

Plant Growth Promoting Abilities of Phosphate Solubilizing Bacteria Native from a High P-Sorbing Ultisol

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Abstract. P adsorption isotherms were used to analyze P-sorption characteristics in a weathered slightly acid savanna ultisol. High P adsorption in this soil indicates significant P limitation in the experimental area. Microorganisms able to dissolve sparingly soluble phosphates of Fe and Al in the ultisol were studied in the rhizospheres of *Galactia jussiaeana* and *Centrosema venosum*, which showed *Burkholderia cepacia* as the dominant P-solubilizing bacteria species. Its promoting plant growth capacity was tested inoculating sterile *Zea mays* seeds; inoculated seeds germinated 100% while non-inoculated showed only 71% of germination. After three weeks, inoculated plants showed 28% increase in root length, higher biomass and foliar concentration of phosphate compared to non-inoculated. Improvement of phosphate availability and a higher production of promoting root growth hormones by bacteria may explain these results. *Burkholderia cepacia*, native from tropical acid soils showed potential as a biofertilizer able to increase plant P nutrition in high P fixing soils.

Keywords: Acid soils, biofertilizers, *Burkholderia cepacia*, plant growth promoting rhizobacteria (PGPR), phosphorus, sustainability

1 Introduction

The soils of the Orinoco savannas are, in general, well weathered due to the high temperature and relatively high precipitation occurring in the zone. Consequently, they are acid soils with low contents in C, N, P and as well low base contents in the exchangeable matrix [21]. Under increasing acidity, soil exchangeable aluminum (Al³⁺) tends to increase, in some cases to toxic levels, with a concomitant deficiency in available forms of phosphorus due to P-adsorption [17,21,40]. Currently, savanna's ecosystems are transformed into agricultural lands, which imply the use of important amount of soluble P-fertilizers to correct the high P-sorption capacities of savanna soils. Thus, in accordance with Casanova [6], about 70 x 106 ha of Venezuelan territory is affected by acidity where P is the main limiting element.

In natural conditions, most of tropical plants are adapted to different ecological niches associated to soil microorganisms. The diversity and abundance of microorganisms, plant and pedofauna influence different functions of the ecosystems which can play key roles in nutrient cycling and in the protection of the plant from environmental stress [4,11,24]. Within the microorganisms that may contribute to increasing nutrient availabilities, phosphate solubilizing bacteria are outstanding. Many of these bacteria have Plant Growth Promoting (PGP) properties that play a significant role in soil [2,13,38]. Plant Growth Promoting Rhizobacteria (PGPR) are soil free bacteria able to colonize and live in the rhizosphere, where they contribute to the growth and development of the plant system through different activities such as: increasing the solubility of inorganic nutrient elements (e.g. P, K,Ca) from insoluble sources, fixing atmospheric N₂, reducing root pathogens (by antagonism or competition), and finally, producing plant growth regulating substances (vitamins, auxins, cytokinins, and gibberellins) that contribute to increasing root growth [2,8,13].

It is well known that some soil microorganisms, including PGPR, have great potential to contribute to amending local fertility problems; consequently, they might be used as biofertilizers [3,38]. Biotechnologies with those biological agents, when found, may be applied at greenhouse level in the production of good quality seeds or in the massive generation of plants [4,11,48]. Therefore, at the level of agroecosystems, the finding, isolation and sustainable management of those microorganisms is an

important desideratum, since they favor ecosystem plant nutrition and consequently, they may be used to reduce the application of high commercial fertilizer doses [47].

In a previous contribution [25], it was reported a survey of the presence of some beneficial plant-microorganism associations in a typical *Trachypogon* savanna, with particular emphasis on arbuscular mycorrhiza populations, symbiotic N-fixing bacteria and free living microorganisms that stimulate plant growth through phosphate solubilization.

In this contribution we analyze, firstly, P parameters of the savanna soil previously studied in a more detailed form, and secondly, from the original plant survey studied, we examined a selection of effective local bacteria strains that will be assayed to determine their capacity to solubilize phosphates, promote plant growth, enhance germination and stimulation of root growth in *Zea mayz*; attributes that can be used to define those PGPR as potential biofertilizers.

