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Endophytic Bacteria Isolated from Angleton pasture (*Dichantium aristatum*, Benth) in Sucre Department, Colombia

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Abstract

Objective: This study evaluated *in vitro* the growth promotion activity of endophytic bacteria extracted from different tissues of *Dichantium aristatum* from livestock farms localized in the zone of Santiago de Tolú, Sucre Department, Colombia. **Methods/Statistical Analysis**: Endophytic bacteria from different tissues were isolated, and the population was determined in CFU/g of tissue. Each isolated morphotype was used to quantitatively and qualitatively evaluate the BNF, PS, siderophore and indole acetic acid production *in vitro*. The bacteria that showed this activity were sequenced for identification for sequencing of the 16S rDNA gene for eubacteria. 62 morphotypes of endophyte bacteria from different Angleton pasture tissues were isolated, of which 28 had siderophore production, five ammonium ion production, 12 soluble phosphates and six indole acetic acid in mg/L. The population of these bacteria varied, from $3.210 \times 10^6 \pm 1.2 \times 10^8$ CFU/g of tissue, with higher quantity found in the Angleton pasture roots. **Findings**: The results of the sequencing showed the presence of a high percentage of similarity with the bacterial species *Serratia marcescens*, *Enterobacter cloacae* and *Bacillus cereus*, presenting phosphate solubilization, reduction of N_2 to ammonium, and production of indole acetic acid and siderophores. **Application**: The findings of this study constitute important alternatives to mitigate the impact of the continuous use of pesticides in the production agricultural; the use of endophytic bacteria associated with vegetable species with the ability to produce multiple benefits has gained attention. Studies have shown the goodnesses of plant growth promoting-bacteria as an alternative for obtaining biofertilizers, with the ability to stimulate the growth and productivity of plants, making it are possible to improve the yield of crops.

Keywords: Biological Nitrogen Fixation, Indole Acetic Acid, Pasture, Phosphate Solubilization, Siderophore

1. Introduction

In the department of Sucre, 84.9% of its territory is dedicated as a main activity, for the dual-purpose livestock. The source of bovine feeding in the Department of Sucre is the grazing of native and introduced grasses and legumes, meaning the animal productivity per surface unit is determined by the nutritional value and production of the fodder that is offered. Among the introduced species, Angleton grass (*Dichantium aristatum*, Benth) ranks third for the largest planted area in the Department of Sucre with approximately 56,200 hectares distributed in 19 municipalities. The municipality of Santiago de Tolú, whose main economic activity is semi-intensive livestock, has the second largest planted area for this species in the department with 9,400 ha [1,2].

Currently, all of the pastures suffer from a series of problems, as described by Cajas-Girón et al.[3]: the production and nutritional quality of the fodder decrease dramatically during the long periods of drought that occur each year in the Caribbean region, which means that livestock systems have low biological and economic

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efficiency, along with an advanced state of soil degradation that is common in most grazing areas. To increase production of the crop, the use of chemical fertilizers seems to be the only alternative, however the continuous use of these chemical compounds has led to an imbalance of the native microbiota that plays an important functional role, causing low yields and increase in production costs for the farmer, so it is proposed as a new alternative, as microbial biofertilizers[4].

In recent years, several studies have indicated that endophytic bacteria associated with plant tissues play an important role in plant nutrition. These bacteria are defined as those microorganisms that reside within the tissues of plants, do not cause any symptoms of disease in their hosts[5], and have properties that promote the growth of plants, remove contaminants, solubilize phosphate and fix nitrogen[6], along with the control of phytopathogens. Studies conducted by Pérez-Cordero et al.[7], on the *in vitro* activity of nitrogen-fixing endophytic bacteria and phosphate solubilizers in colossal grass in the Colombian Caribbean showed that the endophytic bacteria A*eromonas salmonicida* and *Pasteurella pneumotropica* have the ability to simultaneously solubilize phosphates and biologically fix nitrogen.

This study carried out an *in vitro* evaluation of the ability of the endophyte bacteria isolated from Angleton grass to biologically fix nitrogen, dissolve phosphates, produce indole acetic acid and siderophores and become a biological alternative for future use as a biofertilizer in pastures in the Colombian Caribbean.

