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Nitrogen Fixation And Ammonium Ion Production By Endophytic Bacteria Associated With Tropical Pastures

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ABSTRACT

The objective of this study was to evaluate the efficiency of endophytic bacteria with potential activity for nitrogen fixation and ammonium ion production associated with different tissues of kikuyo grass in the Colombian Caribbean. Endophytic bacteria from different kikuyo grass tissues were isolated, and the population density was determined in CFU/g of tissue. Each isolated was used to quantitatively and qualitatively evaluate the BNF and evaluate the production of ammonium ion from nitrogen fixation. The bacteria that showed this activity were sequenced for identification of the 16S rDNA gene for eubacteria at the sequence level. 98 isolated of endophyte bacteria from different kikuyo grass tissues were isolated of which 17 were able to produce ammonium ion from nitrogen fixed in vitro. 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 reduction of N_2 to ammonium and becomes a biological resource for nitrogen fixation in the foliage of kikuyo grass in the department of Sucre and a bio-input of microbiological

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origin for livestock producers and the agrosostainable production of this pasture.

Keywords: Nitrogen fixation, endophytic bacteria, tissues, ammonium, pasture.

1. INTRODUCTION

Bacteria are associated with plants as pathogens, epiphytes and endophytes; many of these form intimate associations with plants and form diverse phylogenetic groups represented by species belonging to major taxa. Plant-associated bacteria typically exchange molecular signals with their host and possess diverse mechanisms for adaptation and colonization (Pérez et al. 2010). Important aspects of bacterial diversity in the ecosystem include the different processes they carry out, the complexity of the interaction and the number of trophic levels they comprise. Recently, there has been increasing interest in aspects related to the composition, structure and function of bacterial communities in different environments (Beneduzi et al., 2013; Tikhonovich and Provorov, 2011).

Currently, all of the pastures suffer from a series of problems, as described by Cajas-Girón et al. (2012): 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 efficiency, along with an advanced state of soil degradation that is common in most grazing areas. The use of chemical fertilizers to combat these difficulties is seen as an alternative, but the continuous use of fertilizers to improve the productivity of the pastures has caused an imbalance in the native microbiota that carry out important functions, resulting in low yields and increased costs for the farmers and ranchers, so the use of microbial inoculants (biofertilizers) has been proposed (Lara, et al., 2011).

There have been no studies in the Department of Sucre that demonstrate the importance of microorganisms as a biological alternative for the sustainable production of pastures; however, lately, research has increased for the search for mechanisms that are more friendly to the environment and that can efficiently metabolize nitrogen and phosphorus, converting them into components that can be assimilated by plants. Biological fixation of atmospheric nitrogen

has been considered the second most important process occurring in nature after photosynthesis (Sylvia et al., 2005).
As a result, in the search for alternatives to mitigate the impact of the continuous use of chemical products in the production of fodder, the use of endophytic microorganisms associated with plant species with the ability to produce multiple benefits has gained attention. Studies have shown the benefits of plant growth promoting bacteria as an alternative for obtaining biofertilizers, with the ability to stimulate the growth and productivity of plants, making it is possible to improve the yield of crops, to diminish the costs of production and to be friendly to the environment; this symbiotic process is the most efficient way to fix atmospheric nitrogen and solubilize phosphate.

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 (Zinniel et al., 2002), and have properties that promote the growth of plants, remove contaminants, solubilize phosphate and fix nitrogen (Rosenblueth and Martínez-Romero, 2006), along with the control of phytopathogens. Studies conducted by Pérez et al., (2014) 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 Aeromonas 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 kikuyo grass the quantification of ammonium ion from fixed nitrogenand become a biological alternative for future use as a biofertilizer in pastures in the Colombian Caribbean.

2. MATERIALS AND METHODS

Sampling. For the isolation of endophytic bacteria, samples were taken from complete plants (roots, stems and foliage) of kikuyo grass in the recovery stage on cattle farms in the municipality of Corozal, from plants in good physiological and phytosanitary condition. The plant tissue samples were stored in Icopor coolers, marked with the sampling date, variety and batch. These were then transported to the Microbiological Research Laboratory of the University of Sucre for the respective microbiological analyses (Pérez et al., 2010).