2 Materials and Methods

The study was carried out at “Estación Experimental La Iguana” (EELI), located in Guárico State in Northeastern Venezuela (8°25'N and 65°24'W). EELI is under the influence of the Orinoco River watershed and corresponds to representative savannas of the Venezuelan Central Plains. These savannas are dominated by *T. plumosus* (Poaceae) with the presence of isolated trees and shrubs such as *Curatella americana* (Dellineaceae), *Copernicia tectorum* (Palmae), *Byrsonima crassifolia* (Malpigheaceae) and *Bowdichia virgilioides* (Papiloneaceae). The climate of the zone is markedly tropical isothermic, with a mean annual precipitation of 1342 mm, most of which falls during the rainy season (May to October), and a mean annual temperature of 27.9°C. EELI has edaphic substrates different in age and genesis [32].

2.1 Soil Sampling and P Characterization

In the experimental site, selected triplicate soil samples of an ultisol were collected. Each replicate was a composite sample integrated by eight soil subsamples collected at random from the 0–10 cm of soil layer and sieved through a 2-mm mesh.

2.2 Determination of Phosphate Sorption Isotherm

The phosphate adsorption isotherm of the ultisol was obtained by a modification of the method described by López-Hernández et al. (1986). Phosphate was adsorbed on 0.5 g soil samples by shaking them for 24 h with phosphate solutions (KH₂PO₄) which give 0, 20, 40, 50, 60, 80 y 100 µgP/g in 0.02 M KCl. After phosphate adsorption the P concentration in the clear solution was determined [26].

2.3 Sampling of Rhizospheric Soils and Selection of Plants with Abundance of PSB

In order to describe, characterize and quantify the association of native plants with Phosphate Solubilizing Bacteria (PSB) living in the rhizosphere of the studied soil plants, in an experimental area of 260 m x 77 m at EELI, three equidistant transects were located [24]. Seven plots of 1 m² were set up, 10 m apart, at both sides of the three transects during the middle of the rainy season (July). All plants inside the 21 plots were collected and preserved for later identification.

Rhizospheric soil and roots from each plant were collected from the root zone (0 – 20 cm) by previously removing plant debris from the soil surface. Soil located at 1 cm from the roots was used to isolate phosphate solubilizing bacteria and to measure rhizospheric pH. pH of bulk and rhizospheric soil samples were determined in a KCl 1M 1:1 soil solution ratio [1].

2.4 Isolation and Identification of Phosphate Solubilizing Bacteria

One g of fresh rhizospheric soil was serial diluted and 0.1 mL of dilution was added to Petri dishes with the following selective media: standard phosphate-solubilizing medium (PS) [50] and yeast extract-dextrose medium (YED) [31], each containing one of the following phosphorus compounds: dicalcium phosphate (CaHPO₄), aluminum phosphate (AlP₀₄) and iron phosphate (FeP₀₄). After bacteria

development, colonies showing a surrounding halo of clearance, or a change of color surrounding the colony (from purple to yellow), were selected as phosphate solubilizers or acidifiers of the surrounding environment, respectively [23,42,50]. Halo clearance showing solubilization capacity of bacteria over Ca, Al and Fe phosphates was measured in three colonies of the bacteria growing in each media; data are mean of three halos.

From the total universe of native plants analyzed, we will choose those which showed a high proportion of PSB in relation to the total bacteria number since we believe that the presence of PSB in the rhizosphere of those species constitutes an important starting material to look for potential biofertilizers [24,26].

2.5 Identification of Bacteria

Once the colonies able to solubilize phosphates were isolated from the plant rhizosphere, different biochemical tests were applied in order to identify and characterize the bacteria [22]. Moreover, to assess them as PGPR, experiments were conducted to determine their capacity to enhance germination, promote plant growth and stimulation of the root and shoot growth in *Zea mayz* (Turén variety) [14, 29].

After the colonies with P-solubilizing capacity were purified, we continued with morphological characterization and biochemical tests for identification [22]. Subsequently, API® reference range identification systems and confirmatory tests for definitive identification were applied [23,30].

2.6 Testing of the Plant Growth Promotion

Promotion of germination: Seeds of maize were sterilized superficially with sodium hypochlorite (10%) and uniformly distributed into sterile Petri dishes. 1 mL of PSB culture was applied to each seed with a 108cells/mL density [31,42]. Control treatments did not carry the bacterial culture, but medium culture without bacteria. Each treatment presented five repetitions. After seven days, the percentage of germination and the size of the radicles were measured.

