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RESEARCH ARTICLE

The Presence of Beneficial Organisms Associated to N and P Economy in the Rhizosphere of Native Vegetation in an Oligotrophic Savanna of Guárico State, Venezuela

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Abstract:

Background:

In natural conditions, tropical plants are adapted to different ecological niches that can be associated to soil microorganisms which play a key role in nutrient cycling like *Arbuscular mycorrhiza* (AM), phosphate solubilizing bacteria (PSB) and/or nitrogen fixing rhizobia.

Methods:

We report a survey of the presence in a Trachypogon savanna located at Estación Experimental La Iguana (EELI) in Central Venezuela, of some beneficial plant-microorganism associations. In this savanna, plants present a high AM symbiosis affinity. The high mycorrhization and the presence of potential PSB suggest a synergic effect in plant P-uptake.

Results:

After screening the rhizospheres of 25 plant species from the zone, we could isolate a high proportion of potential PSB in relation to the total bacteria number from the rhizospheres of *Centrosema venosum* and *Galactia jussiaeana*.

Conclusion:

Therefore, the presence of potential PSB in the rhizosphere of those species constitutes an important finding to discover novel biofertilizers for crop plants.

Keywords: *Arbuscular mycorrhizae*, PGPR, N-fixation, Phosphorus, Nitrogen, Sustainability.

1. INTRODUCTION

In Venezuela, savanna ecosystems occupy 260.000 km^2 (about 29% of the territory), located on dystrophic and well drained soils dominated by grasses such as *Trachypogon plumosus* Ness, locally known as Trachypogon savannas [1]. The dominant grass, *Trachypogon*, is characterized by its low productivity, digestibility and palatability values, so the genus seems to be well adapted to acid and nutrient-depleted soils, particularly in nitrogen and phosphorus [2 - 5].

Savanna soils are frequently burnt as a common agriculture practice, and this kind of management have contributed to accelerate carbon and nitrogen losses in the soil [6, 7]. Moreover, under increasing acidity, that characterizes the well-developed savanna soils, soil exchangeable aluminum (Al^{+3}) tends to increase to toxic levels with a concomitant deficiency in available forms of phosphorus [8, 9]. Therefore, when savanna ecosystems are transformed in intensive

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agricultural lands, large amount of soluble and expensive P-fertilizers must be quenched by the high P-sorption capacities of savanna soils.

In natural conditions, most of the tropical plants are adapted to different ecological niches associated to soil microorganisms such as *Arbuscular mycorrhiza*-AM [10,11], phosphate solubilizing bacteria (PSB) [12] and symbiotic nitrogen fixing bacteria, as rhizobia [13], which can play a key role in nutrient cycling and in the protection of the plant to environmental stress.

The diversity and abundance of microorganisms, plant and pedofauna influence the diverse functions of the ecosystems such as soil nutrient cycling (nitrogen, phosphorus and carbon). The interaction between the specific host and the composition of the microbial communities might be affected by the root exudates [14], so the rhizosphere can be considered as an important domain to attract beneficial microorganisms nevertheless the exact role of the organisms are not yet fully understood [15].

Some soil microorganisms have a great potential to contribute to amend soil fertility problems and consequently they might be considered as promising biofertilizers [16]. Thus, the potential use of biofertilizers in tropical savannas with low nutritional levels are starting to be currently assayed in Venezuelan savannas as a convenient technique to improve plant nutrition and reduce the application of high commercial fertilizer doses [17 - 20].

In this contribution, we report a survey of the presence of some beneficial plant-microorganism associations in a typical Trachypogon savanna located in Central Venezuela, with particular emphasis on the populations of *Arbuscular mycorrhiza*, symbiotic N-fixing bacteria and free-living microorganisms that stimulate plant growth through phosphate solubilization. This preliminary report on beneficial microorganisms will be the basis to follow up more detailed research on specific treatments of savanna´s soils with potential native biofertilizers.

2. MATERIALS AND METHODS

2.1. Study Site

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 area 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 is markedly tropical isothermic, with a mean annual precipitation of 1342 mm, most of which falls during the rainy season (May to August), and a mean annual temperature of 27.9°C. EELI has different edaphic substrates in age and genesis [21].

