors, who declare the absence of any conflict that may place the validity of presented results in risk.

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Como Citar:

Sánchez, D.B.; Gómez, R.M.; García, A.M., Bonilla, R.R. 2014. Phosphate solubilizing bacteria isolated from *Pennisetum clandestinum* associate to livestock systems in the Andean area. Rev. U.D.C.A Act. & Div. Cient. 17(2): 423-431.