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Pseudomona Aeruginosa Isolated From Colosuana Grass (Bothriochloa Pertusa) With Nitrogen Fixing And Phosphate Solubilizing Activity

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ABSTRACT

The aim of this study was to evaluate in vitro the efficiency of endophytic bacteria isolated from different Colosuana grass tissues in the municipality of Corozal, department of Sucre, Colombia. To fulfil this purpose, endophytic bacteria were isolated from different Colosuana grass tissues and the population density was determined in CFU/g of tissue. The isolates obtained were used to evaluate in vitro the activities of biological nitrogen fixation (BNF), phosphate solubilization, siderophore production, indole-3-acetic acid (IAA, 3-AIA) and subsequent identification at the molecular level by amplification and sequence analysis of the 16S rDNA gene. The results show significant differences between the number of bacteria and tissues. The highest amounts of endophytic bacteria were found in roots (5.0 X 10^{10} 3.8 X 10^{10} 2.8 X 10^{10} 2.4 X 10^{10} and 1.5 X 10^{10} CFU/g root, for the villages of La Peñas, El Mamón, Cantagallo, Chapinero and Hato Nuevo, respectively) with respect to stem and leaf. A total of 30 morphotypes of endophytic bacteria were isolated, 11 of which showed in vitro nitrogen fixation capacity, N2 to ammonium reduction capacity, phosphate solubilization capacity, indole acetic acid and siderophore production. In conclusion, this work isolated endophytic bacteria from different tissues of Colosuana grass and tested in vitro, the ability to promote plant growth. The endophytic bacterial species identified as Pseudomona aeruginosa, showed excellent results for its direct and indirect growth promotion capabilities through phosphate solubilization and biological nitrogen fixation.

Keywords. Endophytic bacteria, Colosuana pasture, tissue, growth promotion.

Introduction

Livestock farming covers most of the territory in Colombia (Murgueitio, 2000), being a socio-economic line of great importance for the development of the country's countryside. For Colombia, it represents 1.7% of the National Gross Domestic Product (GDP), 20% of the agricultural GDP and 53% of the livestock GDP (Gaviria et al., 2012). Furthermore, its dual-purpose mode represents the main economic activity in the department of Sucre, where 94.9% of its territory is dedicated exclusively to cattle grazing (Gómez et al., 2010).

Colosuana grass (Bothriochloa pertusa (L.) A. Camus) is an introduced-naturalized species, and is found in different regions of Colombia such as the Andes, Caribbean Plain, Cauca Valley and Magdalena Valley (Giraldo-Cañas, 2013). In the Colombian Caribbean region it is widely distributed (Peters, Franco, Schmidt, & Hincapie, 2011), where it currently covers extensive areas of the tropical dry forest (bs-T) and very dry tropical forest (bms-T) life zones in the departments of Córdoba, Sucre, Bolívar and Magdalena (Pérez eta al., 2010). In the department of Sucre, this species covers a total of 274,005 ha, distributed in 19 municipalities (Aguilera, 2005); where the municipality with the largest planted area is Corozal with 32,223 ha (Viloria, 2003).

This pasture is considered as the forage species with the largest sown area in the department of Sucre and part of the Colombian Caribbean, but due to physiographic factors, anthropogenic actions degenerative of the environment and the use of inadequate technologies, have brought as a consequence, the degradation of the physical, chemical and biological properties of the soil, have limited the supply and quality of this pasture mainly in dry season (Pérez et al., 2010), due to the nutritional requirements of the plants and the nutritional exhaustion of the soil by intensive cultivation techniques, the constant use of chemical fertilizers is necessary to provide nutrients to the soil, which are necessary for the optimal development of the crop. However, despite the fact that the use of fertilizers has yielded excellent results in terms of production yields, it is inevitable to deny the environmental problems caused by their indiscriminate use, especially in developing countries such as Colombia.

Currently all pastures present a series of drawbacks, as described by Cajas-Girón et al. (2012): the production and nutritional quality of forage decreases dramatically during the long periods of drought that occur every year in the Caribbean region, which leads to low biological and economic efficiency of livestock systems, not to mention the advanced state of soil degradation that is common in most grazing areas.