2.2. Soil Characterization

In order to characterize the soil of the experimental site, in an area of 3 ha, samples from the surface (0-14 cm) and subsoil (14-28 cm) were collected in the month of July (middle of the rainy season). Within each depth, one composite mixed sample was taken from at least 6 cores (15 cm depth of sampling, and 9.5 of internal diameter) collected at random and then sieved through 2-mm mesh. After drying, duplicate samples were analyzed for soil pH (measured in a ratio 1:1 soil: water), organic carbon content [22], total nitrogen (micro Kjeldahl method) according to Anderson and Ingram [23]. Available phosphorus was extracted according to Olsen [24] and phosphorus in the extracts determined using the Murphy and Riley method [25]; exchangeable bases were determined by atomic absorption.

2.3. Experimental Design

At the experimental site at EELI, an area of 260 m x77 m was located; within it three 65 m equidistant transects were delimitated (Fig. **1**). In the transects, at both sides, seven quadrats of 1 m^2 were set up, 10 m apart from each other. At the middle of the rainy season (July), sampling was undertaken in the quadrats by collecting all the different plant species and the accompanying soil around the root (rhizospheric soil) inside the 21 chosen plots.

2.4. Sampling of Rhizospheric Soils and Root Systems

Rhizospheric soils and roots from each plant species located in the 21 plots were collected from the root zone (0-20 cm) by previously removing plant debris from the surface of the soil. Triplicate samples (1 g) of this rhizospheric soil located at 1 cm from the roots were used to isolate phosphate-solubilizing bacteria according to Varma [26]. Therefore,

microorganisms were characterized by sampling the rhizospheric soil of each one of the plant species collected following Barillot *et al.* [27]. Plant species were collected and preserved for later identification following Blackwelder [28].

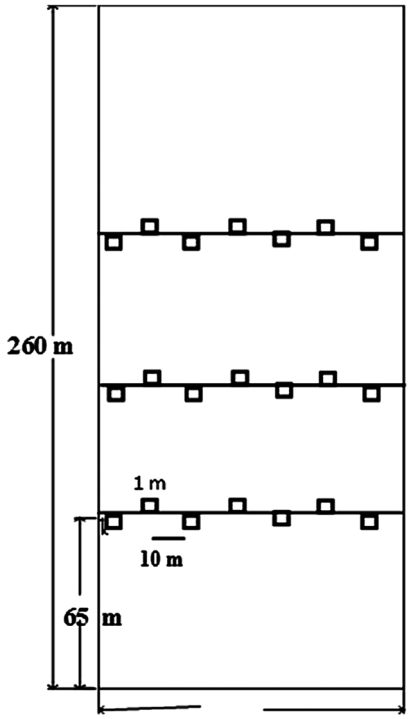

Fig. (1). Scheme of the experimental plot with transects and quadrats sampled.

2.5. AM Staining and Quantification

The whole radical system was extracted to avoid mechanical damage or losses of fine roots. Roots were preserved in a mixture containing 10 mL of formaldehyde, 5 mL of acetic acid, 50 mL of alcohol (95-96%) and 35 mL of distilled water. In order to be stained, roots were firstly, clarified in 10% KOH, washed with tap water, submerged in HCl 1N for 15-20 min, and then finally heated to dye with trypan blue 0.05% for 20 min [29]. AM colonization was quantified using the grid intersect method and expressed as percentage of colonized root length [30]. In the case of the roots of legumes, rhizobial nodules were carefully removed, counted and kept in silicagel vials [31].