2. Materials and Methods

2.1 Sampling

The sampling was carried out on cattle farms planted only with Angleton grass in the municipality of Santiago de Tolú, located in the gulf of Morrosquillo, belonging of the Sucre Department with 35,750 hectares and 45 km of coastline, maximum altitude of 10 meters above sea level. The area corresponds to a tropical dry forest[8], with a dry, warm climate and soils made up of alluvial sediments, marine sediments or combinations of the two. In some areas, there are organic sediments.

The samples were collected between June to December 2016. The sampling was random in zigzag, collecting Angleton pasture plants with the roots, stems and leaves. The samples were labeled with the date of collection, farm and village and were stored and preserved for transport to the microbiological research laboratory of the Universidad de Sucre for the respective microbiological analyzes.

2.2 Isolation of Endophytic Bacteria

The isolation of plant endophytes bacteria was carried out in the following stages:

2.2.1 Surface Disinfection of Tissues

The tissues of each pasture collected were separated by tissues and subjected to the superficial disinfection process protocols described by Pérez-Cordero[9], this process was repeated two times and fitter the disinfection process, the samples were prepared for the isolation and counting of the endophytic bacteria.

2.2.2 Determination of Population Density

After the disinfection process, each tissue was placed on a porcelain plate and macerated with liquid nitrogen until homogenized. The macerate was transferred to a tube with 9 ml of peptonated water and stirred for 24 hours at 30°C. Serial (10^{-1} to 10^{-8}) dilutions were prepared from each sample in triplicate, from which aliquots were taken and deposited on the surface of R2A agar and incubated at 30°C during four days. The population density of bacteria per tissue (CFU/g of tissue) was determined with a direct count of the colonies on the plates. During the count, colonies that differed in shape, texture, color and size were observed and selected[7,10].

2.3 In vitro Evaluation of the Promoting Growth Activity of the Endophytic Bacteria

An each of the morphotypes of endophytic bacteria obtained from the different Angleton pasture tissues were used for the *in vitro* evaluation of the growth promoting activity, well as described below:

2.3.1 Nitrogen to Ammonium Ion Reduction

The qualitative evaluation of the nitrogen biological fixation was carried out with direct seeding of each morphotype on the surface of the Burk's selective agar medium (5 gr mgso$_4$, 20 gr KH_2PO_4, 5 gr K_2HPO_4, 3.25 gr $CaSo_4$, 1.45 gr $FeCl_3$, 0.253 gr NaMoO4, and 1000 ml of sterile distilled water[11], which lacked a nitrogen source, to determine the nitrogen-fixing activity[7].

The quantity of the nitrogen fixed was determined with indirect evaluation of the reduced nitrogen quantity in the form of ammonium ion using the Berthelot colorimetric method. For this test, the isolates were inoculated in the Burk's medium at 30°C during four days at 150 rpm. Afterwards, a KCl medium was added to 2m, followed by incubation a second time under the same conditions for 1 hour. Then, 10 ml of supernatant were taken and stirred in centrifuge at 8000 rpm for 20 min. An alcoholic solution was added, 10% phenol, 0.5% sodium nitroprusside and 1 ml of oxidant solution (20g of citrate sodium, 1 g of sodium hydroxide and 1 ml of 1.5n sodium hypochlorite in 100ml of H_2O). The mixture and the sample were incubated for 1 hour, and the results were analyzed by reading absorbance at 632 nm in a Genesys 10S UV-V is spectrophotometer. The standard curve was standardized by the biotechnology laboratory of the Universidad de Córdoba-Colombia[12]. Each isolate was evaluated in triplicate.

2.3.2 Phosphate Solubilization

The qualitative evaluation of the phosphate solubilization was carried out with direct seeding of each morphotype on the surface of the NBRIP agar medium (10 gr glucose, 5 gr $CA_3(PO_4)2$, 5 gr $MgCl_2 * 6H_2O$, 0.25 gr $MgsSO_4 * 7H_2O$, 0.2 gr KCl, (NH_4) SO_4 in 1000 ml of distilled water) following the protocol proposed by[7]. The isolates that showed positive activity through halo production were used to determine the capacity of the bacteria to solubilize phosphate in the hybrid culture medium using indirect measurement of the dissolved phosphate[12] according to the protocol proposed by Rodríguez et al.[13]. The data were analyzed using the calibration curve standardized in the biotechnology laboratory of the Universidad de Córdoba–Colombia by Lara et al.[14].