Promotion of radical growth: Pots were filled with steamed sterilized sand, and, then, one sterilized maize seed per pot was introduced. Four days later the emerged seedlings were inoculated with 1 mL of PSB culture (108 cells/mL). Inoculation was repeated a week later. Control did not carry the bacterial inoculum but the medium culture without bacteria. All treatments were irrigated with Hewitt's nutritive solution [8]. The experiment took place in greenhouse conditions of sterility for 21 days. After this time the plants were cut, the length of root was measured by the Newman method [27] modified by Tennant [41]. Each treatment had 5 repetitions.

2.7 Plant Determinations

After harvesting, plant materials were washed out with distilled water and dried at 60°C for 24 hours; then the biomass was determined. Phosphorus foliar content was quantified after an acid digestion with a binary mixture (hydrochloric and perchloric acids) [26].

2.8 Statistical Analysis

A test of homogeneity by applying a one-way ANOVA (95% confidence) was performed to analyze the plant variables: P content, root length and stem dry weight. Mean comparisons were made between inoculated and non-inoculated treatments. Significant differences are given by the standard deviations.

3 Results and Discussion

3.1 General Characteristics of the Studied Soil

The soil used for the determination of the adsorption isotherm is slightly acid, with low natural fertility and low cation exchange capacity (CEC); main soil characteristics were already presented by Lopez

Gutierrez et al. [15]. The organic matter and nitrogen contents were moderately corresponding to a C/N ratio of 23.6 (Table 1). The total P content (Pt) and P availability (P-NaHCO₃) were also low (Table 1). On the contrary, this ultisol presented a high amount of exchangeable iron and aluminium and, according to the P adsorption isotherm (Fig. 1), a very high P-sorption capacity; a similar result has been already presented in an ultisol corresponding to the serie Tamaca, Venezuela, with high concentrations of exchangeable aluminum and free iron [19].

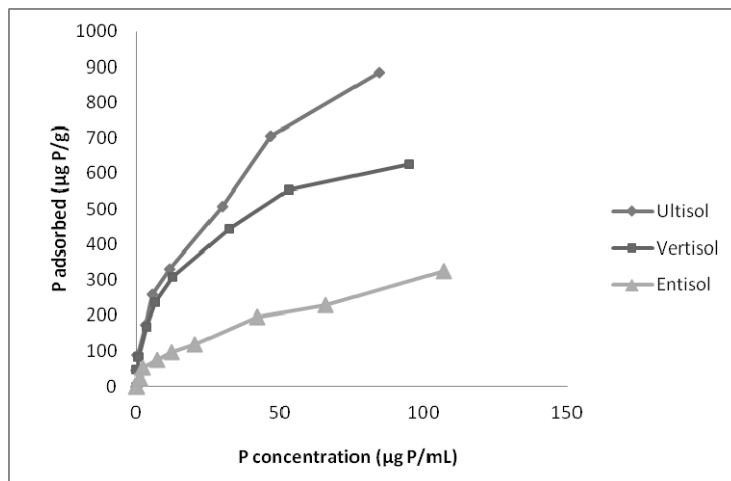


Figure 1. Phosphate adsorption isotherms in selected soils of Estación Experimental La Iguana

The high P adsorption in the soil herein analyzed, seems to be more related to the Fe content, than to the exchangeable aluminum and is concomitant with their low available P content (Table 1).

Table 1. General characterization of a savanna ultisol in Estación Experimental La Iguana. Modified from López-Gutiérrez et al [15].

Soil Characteristic	
pH	5.4
Sand (%)	73.2
Silt (%)	13.2
Clay (%)	13.6
Texture	Sandy loam
P-Total (mg kg ⁻¹)	102.1
P-NaHCO ₃ (mg kg ⁻¹)	2.6
N (%)	0.11
C (%)	2.36
Fe (mg kg ⁻¹)	109.6
Al (cmol kg ⁻¹)	0.70
H (cmol kg ⁻¹)	0.20
Ca (cmol kg ⁻¹)	1.88
Mg (cmol kg ⁻¹)	2.41
Na (cmol kg ⁻¹)	0.09
K (cmol kg ⁻¹)	0.22
CEC (cmol kg ⁻¹)	8.28

In Figure 1, for comparative reasons, the adsorption isotherm of the ultisol is presented, together with two isotherms corresponding to a very low sorbing entisol and a moderate P-retention vertisol, located in the same savanna zone at EELI. All these aspects indicate a significant P limitation in the

experimental area covered by the ultisol. A situation that might make particularly relevant the influence of the root environment (and associated processes) as important locus for mechanisms of biological origin which help to cope with P deficiencies [21,45, 46].