Disinfection processing of the samples. Each sample was subjected to a surface disinfection process using the protocol proposed by (Pérez et al., 2010). The process begins with 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; immersed for 1 min in 70% alcohol; shaken for 5 min in 5% sodium hypochlorite solution and dipped in Tween 80; again immersed for 1 min in 70% alcohol followed by shaking for 15 min in potassium phosphate buffer 0.05 mol.L-1, pH 7.0; finally, washed four times in sterile distilled water. The process was repeated twice.

Quantification in (CFU/g tissue) of endophytic bacteria associated with different tissues of kikuyo grass. To quantify endophytic bacteria with nitrogen-fixing capacity, the following steps were followed: 1 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 peptonized water and shaken vigorously for two hours and incubated at 37°C. From this solution, serial dilutions (10^{-1} to 10^{-8}) were made 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 of tissues: root, stem and foliage) was estimated by direct colony counting on plates. During counting, colonies distinguishable in shape, surface appearance, color and size were observed and selected. Selected morphotypes were purified and maintained on R2A agar for further analysis**.**

In vitro evaluation of biological nitrogen fixation activity. For this purpose, a direct surface seeding of the isolated bacteria was carried out on selective BURK agar medium containing the following constituents: 5 g MgSO4, 20 g KH2PO4, 5 g K_2HPO_4, 3.25 g CaSO4, 1.45 g $FeCl_3$, 0.253 g $NaMoO_4$, 1000 ml sterile distilled water (Park et al., 2005; Tejera et al., 2005) devoid of nitrogen source as an indicator of nitrogen fixing activity. The results were observed according to the growth of the bacteria in the medium**.**

Amount of nitrogen fixed. Once the bacteria had been selected for their optimal growth in culture medium, the amount of nitrogen fixed was evaluated by indirect evaluation of the amount of nitrogen reduced in the form of ammonium ion, using the Berthelot colorimetric method. For this test, the isolates were inoculated in Burk's broth medium at 30°C for 72 hours at 150rpm. After this time, 2M KCl was added to the medium and incubated again at the same conditions for 1 hour. Then, 10 ml of supernatant were taken and centrifuged at 8000 rpm for 20 min. At the end of this time, an

alcoholic solution of 10% phenol, 0.5% sodium nitroprusside and 1 ml of oxidizing solution (20 g sodium citrate, 1 g sodium hydroxide and 1 ml 1.5N sodium hypochlorite in 100 ml H2O), the mixture plus the sample was incubated for 1 hour and the results analyzed by absorbance reading at 632 nm in a Genesys 10S UV-Vis spectrophotometer. The standardized standard curve followed the model recommended by (Lara et al., 2007). Each isolate will be evaluated in triplicate.

DNA extraction from endophytic bacteria. Genomic DNA extraction was carried out according to the protocol recommended by (Green and Sambrook, 2012; M. N. Oliveira et al. 2013). The endophytic bacterial isolates were purified and activated in LB medium for 18 hours, after which time 1 ml of the bacterial suspension was taken and centrifuged at 12000 rpm to obtain a bacterial precipitate; this precipitate was resuspended in 0.5M EDTA and centrifuged at 12000 rmp for 20 minutes; the precipitate was treated with lysis buffer (0.5m EDTA and 0.25% SDS) and the mixture was incubated at 60°C for 1 hour. After this time, 5M NaCl was added with incubation at 4°C for 5 minutes, and the samples were centrifuged at 12000rpm. To the resulting suspension, an equal volume of phenol-chloroform-isoamyl alcohol 25:24:1 was added and centrifuged at 12000rpm, and half a volume of isopropanol was added to the suspension and then incubated for 14 hours. Finally, the samples were added ethanol until the DNA dried and then resuspended in 0.5X TE buffer.

DNA amplification of endophytic bacteria. Universal primers of the 16S rDNA region, the gene encoding the 16S rRNA small ribosomal subunit molecule, were used for the molecular identification of growth-promoting endophytic bacteria. Specific primers for each of the classes belonging to the bacterial domain such as alpha, beta, gamma proteobacteria and firmicutes (Oliveira et al., 2013).