2.6. Identification and Frequency of Plant Species

All the plant samples collected in the experimental area were identified by using the corresponding taxonomy keys and compared with the collections already deposited at the Botanical Garden Herbarium, Caracas, Venezuela. The frequency of the different species and their families were established by the following formula:

Frequency (*f*): $fi = \frac{ji}{k}$

ji the number of quadrats where the species appear

k total number of quadrats

2.7. Determinations of AM Infective Potential

AM propagules in the native soil were quantified using the most probable number (MPN) method [32, 33]. A composite mixed sample of, at least, 5 subsamples were collected at random from the experimental area to obtain five kg of EELL soil (0-20 cm); it was sieved (< 1 cm) and steam sterilized for 1 h on 3 consecutive days. Ten-fold serial dilutions (1x up to a 10^{-9} dilution) of sterile/non-sterile soil were placed, by quintuplicates, in 250 g pots. A surface

sterilized seed of *Sorghum vulgare* (Poaceae) was planted at a 2 cm depth in each pot and allowed to grow for 40 days. Sorghum roots were separated, washed and preserved as previously described for rhizospheric sampling to apply the trypan blue staining method [29] to observe and register the presence or absence of AM structures [32, 33]. Data are expressed as the number of infective AM propagules in 100 g of dry soil; confidence limits were assigned according to Fisher and Yates [34].

2.8. AM Root Colonization and Glomeromycota Spores Number

The roots of each one of the plant species collected at the experimental site were dyed [29] and the root colonization was quantified using the grid intersect method [30]. In the case of the plants detected with a higher frequency (>0.50) and with a higher percent of infected root length (>50%) Glomeromycota fungal spores were counted. For technical reasons the spore number associated with each plant species was assessed by wet sieving and decanting [35] in only one rhizospheric soil sample and expressed as the number of viable AM spores per g of dry soil.

2.9. Isolation of Phosphate Solubilizing Bacteria (PSB)

The presence of calcium phosphate ($CaHPO_4$) solubilizing bacteria in the rhizospheric soils was determined in Petri dishes by using the method of serial dilutions from 1 g of rhizospheric soil on two selective media; YED (0.5% yeast extract, 1% glucose, 0.2% calcium phosphate and 2% agar), according to Thomas *et al.* [36], and PS (sucrose 0.5%, 0.05% magnesium sulphate, 0.05% potassium chloride, 0.1% potassium nitrate, calcium phosphate 0.3% and 1.5% agar), according to Wenzel *et al.* [37], in both media 30 mg/L of cycloheximide was added in order to minimize the growth of other contaminant microbial groups, such as yeasts and fungi.

The plates were incubated between 4 and 15 days up to the emergence of a clear halo around the colony in the case of YED, whereas in the PS medium, a change of color from blue to yellow indicates the acidification and solubilization of phosphates. The total number of colony forming units was quantified per gram of rhizospheric soil (cfu/g rhizospheric soil), and the proportion of phosphate solubilizers, with respect to the total number of bacterial colonies present was calculated.

3. RESULTS

3.1. General Soil Characteristics and Plant Community of the Experimental Area

3.1.1. Soil Characteristics

The main physical and chemical characteristics of the soil in the experimental area are presented in Table **1**. The soil is a Typic Plinthustuls, sand loamy, kaolinitic, isohyperthemic, with low natural fertility and organic matter, and acidic pH (4.15-4.60).

Table 1. Main physical and chemical characteristics of the soil in the experimental area.

Soil Depth	pH	Inorg. N	P	K	Ca	Mg	Na	Exchan. Al	CEC	Organic Matter	Texture
(cm)		($mg.kg^{-1}$)						($cmol+.kg^{-1}$)		%	
0-14	4.60	20.4	7.43	44.0	61.6	28.4	15.0	0.39	3.92	1.33	sL
14-28	4.15	16.3	4.86	30.8	57.2	37.2	20.0	0.56	4.22	1.23	sL

sL= sandy Loam; Inorg. N= inorganic N; Exchan. Al = exchangeable Al; CEC= Cation Exchange Capacity

3.1.2. Identification and Frequency of Plant Species

Dominant species in the experimental area (*e.g.* with a presence above 75%) are: *Trachypogon sp., Fimbristylis sp., Hyptis sp., Rynchospora barbata, Mimosa pudica, Cassia cultrifolia*. In the sampling, 25 species distributed in 10 families were found (Table **2**) where the Poaceae, Ciperaceae and Leguminosae are the most abundant families.

3.1.3. AM Infective Potential in Savanna Soils

The number of AM infective propagules in the serial dilutions assay was of 4571 in 100 g of soil (intervals 2141-9765 at 95% confidence). This represents the presence of AM propagules like spores, Glomeromycota fungi mycelium and AM colonized rootlets that potentially can colonize new roots in soil.