Faced with this situation, the use of chemical fertilizers to overcome these difficulties seems to be an alternative, but the abuse of fertilizers to improve pasture productivity has caused an imbalance of the native microbiota that performs important functions within these, causing low yields and increased costs for the farmer and livestock farmer, so the use of microbial inoculants (biofertilizers) is proposed (Lara et al., 2011).

In addition, there are no studies in the department of Sucre that demonstrate the importance of endophytic bacteria as a biological alternative for the sustainable production of fodder, and in response to this situation, research has recently increased on the search for more environmentally friendly mechanisms that can efficiently metabolize nitrogen and phosphorus, converting them into components that can be assimilated by plants. In this search, one of the mechanisms found has been the discovery of micro-organisms, endophytes with the capacity to produce multiple benefits for higher plants. Studies in this respect have generated another alternative for plant fertilization, using plant growth-promoting bacteria to produce biofertilizers, with the capacity to stimulate plant growth and productivity, thereby improving crop yields, reducing production costs and being environmentally friendly; this symbiotic process is considered to be the most efficient way of fixing atmospheric nitrogen and solubilizing phosphate.

In recent years, several studies indicate that endophytic bacteria associated with plant tissues play an important role in plant nutrition. Bacteria are defined as those microorganisms that reside within plant tissues and do not cause any disease symptoms to their hosts (Zinniel et al., 2002), and have properties to promote plant growth, remove contaminants, solubilize phosphate and fix nitrogen (Rosenblueth and Martínez-Romero, 2006), as well as control phytopathogens. Studies conducted by Pérez et al., 2014, on the in vitro activity of nitrogen-fixing and phosphate-solubilizing endophytic bacteria associated with Colosoana grass in the Colombian Caribbean, identified the presence of Aeromonas salmonicida and Pasteurella pneumotropica as two species of endophytic bacteria with simultaneous phosphate-solubilizing and biological nitrogen-fixing capabilities. The aim of the present study was to isolate endophytic bacteria from different tissues of Colosuana grass and to evaluate their growth promoting activity.

MATERIALS AND METHOD

Sampling. Based on information provided by livestock farmers in the region, areas established only with Colosuana grass were selected for sampling (root, stem and leaves). The samples deposited in sterile bags, labelled and preserved, were transferred to the Microbiological Research Laboratory of the University of Sucre for their respective analysis.

Isolation of endophytes. Each sample (root, stem and leaves) was subjected to a surface disinfection process (Pérez et al., 2010). The process consisted of: two washes of the root in sterile distilled water, followed by shaking for 15 min in potassium phosphate buffer solution 0.05 $mol.L^{-1}$, pH 7. 0; immersion for 1 min in 70% alcohol; shaking for 5 min in 5% sodium hypochlorite solution and Tween 80 %; again immersion for 1 min in 70% alcohol followed by shaking for 15 min in potassium phosphate buffer solution 0.05 mol. L^{-1}, pH 7.0 and, finally, washing four times in sterile distilled water. The process was repeated twice.

For confirmation of the surface sterilization process of the root, stem and leaf tissues, the aliquot of the last wash was spread on a plate containing R2A agar medium and incubated at 28°C for 72 hours. Each separately sterilized tissue was then transferred to tubes containing R2A broth and incubated at 28°C for 72 hours to confirm the absence of epiphytic microorganisms on the surface of the tissues.

Bacterial count. To quantify the total number of endophytic bacteria, one g of each disinfected tissue was taken and crushed in liquid nitrogen. The homogeneous macerate obtained was transferred to a tube containing 9 mL of peptone water and shaken vigorously. From this solution, serial dilutions (10^{-1} to 10^{-8}) were prepared in triplicate and inoculated by surface seeding on R2A agar medium and incubated at 28°C for 72 hours. The population density of endophytic bacteria (CFU/g root-1) was estimated by direct colony counting on plates. During counting, colonies that were distinguishable in shape, surface appearance, color and size were observed and selected. Selected morphotypes were purified and preserved on R2A agar for further biochemical analysis.