3. RESULTS AND DISCUSSION

Table 1 describes the number of isolates obtained from the different samples taken from livestock farms in the municipality of Corozal. Of the 96 isolates evaluated for N2 to ammonium reducing capacity, all showed ammonium ion production at different concentrations, however 17 of the isolates from different plant tissues (root, stem and foliage) showed ammonium ion production at concentrations higher than 0.3 mg/L. Of the 96 isolates evaluated for N2 to ammonium reducing capacity, all showed ammonium ion production at different concentrations, however 17 of the isolates from different plant tissues

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Figure 1 shows the in vitro nitrogen fixation activity of endophytic bacteria on selective BURK agar medium.

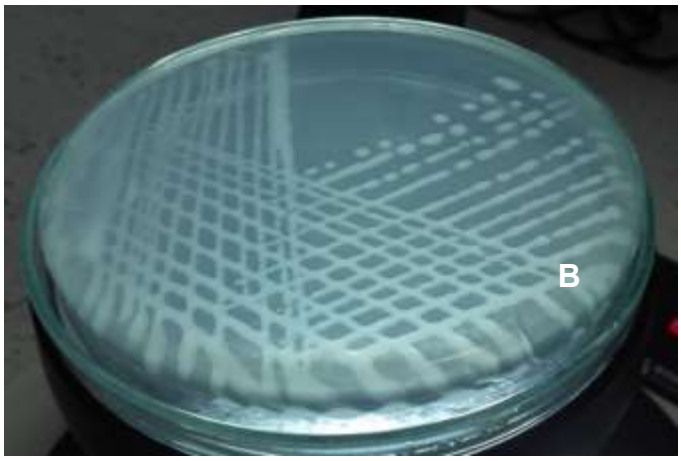

Figure 1. Qualitative in vitro evaluation of the activity's biological nitrogen fixation, in the endophytic bacteria.

Currently, several studies have focused their efforts on the knowledge, search and application of endophytic bacteria, which colonize intracellular spaces without the formation of symbiotic relationships and which have the capacity to take advantage of N2, abundant in the atmosphere but not available to the plant, reducing it to assimilable forms such as NH4 or NO3, on crops of economic importance, including pastures for the production of foliage for cattle feed worldwide (Barraquio et al., 1997; Mia et al., 2013; Stoltzfus et al., 1997).

En the table 1 lists the isolates extracted from different plant tissues of kikuyo grass from livestock farms in the municipality of Corozal.

Table 1. Nitrogen fixing isolates from each of the cattle farms sampled by tissue in the municipality of Corozal, Colombia, 2022.

Livestock farms	Roots	Stems	Foliages	TOTAL
1	12	4	2	18
2	11	6	2	19
3	13	14	4	31
4	14	10	4	28
TOTAL	**50**	**34**	**12**	**96**

Of the 96 morphotypes evaluated in the N2 to ammonium reducing capacity, all showed ammonium ion production at different concentrations, however 17 of the isolates belonging to different tissues of kikuyo grass showed ammonium ion production at concentrations higher than 0.3 mg/L. Thus, according to the isolate and type of tissue, the highest average concentrations in ammonium ion production were shown by isolate A4FoliageFarm1 with a value of 1.47 mg/L (figure 2).