Table 2. Frequency of native plant species in the experimental area.

Species	F
Trachypogon sp.	1.000
Fimbristylis sp.	0.905
Hyptis sp.	0.810
Rynchospora barbata	0.810
Mimosa pudica	0.810
Cassia cultrifolia	0.762
Hyptis suaveolens	0.762
Rynchospora cephalotes	0.762
Borreria sp.	0.762
Panicum sp.	0.762
Paspalum sp.	0.714
Polygala glochidiata	0.619
Diodia teres	0.619
Aeschynomene sp.	0.619
Desmodium sp.	0.524
Indigosfera pascuorum	0.476
Annona sp.	0.381
Egletes florida	0.381
Sida sp.	0.381
Stylosanthes sp.	0.238
Galactia jussiaeana	0.190
Ruellia geminiflora	0.095
Phaseolus vulgaris	0.048
Centrosema venosum	0.048

3.1.4. Arbuscular Mycorrhizae Colonization (AM)

The levels of arbuscular mycorrhizae root colonization found allowed us to establish two categories among the species: a group with a high percentage of AM colonization (≥ 50%) (Table **3**) and another with an intermediate percentage (30-50%) (Table **4**). The Fabaceous-leguminous *Desmodium* sp. shows the highest colonization percentage followed by *Hyptis suaveolens* of the Lamiaceae family, whereas the leguminous *Phaseolus vulgaris* also shows a high mycorrhizal colonization percentage (Table **3**).

Table 3. Plant species of the experimental area with a % AM root colonization of ≥ 50%.

Species With % AM Root Colonization ≥ 50%.	% AM Root Colonization
Desmodium sp.	79.0
Hyptis suaveolens	76.8
Phaseolus vulgaris	70.0
Trachypogon sp.	68.0
Hyptis sp.	63.3
Ruellia geminiflora	61.0
Annona sp.	60.0
Rynchospora cephalotes	60.0
Polygala glochidiata	57.9
Diodia teres	57.1
Stylosanthes	55.7
Borreria sp.	55.9
Indigosfera pascuorum	54.7
Galactia jussiaeana	54.0
Egletes florida	53.4
Paspalum sp.	53.3
Rynchospora barbata	51.0
Centrosema venosum	51.0

Within the Poaceae, *Trachypogon* sp. presents the higher degree of colonization (68%). In the second category (30-50% root colonization), dominate the legumes *Cassia cultrifolia* and *Mimosa pudica* (Table **4**).

Table 4. Plant species of the experimental area with a % AM root colonization of 30-50%.

Species With Intermediate % AM Root Colonization (30-50%)	% AM Root Colonization
Cassia cultrifolia	49.3
Mimosa pudica	48.8
Fimbristylis sp.	47.0
Panicum sp.	35.8
Sida sp.	32.0
Aeschynomene sp.	32.0

3.1.5. Rhizobial Symbiosis

Legumes represented a high proportion of the plant species collected in the experimental area (43.5%); most of them were individuals of *Cassia cultrifolia* and *Mimosa pudica*. In total 10 leguminous species were collected, from which, 50% showed the presence of nodules located in the lateral roots. Nodulated species were: *Indigosfera pascuorum* and *Stylosanthes sp.* with the highest number of nodules followed by *Cassia cultrifolia* and *Desmodium intortuo* and *Mimosa pudica*. 50% of the legumes collected presented double symbiosis, *Arbuscular mycorrhiza* and rhizobia (Fig. **2**).

3.1.6. Evaluation of Glomeromycota Fungi Spores Number Present in the Rhizosphere of Native Plants

Quantification of Glomeromycota spore number in the rhizospheric soil (soil around the root) was performed only in the plant species, which were detected in a higher frequency (> 0.50) and with % AM root colonization higher than 50%. The number of spores ranged from 100 to 1700 per 100 g soil (Fig. **3**).