Biological nitrogen fixation. For this technique, a direct sowing of the bacteria on the surface was carried out. A selective culture medium devoid of nitrogen (ASHBY medium) was used, because it uses a combined carbon source that allows a greater number of micro-organisms to be recovered and where only those that possess the enzymatic system that allows them to reduce atmospheric nitrogen and use it in their metabolism will grow.

Phosphate solubilization. Direct sowing of the bacteria on the surface was carried out. For the isolation of these microorganisms, the solid culture medium according to Sundara, Rao and Sinha (SRS) was used. This culture medium contains calcium phosphate salts and bromocresol purple as pH indicator. Approximately 6 days after sowing, bacterial colonies that grew acidifying the medium (turning from red to yellow) and forming a transparent halo, indicating phosphate solubilizing activity, were selected.

Identification of endophytic bacteria. Bacteria with positive activity for biological nitrogen fixation and phosphate solubilization were identified by API 20E biochemical test kit. The API20E test battery is a rapid identification system for bacteria of the Enterobacteriaceae family and other Gram-negative bacteria, consisting of 21 standardized and miniaturized biochemical tests and a database. This system has the advantages of being fast, efficient and allowing numerous tests to be performed at the same time.

Results and discussion.

Isolation and density of endophytic bacteria. A total of 51 morphotypes of endophytic bacteria were isolated from different tissues of Colosuana grass. The number of bacteria ranged from $2.45 \times 10^{8} \pm 4.48 \times 10^{10}$ of tissue. Studies by Perez et al., (2014) on population density of endophytic bacteria associated with Colosuana grass species in three localities in the department of Sucre, found average densities of 3.24 x 10^9 ± 2.2 x 10^{10} CFU/g per g of tissue for this grass species. The results of the multifactorial analysis between bacterial numbers as a function of zone and tissue show highly significant differences for each variable (Figure 1).