2.3.3 Production of 3-indole Acetic Acid (IAA)

The evaluation of the production of 3- indole acetic acid (IAA) was carried out in a liquid Burk's medium (0.41 gr KH_2PO_4, 0.52 gr K_2HPO_4, 0.05 gr Na_2SO_4, 0.2 gr $CaCl_2$, 0.1 $MgSO_4 * 7H_2O$, 0.01 gr $FeSO_4 * 7H_2O$, 0.0025 gr $NaMoO_4$, in 1000 ml of distilled water), supplemented with 0.1 g of tryptophan (precursor of indole acetic acid) with the techniques proposed by Dawwam et al.[15], Gordon and Weber[16]. For the quantitative determination, the curve standardized with standard solutions of pure 3-indole acetic acid in the biotechnology laboratory of the Universidad de Córdoba-Colombia, by Lara et al.[14] was used.

2.3.4 Siderophores Obtaining

The evaluation of the obtaining of side rophores was carried out with direct seeding of each morphotype on the surface of the chrome azurol S medium, as proposed by Schwyn and Neilands[17].

2.4 Molecular Identification of Endophytic Bacteria with Positive Growth Promotion Activity

Bacterial isolates with positive activity for growth promotion: Biological nitrogen fixation, phosphate solubilization, 3-indole acetic acid and siderophore production, were purified and identified by gram stain. The extraction of genomic DNA from mercury-resistant endophytic bacteria was carried out using the protocol proposed by Oliveira et al.[10]. For amplification of the 16R ARNR gene, three sets of specific primers were used: fbls342 and r1392 for the firmicute group; f948β and r1492 (5 'tacgg (c / t) for the beta-proteobacteria group; and fd2 and rp1, for the gamma-proteobacteria group. The conditions used in each amplification reaction[9] were based on the protocol described by Oliveira et al.[10], using a mastercycler nexus eppendorf thermal cycler. The products obtained from the amplification were purified and sequenced in Macrogen. The sequences were compared with those stored in the GenBank. The base alignment was done with the Crustal software, and the analysis and correction were done with the Mega 6.0 program[18]. The phylogenetic inferences that were evaluated were determined using the same program.

2.5 Statistical Analysis

To assess the amount of endophytic bacteria in relation to the area and tissue, a two-way multivariate analysis of variance was carried out. The multiple range tests were used to establish significant differences between means with the Bonferroni method at 95% confidence. The data were analyzed using the R environment for statistical computing[19].

3. Results and Discussion

A total of 35 cattle ranches planted with Angleton pasture were sampled, distributed in seven townships belonging to the municipality of Santiago de Tolú in the department of Sucre, Colombia. A total of 62 isolated of endophytic bacteria from different tissues of Angleton pasture were obtained. The population density of these bacteria varied, starting from $3.210 \times 106 \pm 1.2 \times 108$ CFU/g of tissue. The results of the multifactorial analysis between the population density of the areas and tissues showed highly significant differences for each variable. The averages of the endophytic bacteria by area showed that the greatest presence of these bacteria was found in the old port area, and the smallest one was found in the municipal seat (Figure 1). Figure 2 shows that the highest amount of these bacteria was found in the roots of the angletón pasture in the 7 townships (6.4×10^8; 4.17×10^8, 2.12×10^8, 3.4×10^8, 1.8×10^8, 1.4×10^8, 1.2×10^8 and 8.2×10^7 CFU/g of tissue for the municipalities of Puerto Viejo, Santa Lucia, Coveñas, Nueva Era, Pita Arriba, Pita Abajo, and the municipal seat, respectively), followed by the stem tissue and the leaf tissue.