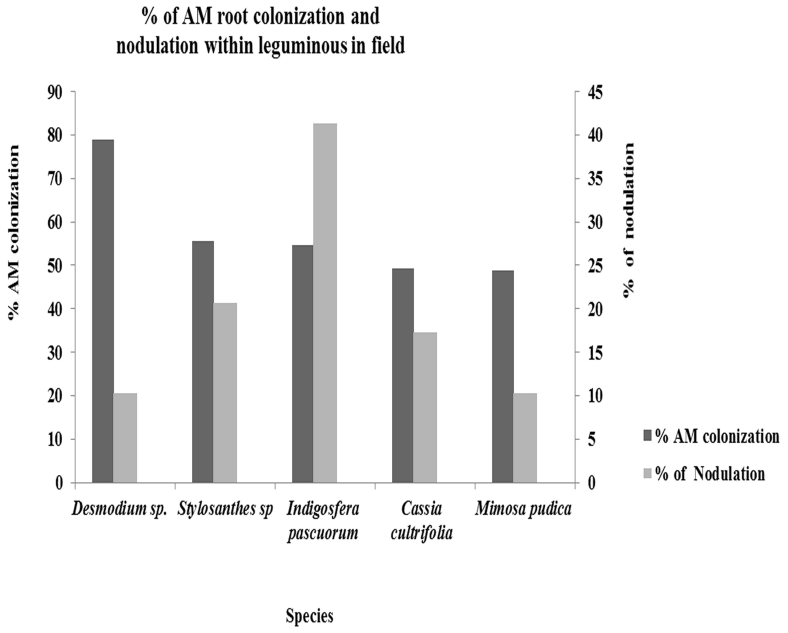

Fig. (2). Percentage of AM root colonization and nodulation within leguminous.

3.1.7. Phosphate Solubilizing Bacteria (PSB)

The isolation of PSB was done in a total of 25 rhizospheres corresponding to the most important plant species present in the experimental area. From those rhizospheres, 8 were positive with the presence of potential PSB and correspond to the following species: *Centrosema venosum, Galactia jussiaeana*, *Fimbristylis* sp., *Mimosa pudica*, *Ruellia geminiflora*, *Aeschynomene* sp., *Trachypogon* sp. and *Indigosphera pascuorum*. When analysing the total amount of colony forming units per Petri dish with respect to the PSB we found that *Centrosema venosum* and *Galactia jussiaeana* present a high proportion of PSB (75% and 43%, respectively).

4. DISCUSSION

4.1. N-Fixation and Rhizobium-Legume Symbiosis

African and South American savannas are characterized by their great diversity of herbaceous and woody leguminous species and the proportion of leguminous tends to increase under moderate and over grazing [6]. In Orinoco´s savannas, few studies have done to document nitrogen fixation by native legumes under natural conditions, although evidence through natural abundance of ^{15}N and relative abundance of ureids suggest N-fixation for a few species [38, 39].

In the experimental area at ELLI the legumes represented almost half of the plant species collected that account for a good N-fixing plant presence; most of them were individuals of *Cassia cultrifolia* and *Mimosa pudica*. In total, ten leguminous were collected, from which, 50% showed the presence of nodules; Aristeguieta [40] reported also that Poaceae and Leguminosae were the more abundant families in a savanna located in Central, Venezuela with a proportion of 27 and 26%, respectively. Thus, nitrogen fixation by different mechanisms existing in savannas appears as an option to supply N to this ecosystem [6,7,41,42]. Consequently, under natural conditions the studied savanna presents a microbial community which might be metabolically adapted to different mechanisms able to profit from the scarce sources of N and P [2,3,15,43].

Although, 50% of the legumes presented nodules and AM (Fig. **2**), the lower nodulation reported, may be a consequence of the acidity and low fertility of the ultisols since rhizobia do not growth efficiently in acid soils [6,7], whereas, on the contrary, mycorrhiza are more adapted to those environments [11, 43 - 45]. Moreover, nodulation was lower, even though sampling was performed at the peak of the rainy season (July), it is well established that nodulation and nodule numbers in savannas are favoured during the wet season [13,46].