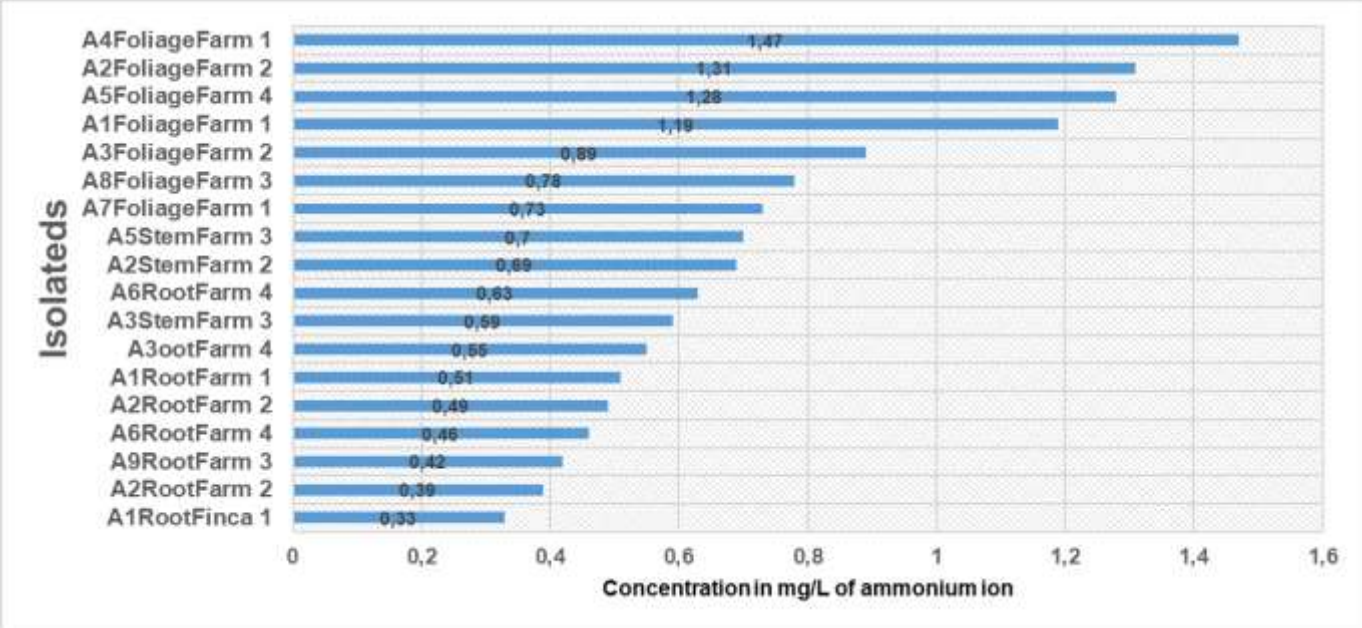

Figure 2. Concentration in mg/L of ammonium ion produced by endophytic bacteria isolated from different tissues of kikuyo grass.

The production of ammonium ion was determined using the Berthelot method, which consists of a change in color to an intense indophenol blue due to the reaction produced between the ammonium ion and phenolic compounds in the presence of an oxidizing agent. Additionally, a nitrogen-free Burk's medium was used for the test, which allows observing the capacity of the endophytic bacteria to convert atmospheric nitrogen and expel it to the medium. A total of 96 isolates of endophytic bacteria isolated from different tissues of kikuyo grass grown in different cattle farms in the municipality of Corozal, department of Sucre, Colombia, were evaluated.

The closest studies in the Colombian Caribbean region on fixation bacteria with biofertilizers potential on different crops have been carried out by Lara et al. (2007) where they evaluated the ammonium ion production of rhizosphere symbiotic bacteria evaluated in minimal salt and nitrogen-free medium, obtaining concentrations between

0.17 mg/L and 0.25 mg/L, data that do not exceed the results found by isolated endophytic bacteria from kikuyo grass foliage evaluated here, whose concentrations reach up to 1.47 mg/L of ammonium ion.

From the sequences of the amplified products and the homologous sequences obtained at NCBI, analyzed by Clustal W and Mega 4, a phylogenetic identification deduced from the Maximum Similarity method based on the Kimura 2 Parameter model with a gamma distribution was obtained for the modelling of evolutionary differences. A phylogenetic inference of 1000 replicates was used for the bootstrap consensus tree. The phylogenetic results show the divisions of two major taxonomic groups, placing the bacterial isolates mainly within two groups. The first is firmicutes, which have sequence homology with the bacterial phylotypes Bacillus cerus and Bacillus thuringiensis. The second group corresponds to the bacterial class proteobacteria, finding isolates with homologues to the phylotypes Serratia marcescens, Serratia nematodiphila, Enterobacter cloacae, Aeromonas hydrophila (figure 3).