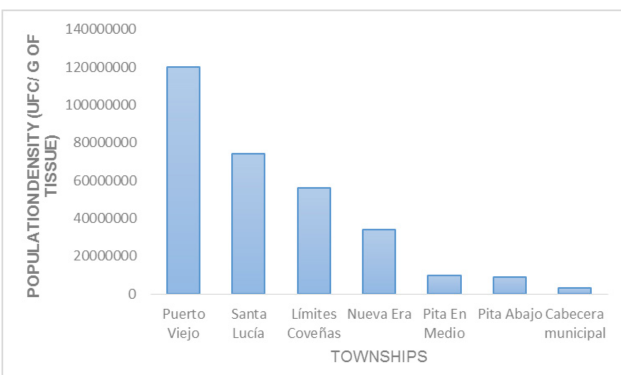

Figure 1. Density of endophytic bacteria isolated from Angleton pasture for townships in the municipality of Santiago de Tolú, Department of Sucre, Colombia.

Studies on the diversity of endophytic bacteria associated with Angleton pasture in the department of Sucre, Colombia are scarce. There is no specialized literature based on data for the presence of these bacteria in gulf of the Morrosquillo; however, the average values of the amount of the endophytic bacteria found in the present study are lower than those found in research carried out on colosoana grass (*Bothrichloa pertusa* (L.) A. Camus) in the Department of Sucre by[9]. The results of this study

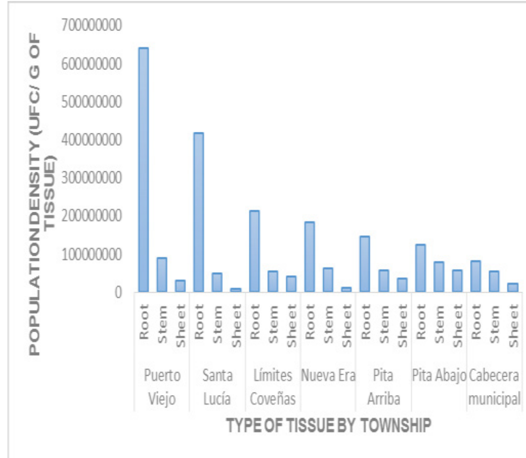

Figure 2. Density Averages of the endophytic bacteria from the Angleton pasture tissue in the sampled townships. Municipality of Santiago de Tolú, Department of Sucre, Colombia, 2016.

determined average amount of $3.21 \times 10^6 \pm 1.2 \times 10^8$ CFU/g of tissue, while the study on colosoana pasture had averages of $3.24 \times 10^9 \pm 2.2 \times 10^{10}$ CFU/g of tissue.

The difference in the average value of the amount of the endophytic bacteria was possibly due to the fact that, the colonization of endophytic bacteria varies with the amount of inoculum and the species[20], the environmental conditions of the study area, the type of colonized plant tissue[21] the species and its different cultivation methods, the interactions of the plants with other beneficial microorganisms[22] and the colonization process used by the endophytic microorganism.

3.1 In vitro Evaluation of Growth Promoting Capacity

Of the 62 Angleton grass morphotypes, 28 showed a growth promoting capacity: Biological nitrogen fixation, phosphate solubilization, 3-indole acetic acid production and siderophore production (Table 1), equally shows the distribution of the number of morphotypes by area and by growth promoting activity.

The results reported in Table 1 indicate that the highest presence of morphotypes with capacities to biological nitrogen fixation (12), followed by production of 3-indole acetic acid (6); meanwhile, the phosphate solubilization and production of siderophores presented the same number (5). Likewise, the results show that Puerto Viejo had the largest number of morphotypes with growth promoting activity.

Table 1. Number of morphotypes with growth promoting activity of the endophyte bacteria isolated from Angleton pasture in seven townships of Santiago de Tolú, Department of Sucre, 2016

Townships	Biological nitrogen fixation	Phosphate solubilization	3-indole acetic acid production	Siderophores	Total
Puerto Viejo	4	2	1	1	8
Santa Lucía	2		2	1	5
Límites Coveñas	2	1			3
Nueva Era	1		1	1	3
Pita En Medio	1	2			3
Pita Abajo	1		1	1	3
Cabecera municipal	1		1	1	3
Total	12	5	6	5	

Figure 3 shows the qualitative *in vitro* evaluation of the biological nitrogen fixation, phosphate solubilization and siderophore production of the endophytes bacteria isolated from Angleton pasture.