As far as the pHs of the rhizospheric soils are concerned, their values were always lower than in the bulk soil with a difference ranging from 0.13 to 0.98 for *Sida* sp. and *Stylosanthes* sp., respectively (Table 2), which indicate the influence that may have the acidification of the root environment and their associated processes such as organic acid secretions on the improvement of available P [5,18,20,37].

3.2 Isolation of Phosphate Solubilizing Bacteria (PSB)

The isolation of PSB was done in a total of 22 rhizospheres corresponding to the most important plant species present in the experimental area (Table 2); from the total, eight rhizospheres were positive to the presence of PSB (Figure 2).

Table 2. pH (KCl) of rhizospheric soils of chosen plant species in the experimental area and differences between pH of bulk (4.86) and rhizospheric soils

Species	pH rhizospheric soil	Δ pH
<i>Desmodium</i> sp.	3.93	0.93
<i>Indigosfera pascuorum</i>	4.59	0.27
<i>Phaseolus vulgaris</i>	4.14	0.72
<i>Stylosanthes</i> sp.	3.88	0.98
<i>Galactia jussiaeana</i>	4.16	0.70
<i>Mimosa pudica</i>	4.20	0.62
<i>Trachypogon</i> sp.	4.18	0.68
<i>Panicum</i> sp.	4.06	0.70
<i>Rynchospora cephalotes</i>	4.05	0.81
<i>Rynchospora barbata</i>	3.91	0.95
<i>Fimbristylis</i> sp.	4.27	0.59
<i>Polygala glochiata</i>	4.09	0.77
<i>Hyptis</i> sp.	3.99	0.87
<i>Sida</i> sp.	4.73	0.13
<i>Borreria</i> sp.	4.22	0.64
<i>Diodia teres</i>	4.06	0.80
<i>Ruellia geminiflora</i>	4.56	0.30
<i>Aeschynomene</i> sp.	4.40	0.46
<i>Cassia cultrifolia</i>	4.30	0.56
<i>Centrosema venosum</i>	4.35	0.51
<i>Desmodium</i> sp.	3.93	0.93
<i>Indigosfera pascuorum</i>	4.59	0.27

When analyzing the total amount of bacteria colony forming units in relation to PSB, we found that the rhizospheres of *Centrosema venosum* and *Galactia jussiaeana* present a high proportion of PSB in relation to the total bacteria (ToB) number (75% and 43%, respectively), therefore the presence of PSB in the rhizosphere of those two species might constitute an important starting material to look for potential biofertilizers (Figure 2).

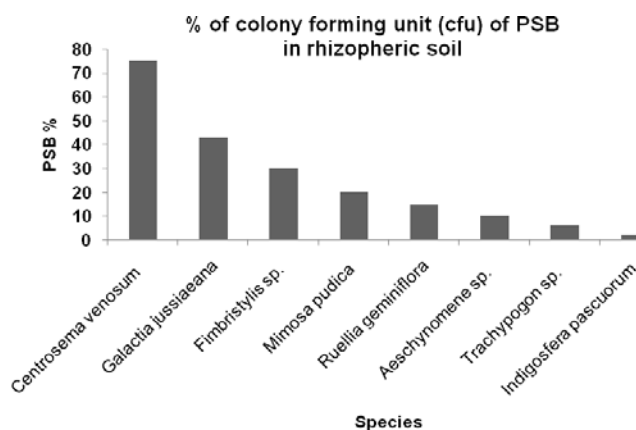


Figure 2. Percentage of PSB (% cfu PSB) respect to total bacterial counts (cfu ToB) in rhizospheric soil of field plants.

3.3 Isolation and Identification of PGPR

From the rhizosphere of *Centrosema venosum* and *Galactia jussiaeana*, bacteria strains were isolated based on their capacity to solubilize phosphates of calcium, iron and aluminum.

According to the morphological characteristics of bacteria in different media and the preliminary biochemical tests, we concluded the predominance of *Burkholderia cepacia* in the studied rhizospheres. The later tests with specific galleries [35] corroborated such identification. *Burkholderia cepacia*, the phosphate solubilizing bacteria isolated, presents the capacity to acidify the medium and to solubilize inorganic phosphate forms (Table 3).

Table 3. Capacity of solubilization of phosphates of calcium, iron and aluminum and acidification of the surrounding media by *Burkholderia cepacia*.