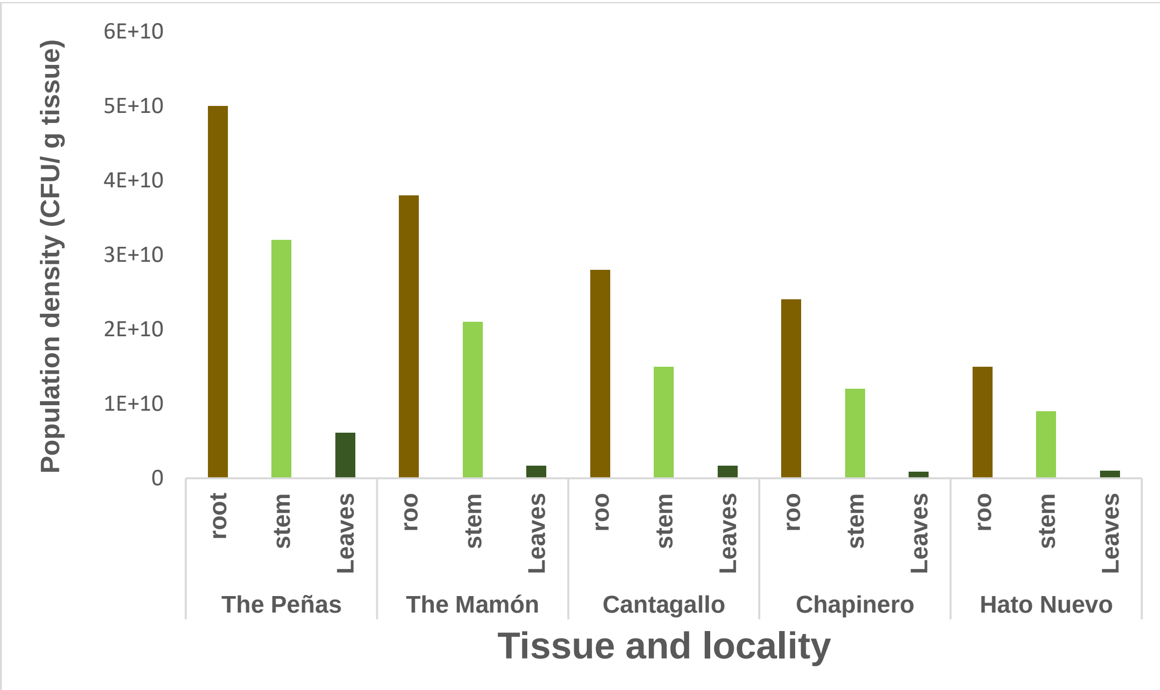

Figure 1. Quantity of endophytic bacteria in Colosuana grass tissues in some localities of the municipality of Corozal, Sucre, Colombia. 2022.

The multiple range test for population density of endophytic bacteria with respect to plant tissue shows significant differences, indicating higher population densities in roots ($1.0x10^{11}$ CFU/g tissue) and lower densities for stem and leaf respectively ($1.82x10^{10}$ and $1.15x10^{10}$ CFU/g tissue).

Nitrogen fixing activity. The isolates of endophytic bacteria showed positive activity for biological nitrogen fixation in vitro (figure 2). The highest presence of morphotypes was observed in roots, followed by stems and leaves. Figure 3 shows that the localities with the highest presence of nitrogen-fixing endophyte bacteria is Las Peñas, where they are more active in the different tissues than in the other localities.

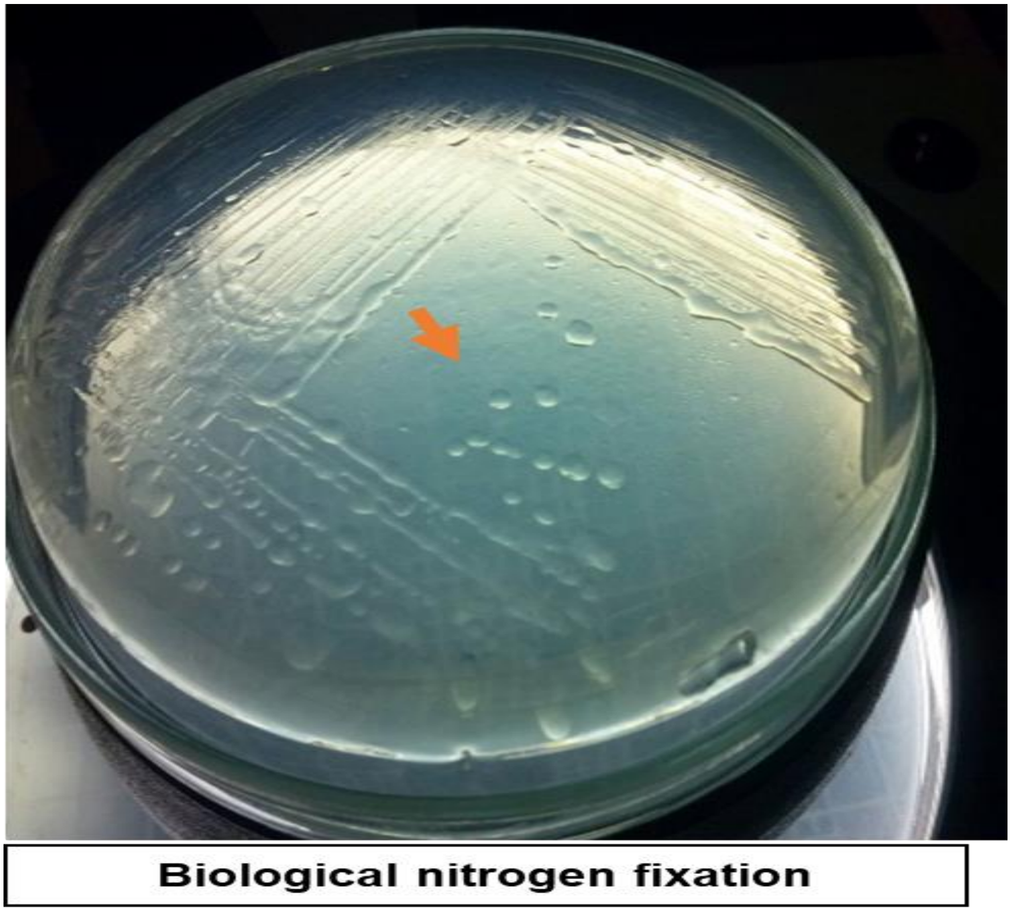

Figure 2. Biological nitrogen fixation activity in vitro of endophytic bacteria isolated from Colosuana grass (Bothriochloa pertusa) belonging to cattle farms in the municipality of Corozal, Sucre, Colombia 2022.