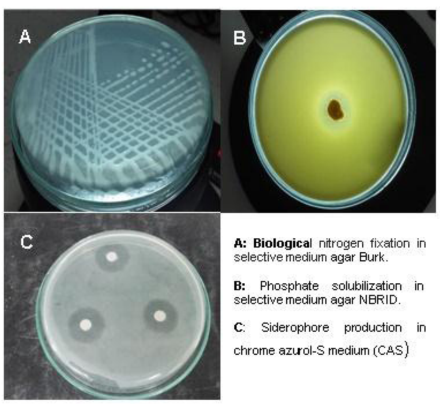

Figure 3. Qualitative *in vitro* evaluation of the activities biological nitrogen fixation, solubilization of phosphates and production of siderophores in the endophytic bacteria.

The efficiency of the biological nitrogen fixation for the production of ammonium ions was determined with the Berthelot method, which consists of a change in color to an intense blue resulting from phenol-idol because of the reaction produced between the ammonium ion and phenolic compounds in the presence of an oxidizing agent. Figure 4A shows that the highest production of ammonium ions occurred in the morphotype M4HPV (morphotype 4, from the leaf, isolated from the Puerto Viejo Township) with a value of 2.8 mg / L.

For the nitrogen efficiency of the endophyte bacteria isolated from Angleton pasture, it was found that 12 morphotypes had an ammonium ion production capacity in concentrations of 0.2 to 2.8 mg/L. Similar studies on Angleton pasture have not been carried out to date, but, according to Mantilla et al.[12], symbiotic biological nitrogen fixing bacteria from the agricultural zone of San Carlos, Córdoba, Colombia, provided 14 isolates that produced concentrations of 0.9 to 5.2 mg/L ammonium ion. In the Azotobacter group, A16PG and A26M1P produced 5.1545 and 5.1743 mg/L ammonium, respectively, and, in the Azospirillum group, A5M1G produced 4.6741 mg/L ammonium ion. The biological nitrogen fixation (BNF) of rhizospheric bacteria has contributed to increased yields in crops, reducing the quantity for nitrogen fertilizers and the emission of toxic gases such as N_2O, obtaining economic and environmental benefits for farms.

For the phosphate production efficiency, five morphotypes had this capacity *in vitro* in a range of 685 (pH: 6.5) to 1590 mg/L (pH: 3.1) (Figure 4B). The results found in the present study show a decrease in pH as the concentration of soluble phosphate increased in the culture medium. In the test, the initial measurement of the pH (7.0) of the NBRIP medium was carried out before the inoculation with the tested isolates morphotype, and, at the end of the experiment, the initial pH value was compared with the pH value at the end of the experiment, showing a decrease as the concentration of soluble phosphate was higher[23], who evaluated the efficiency of phosphate production by isolated endophytic bacteria from different rice varieties in the department of Córdoba, observed a decrease in pH values as soluble phosphate concentrations increased. The decrease in pH was possibly due to the direct relationship between the production of acids and the production of phosphate-like compounds. However, there is not always a decrease in pH values because not all bacterial species use the same mechanism to release phosphate and make it available for plants[24]. Phosphate solubilizing bacteria dissolve insoluble phosphate with the production of organic and inorganic acids and/or by decreasing pH; consequently, phosphate that can be captured by plants is produced[25–27].

Figure 4C shows IAA production values of 3.1 to 11.2 mg/L for the 6 evaluated morphotypes. As reported by Lara[28], although research on the evaluation of IAA production show high and low values, it has been demonstrated that low concentrations of phytohormone are able to stimulate plant development, and high concentrations inhibit and reduce the area of elongation. Since native microorganisms are adapted to their own conditions and environments, the appropriate dose and the effect exerted on crops can only be verified by performing *in vitro* bioassays. The auxin indole acetic acid (IAA) induces the deformation and increase of root hairs, achieving greater uptake of nutrients and promoting growth and yield in crops. It has been established that the plant growth promoting rhizobacteria are important in crops production, allowing the use of chemical fertilizers to be decreased, increasing yields, shortening cycles and, consequently, reducing environmental pollution[29].