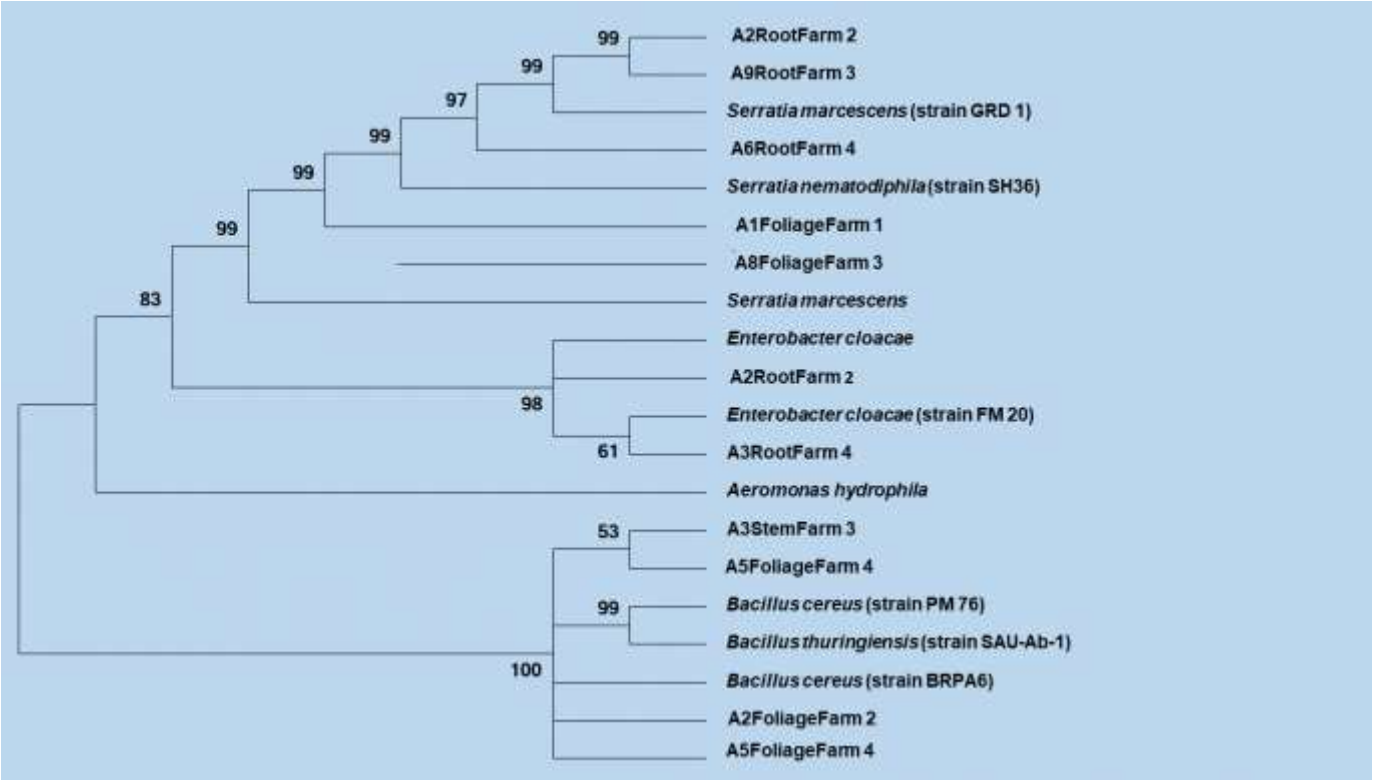

Figure 3. Maximum phylogenetic tree parsimony of gamma-proteobacteria and Firmicutes sequences of 16S rRNAr genes from endophytic growth-promoting bacteria of grass.

Recent studies carried out by Baoyu et al., (2017), reported Bacillus cereus as an endophytic bacteria associated with roots of tomato plants with functional potential as biological control agents and the production of siderophores for the acquisition of nutrients (iron) and activities to promote growth by fixing nitrogen in culture mediums for these plants. E. cloacae was initially identified as a phytopathogenic bacterium of maize plants and, later, as a endophyte bacteria of rice plants (Prakamhang et al., 2009). 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 (English et al., 2010). Work done by Hardoim, et al., (2009) showed that E. cloacae is an endophytic bacteria of rice plants with growth promoting activity.

4. CONCLUSION

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 ability to promote plant growth. In the in vitro tests carried out in the present study, it was confirmed that these endophytic bacterial species have the ability to produce produce ammonium ions, mechanisms used by microorganisms to promote growth in the analyzed grass species.

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6. AUTHORSHIP CONTRIBUTIONS

All authors have jointly and equally contributed to the argumentation and writing of the manuscript.

7. FUNDING. None.

8. CONFLICT OF INTEREST. None.

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