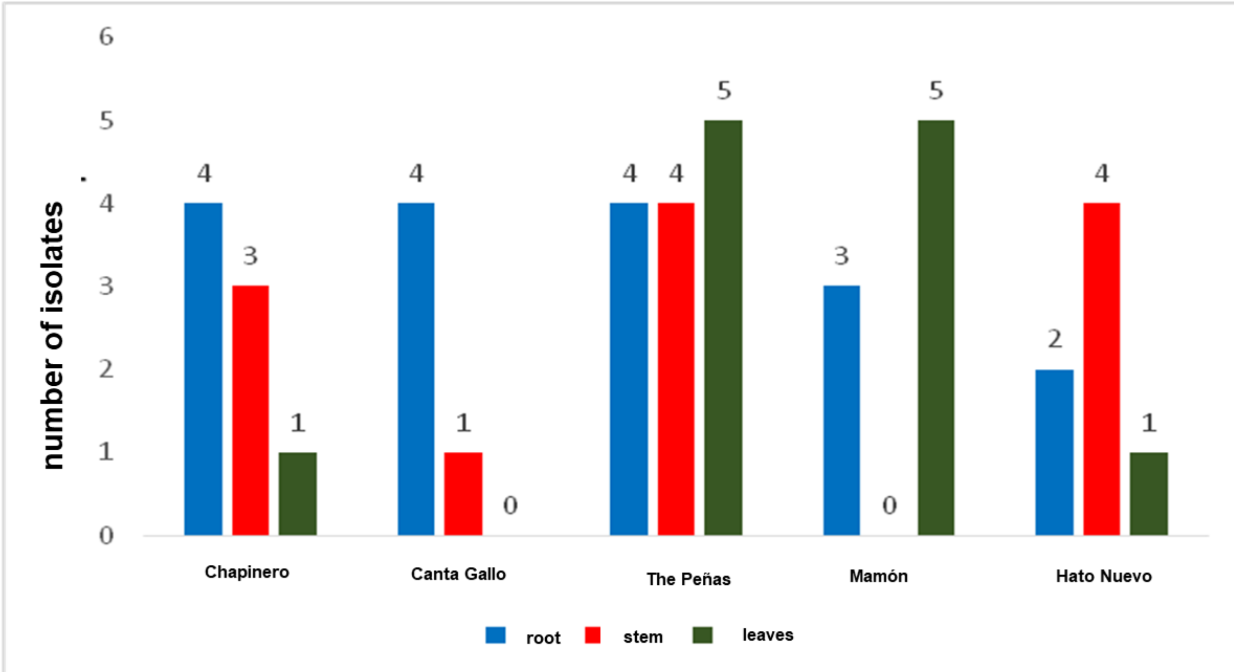

Figure 3. Endophytic bacteria with nitrogen-fixing activity according to the tissue in the different villages of the municipality of Corozal-Sucre.

Phosphate solubilizing activity. Figure 4 shows the phosphate solubilizing activity. For phosphate solubilization, 6 morphotypes were observed with this activity. Phosphorus is the second nutrient that limits plant growth and development. Phosphorus, although one of the main macronutrients essential for any living organism, is poorly available especially for plants, because most of the phosphorus is found in the soil in mineral form, which makes it insoluble and unavailable; only the soluble forms of this, make it available to both plants and microorganisms (Behera et al., 2014). One of the characteristics of endophytic bacteria is the ability to increase phosphate mobilization by developing mechanisms that allow them to capture the insoluble forms of phosphate, releasing it and making it available to the plant (Ryan et al., 2008). One of the mechanisms used by bacteria to solubilize phosphate is through organic acids. The vast majority of acids produced by these bacteria have been previously identified, including malic, oxalic, citrate and gluconate acids, among others, which form complexes with phosphates of aluminium, iron or in this case calcium, resulting in an increase in cations (H+), which reduces the pH, releasing available or soluble phosphate (Jones and Oburger, 2011).