Medium	Initial pH	Final pH	Halo average diameter of three colonies (mm)		
			CaHP04	Al PO4	Fe PO4
YED [31]	7.0	6.0	6	----	----
PS [50]	7.0	5.5	5	9	10

3.4 Testing of the Plant Growth Promotion Ability

Inoculation of *Burkholderia cepacia* to *Zea mayz* seeds favored 100% the germination, while in the control treatment (without bacteria) the germination was 71%; the radicle length averaged 2.17 cm in the inoculated seeds and 0.72 cm in the control treatment. This indicates the important increase in growth together with a clear promotion of germination of the radicle in inoculated seeds of *Zea mayz*.

Tests for growth promotion showed that after 3 weeks of inoculation, plants inoculated with *Burkholderia cepacia* showed higher values for root length (28%), biomass (52%) and foliar P content (50%) over non-inoculated treatments. Increases are statistically significant for biomass and foliar P content, whereas in the case of the root length due to the high variability of results there was not significance between means. The results from growth parameters allow to characterize *Burkholderia cepacia* as a maize growth promoter bacterium (PGPR) under greenhouse conditions.

Our results agree with Torriente [44], who noted that *B. cepacia* was able to solubilize inorganic phosphates and produce 25% yield increases in sugar cane. Our results are also in line with Moreno and Galvis [25], who found that a strain, identified as *B.cepacia*, promoted 30% of germination and root growth in maize over non inoculated controls.

Table 4. Parameters of plant growth of *Zea mayz* inoculated or not with *Burkholderia cepacia*.

	P foliar content ($\mu\text{g/plant}$)	Biomass (g/plant)	Root length (m/plant)
Inoculated plants	5.87 (1.20)	0.420 (0.09)	7.480 (0.88)
Non inoculated plants	3.09 (1.36)	0.276 (0.12)	5.474 (2.24)

Data are average of 5 repetitions, and standard deviations are in brackets.

In acid soils phosphorus is rendered unavailable due to their precipitation and/or adsorption as phosphates of iron and aluminum [17]. Therefore, phosphorus deficiency is an edaphic characteristic most common in the acid and well weathered tropical soils. The soil solution is usually enriched in P through the chemical solubilization of inorganic sources of phosphates, or through the microbial mineralization of organic phosphates, both mechanisms are particularly important in well weathered savanna soils [12,15,16,33]. According to Sanchez and Salinas [36], 82% of the extension of the tropical American land is P-limited; therefore, it is important to highlight the ability of soil microorganisms such as *B. cepacia* capable of solubilizing the main types of unavailable phosphates present in well weathered acidic soils, through its capacity to acidify the surrounding environment (Table 3). This ability may be due to generation of organic acids which release inorganic phosphate sources, or through the competition of organic acids with orthophosphate for P-sorbing places [10,20, 28].

3.5 Diversity of *Burkholderia Cepacia* in the Environment

B. cepacia possess an unusually complex and plastic genome which may favor their adaptation to different environments, and it is not surprising that the different strains of the complex are found in habitats as diverse as soil, water, plant, and animal surfaces and rhizosphere. This remarkable versatility is well exemplified by the wide array of properties that *B. cepacia* possess [34]. Thus, it is now well settled that *B. cepacia* can act both as plant growth promoting biocontrol and bioremediation agent and as opportunistic human pathogen in immune-compromised patients [39, 43]. Despite the useful properties those organisms may offer to bioremediation or as an alternative to pesticides and fertilizer in agriculture, commercial application has been hampered by the risk *B. cepacia* complex may pose to human health. In conclusion, it seems possible that this bacteria due to its diversity can play an important role in ameliorating agriculture practices in tropical ecosystems, nonetheless, before using it as mass inoculum previous tests should be done, in order to discard harmful effects on human health [38,39].

4 Conclusions

The results obtained show *Burkholderia cepacia* as a bacterium capable of promoting the growth and development of plants. Although in this work the bacteria effect was only tested on *Zea mayz*, its promoting capacities must be proved on other tropical crops. Characterization and isolation of *Burkholderia cepacia* as promoter bacteria of plant growth allows us to suggest its use as a biofertilizer for tropical crops developed in acidic soils. *Burkholderia cepacia* showed the ability to promote the growth, germination, elongation of root and production of biomass of *Zea mayz*. These features indicate its potential application as biofertilizer and/or as biological control in crops of agricultural interest for the acidic soils of savanna. However more tests should be done, in order to discard any harmful effects on human and environmental health.

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