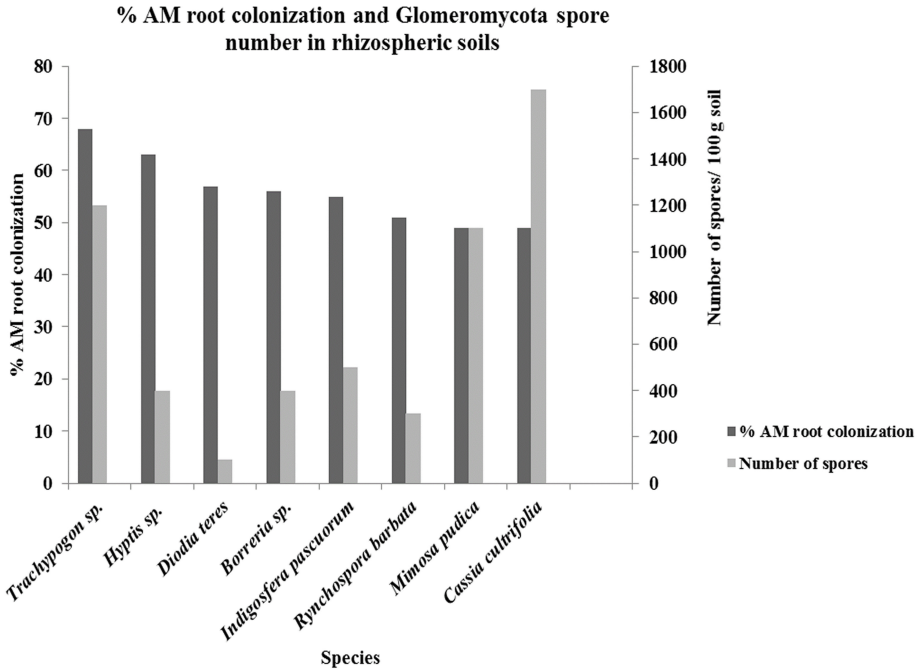

Fig. (3). AM root colonization and glomeromycota spore number in rhizosperic soil's of the native species.

4.2. P-Uptake and Mycorrhizal Associations

Concerning the parameters related to mycorrhizal association, AM associations are relevant in this savanna soil, since the native plants present a high symbiosis affinity. Moreover, as expected, a good AM colonization was found in an important number of collected plants. As low fertility is reported for this soil by classical standard chemical methods [47, 48], the presence of phosphate solubilizer organisms and rhizobia are considered as good fertility indicators, which, in turn, can be considered an indication of "good soil quality". In addition, the number of Glomeromycota fungi spores (100-1700 in 100 g soil) reported for the experimental area can be considered high for soils under natural conditions. In

fact, López-Gutiérrez *et al.* [3] reported values between 80 and 290 spores in 100 g soil in a nearby area, whereas Lovera and Cuenca [49] presented 120 spores in 100 g soil during the dry season in a natural savanna located in Gran Sabana, Venezuela. Similar low results (0-196 in 100 g soil) have been presented by Collins *et al.* [50] in an evaluation of AM in soybean and maize, and by Douds *et al.* [51] for cropping systems (wheat, soybean and maize) under different tillage managements. However, a higher value was also reported (1000 spores in 100 g dry soil) by Howeler *et al.* [52] for introduced *Brachiaria decumbens*. In conclusion, although under controlled conditions spore populations are high, the results presented in this survey indicate also a good establishment of Glomeromycota fungal populations in savanna under acid conditions.

In some cases, a good correlation has been reported between the number of spores of Glomeromycota fungi in the rhizosphere and the percentage of colonized root length [52 - 54]. In the experiments here presented such association was found only in the case of the *Trachypogon sp.*, which showed a high % CRL and also an important spore number (Fig. **3** and Table **3**), no doubt those traits account for the remarkable adaptation of this species in the unfertile Orinoco's savannas [2,3,5]. The density of spores depends on climatic conditions, on the physiology of the plant and the phosphorus availability at the moment of collection [55, 56].