Figure 4. Endophytic bacteria with Phosphate solubilizing activity according to the tissue in the different villages of the municipality of Corozal-Sucre.

Identification of endophytic bacteria. According to the API 20E identification system, 2 species and/or group of bacteria isolated following the described methodology were identified and showed positive activity for biological nitrogen fixation and phosphate solubilization. The identification of Pseudomona aeruginosa with growth promoting activities was confirmed.

Among the results obtained, the bacterium Pseudomona aeruginosa was identified, according to the literature this bacterium belongs to the Pseudomonadaceae family and is an aerobic gram-negative bacillus, it is a common microorganism in the environment and can be found in faces, soil, water and sewage. It can proliferate in aquatic environments, as well as on the surface of favorable organic matter in contact with water (DE Victorica and Galvan, 2001).

Based on the analysis, Pseudomona aeruginosa was identified as an endophytic bacterium isolated from Colosuana grass. The in vitro biological nitrogen fixation test showed that this bacterium, in addition to fixing nitrogen biologically, had the capacity to solubilize phosphate, and it was found that this activity was observed in morphotypes isolated from roots. In studies by Zhang et al., (2002), bacteria of the genus Pseudomona were found to be abundant on root surfaces as they are versatile in their metabolism. Their mechanisms of action include increased water and nutrient uptake by the plant, phosphate solubilization and production of plant growth regulators. Muleta et al., (2013), identified Pseudomonas sp genera in two climatic periods, drought and rain, and determined the presence of Pseudomona sp with the ability to solubilize phosphates in young sour guava plants (Psidium araca Swartz) and coffee plants growing in natural forests.

Loredo et al., (2004), reported bacteria of the genus Pseudomonas, Bacillus and Klebsiella as plant growth promoting bacteria on grasses. Pseudomonas are also an example of the resistance that can be acquired by the plant, as their participation in association with the plant increases not only the speed but also the levels of synthesis of phytoalexins, this group of heterogeneous compounds of low molecular weight, can be induced in the plant either by biotic or abiotic molecules, Some of these molecules correspond to glycoproteins and lipids found in the cell wall of some microorganisms such as bacteria or fungi that can release the action of an inducer such as endopolygalacturonase, which in turn leads to the activation of another inducer present in the cell wall of the plant (ANAYA, 2003).

In biofertilizers, microorganisms belonging to the general Azotobacter sp, Azospirillum sp, and Pseudomonas sp, have been shown to increase crop yields, save mineral fertilizers and reduce environmental pollution (MANTILLA and ZUMAQUE, 2008). For example, in a study carried out in wheat plantations using commercial biofertilizers with Azospirillum brasilence (Az1 and Az2) and Pseudomonas fluorecens (Pf), it was found that grain yields increased by 17, 14 and 19% with the Az1, Az2 and Pf treatments, respectively, compared to the controls without inoculation (Mantilla and Zumaque, 2008).

CONCLUSIONS

The highest population densities of endophytic bacteria were found in the village of Las Peñas in the municipality of Corozal-Sucre. A total of 30 endophytic bacteria were isolated from Colosuana grass, of which 11 bacteria showed activity in promoting plant growth through nitrogen fixation and phosphate solubilization tests. The bacteria identified as Pseudomona aeuroginosa showed growth promoting activities, indicating that these two bacterial species could be used as a biological alternative for nitrogen and phosphorus fertilization of pastures, which would increase nutritional content and thus provide better protein for the livestock sector. It is important to bear in mind that molecular studies should be carried out to verify their identification and compare them with the databases that exist worldwide. It should also be taken into account for future experiments to carry out field tests and thus demonstrate the activity of these bacteria and thus show a biological alternative to chemical fertilizers that damage the environment so much.

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