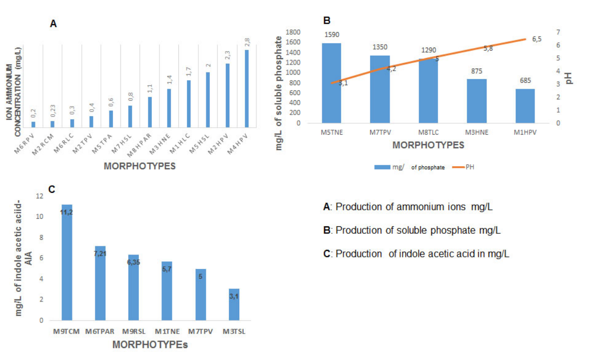

Figure 4. Production of ammonium ions (mg /L), soluble phosphate and 3-indole acetic acid in (mg/L) of the morphotypes of the endophytic bacteria isolated from Angleton pasture in the municipality of Santiago de Tolú, Department of Sucre, Colombia, 2017. M: morphotypes; R: root; T: stem; H: leaf; CM: Municipal seat; LM: Coveñas; NE: Nueva Era; PAR: Pita Arriba, PR: Pita Abajo; PV: Puerto Viejo and SL: Santa Lucia.

A total of five morphotypes showed a qualitative capacity of siderophores in the chrome azurol S medium (CAS) (Figure 3C). Endophytic bacteria have several indirect mechanisms for the promotion of plant growth. The main mechanisms involved are the production of antimicrobial substances and lytic enzymes, the induction of systemic resistance, competition for nutrients and saturation of ecological niches[30]. Mechanisms for competing for nutrients include the production of siderophores. Siderophores produced by bacteria in environments with iron limitations can act in two ways: first, by sequestering iron to make it unavailable for pathogenic microbial communities (considered a biocontrol mechanism) and, secondly, by providing Fe-siderophore complexes to plants that are capable of acquiring it[31].

3.2 Identification of Growth Promoting Endophytic Bacteria

For the identification by molecular sequence of the endophytic bacteria with positive activity for growth promotion, those morphotypes that showed more than one simultaneous activity were analyzed. The phylogenetic analysis of the 16S rDNA gene of endophytic growth-promoting bacteria associated with Angleton pasture is shown in Figure 5.

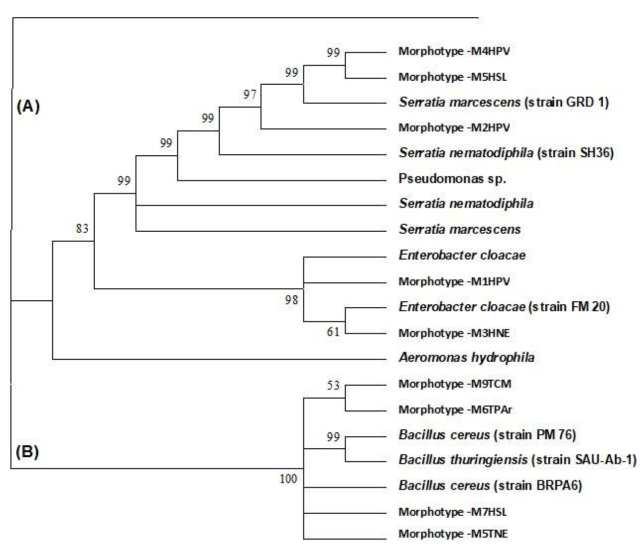

Figure 5. Maximum phylogenetic tree parsimony of (A) gamma-proteobacteria and (B) Firmicutes sequences of 16S rDNA genes from endophytic growth-promoting bacteria of Angleton pasture.

M: morphotypes; 1-7: morphotype identification; T: stem; H: leaf; CM: Municipal seat; NE: Nueva Era; PV: Puerto Viejo; PAr: Pita Arriba; SL: Saint Lucia; +: positive activity; –: negative activity. BNF: biological nitrogen fixation of nitrogen; IAA: indole acetic acid.