The number of AM infective propagules measured was of 4571 in 100 g of dry soil (intervals 2141 – 9765 at 95% confidence). These values were six times higher than those reported for other natural savannas located near the experimental area [57]; however they are similar to the values presented by Toro and Sieverding [58] in Colombian savannas under management, which favor AM potentiality, those results suggest that the studied savanna has enough AM propagules to colonize native plant species. In addition, the presence of potential PSB and high root colonization by AM in this savanna soil suggest that a synergic effect might work in the plant uptake of phosphorus, as previously reported by Barea *et al.* [59]. If that constitutes an important nutritional mechanism of biological origin in this dystrophic soil [5, 60, 61], it deserves further research. Moreover, we have found that the rhizosphere of *Centrosema venosum* and *Galactia jussiaeana* presents a high proportion of PSB, specifically for *Burkholderia cepacia*, which constitutes an important material to look for potential biofertilizers; an information that is presented in a forthcoming publication [62].

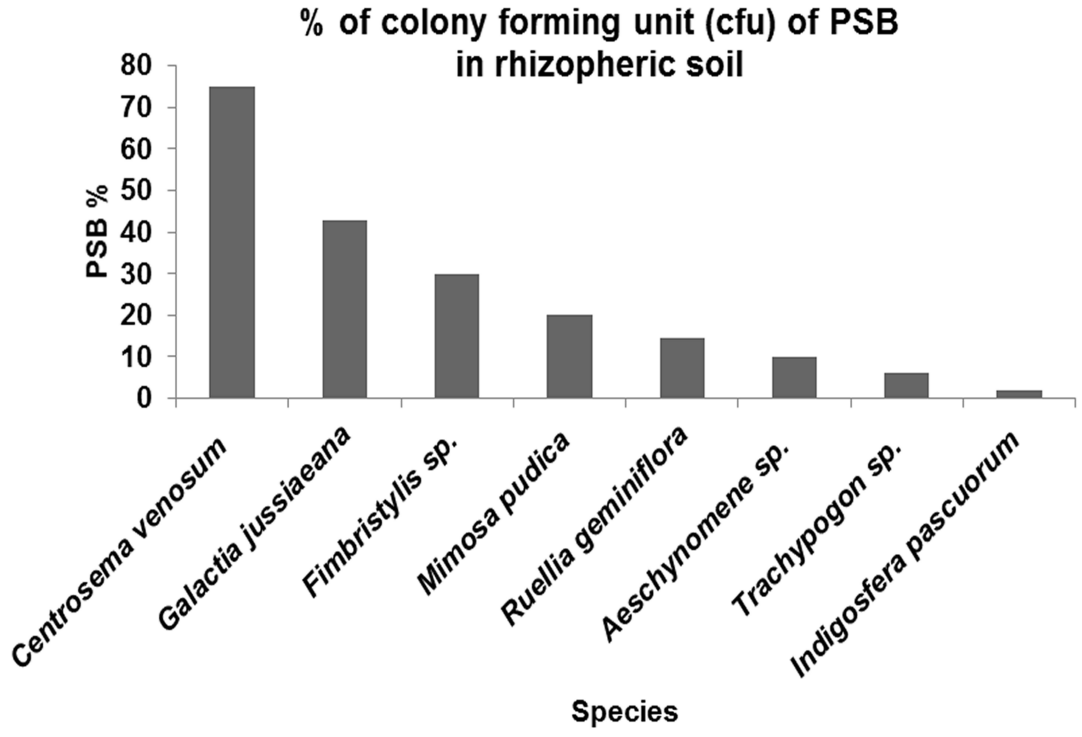

Fig. (4). Percentage of colony forming unit (cfu) of solubilizing bacteria respect to total bacterial counts (cfu ToB) in rhizopheric soil of field plants.

CONCLUSION

In a descriptive study of the soil microbial community in a typical Trachypogon savanna located in Central Llanos, Venezuela, we have found that AM associations are relevant in this savanna soil, since plants present a high AM symbiosis affinity. The isolation of PSB was performed in a total of 25 rhizospheres of the plant species present in the experimental area. From those rhizospheres, 8 were positive to the presence of potential PSB. When analyzing the total amount of bacteria colony forming units respect the PSB we found that the rhizosphere of *Centrosema venosum* and *Galactia jussiaeana* present a high proportion of PSB, therefore the presence of PSB in the rhizosphere of those species constitutes an important material to look for potential biofertilizers.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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