The phylogenetic analysis showed a probability of 99% for the morphotypes MAHP and M5HSL and 97% for M2HPV, similar to the species *Serratia marcescens* (GRD 1 strain) (Figure 4). *S. marcescens* is considered a potential pathogenic bacterium for humans and animals. However, other studies have indicated that Serratia spp. species have been isolated from cotton crops, sweet corn[32], and rhizosphere[33] and rice seeds[34]. Non-pathogenic isolates of *S. marcescens* have been used as biological control agents in agricultural crops[33–35] and the ability to induce systemic resistance in vegetal[36,37], reported *S. marcescens* as an endophytic bacterium from the roots and stems of rice varieties with the capacity to fix nitrogen. The results of the *in vitro* test sample (Table 2) show that *S. marcescens* has the ability to fix nitrogen (M4HPV, M5HSL, M2HPV) solubilize phosphates and produce IAA (M4HPV) and siderophores (M5HSL, M2HPV).

The M1HPV morphotype showed 98% homology with sequences with the *Enterobacter cloaceae* bacterium species. *E. cloacae* were initially identified as a phytopathogenic bacterium of maize plants and, later, as endophyte bacteria of rice plants[38]. Other studies have identified it as an endophytic bacterium in rice crops with the capacity to promote growth through the production of Indole-3-pyruvate descarboxylase genes[39]. Work done by Hardoim[40] showed that *an E. cloaca is* an endophytic bacterium of rice plants with growth promoting activity. On the other hand[41] reported *E. cloacae* as an obligate endophytic bacterium associated with mature pollen grains of various pine species in the Mediterranean, with the ability to produce the indole-3-acetic acid-3 experimentally. According to the results shown in Table 2, the morphotypes identified as M1HPV and M1HNE, identified as *Enterobacter cloacae* bacteria, have the ability to produce siderophores and solubilize phosphate; the latter also has the ability to biological fix nitrogen.

Finally, the M7HSL and M5TNE morphotypes, with 100% similarity to the *Bacillus cereus* bacterium species (strain BRPA6), showed capacity to biological nitrogen fixation, produce siderophores (M7HSL) and IAA, and solubilize phosphate (M5TNE). Recent studies carried out by Baoyu et al.[42] reported *Bacillus cereus* as an endophytic bacterium associated with roots of tomato plants with functional potential as biological control agents and the production of siderophores for the iron acquisition and activities to promote growth by fixing nitrogen in culture mediums for these plants.

4. Conclusion

In this study, 62 isolated of endophytic bacteria from different tissues of Angleton pasture cultivated on livestock farms in the municipality of Santiago de Tolú, department of Sucre, Colombia, were isolated. A total of 28 morphotypes showed a capacity for siderophore production (28/5), 12 for ammonium ion production (28/12), five for phosphate solubilization (28/5) and six for indole acetic acid production (28/6) in mg/L. Angleton pasture is the second largest pasture fodder in the department of Sucre, which serves as an alternative animal feed on livestock farms.

Table 2. Morphotypes, sequence affiliation and growth promoting activity of endophytic bacteria isolated from Angleton pasture in the municipality of Santiago de Tolú, Department of Sucre, Colombia

Morphotypes	Group bacteria	Sequence affiliation (GenBank ID)	BNF	IAA	Sidephore	Soluble phosphate
M4HPV	Gamma proteobacteria	Serratia marcescens (strain GRD 1)	+	+	–	+
M5HSL			+	–	+	–
M2HPV			+	–	+	–
M1HPV		Enterobacter cloacae (strain FM 20)	–	–	+	+
M3HNE			+	–	+	+
M9TCM	Firmicutes	Bacillus sp	–	+	–	–
M6TPAr			–	+	+	–
M7HSL		Bacillus cereus (strain BRPA6)	+	–	+	–
M5TNE			–	+	+	+

However, the production and nutritional quality of fodder decreases during the long periods of drought that occur each year in the Caribbean region, which means that the livestock systems have low biological and economic efficiency, accompanied by an advanced state of degradation and low nutrients of the soils of the cattle farms, which resort to the continuous use of chemical compound to improve the productivity of these grass species.

The results of the sequencing showed a high percentage of similarity with the endophytic bacterial species *Serratia marcescens*, *Enterobacter cloacae* and *Bacillus cereus*, which, according to reports from several studies, have the capacity to promote plant growth. In the experiment carried out in the present study, it was confirmed that these endophytic bacterial species produced siderophores, ammonium ions, solubilize phosphates and produce indole acetic acid in mg/L, mechanisms used by bacteria to promote growth in the analyzed pasture species.

5. Acknowledgement

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