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Study of endophytic bacteria isolated from commercial rice cultivation with growth promoting activity properties

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ABSTRACT

In this study, the growth-promoting activity of endophytic bacteria isolated from different tissues of rice varieties from the municipality of Montería, department of Cordoba, Colombia, was evaluated in vitro. The population of endophytic bacteria was isolated on R_2A agar culture medium, counted and qualitatively evaluated for nitrogen fixation, phosphate solubilization and siderophore production. Endophytic bacteria with positive activities were identified by 16S rRNA gene sequencing with 5 sets of oligonucleotides specific for the class firmicutes, beta-proteobacteria; gamma-proteobacteria; alpha-proteobacteria and actinobacteria. A total of 107 isolates of endophytic bacteria were isolated, with the highest occurrence in variety 2000 and the lowest in variety 67; the most colonized tissue was the root. Of the 22 isolates, 11 showed the ability to solubilize phosphate, 6 to produce siderophores, and 5 to fix nitrogen. Sequencing results proved the identity of Bacillus cereus and Bacillus thuringiensis, which have the ability to promote growth in rice by phosphate solubilization, nitrogen fixation and siderophore production.

Keywords: Endophytic bacteria, nitrogen fixation, nitrogen production, siderophore production, phosphate solubilization

INTRODUCTION

Rice is a cereal considered as one of the main foods in the family basket in densely populated areas, contributing approximately 20% of the world's food energy supply (Baranoa, 2010; FAO, 2013). To maintain the yield and productivity of rice cultivation, the application of chemical fertilizers is necessary, which provide soils with nutrients that are required to promote optimal plant development and good crop yields (Liang et al., 2013). However, many of these chemical fertilizers are causing environmental problems (Izquierdo, 2017).

As an alternative to replace chemical fertilizers, emphasis is currently being placed on the study of endophytic bacteria, which contribute to plant health through the secretion of growth regulating factors, production of phytohormones and microbial metabolites, allowing the reduction of nutritional and pathogenic stress in the plant (Hardoim et al., 2008; Porras and Bayman, 2011). Endophytic bacteria are considered a great biotechnological tool due to their ability to fix nitrogen, solubilize phosphate and produce siderophores, which has favored crop production and yield (Senthilkumar et al., 2011).

Likewise, studies have shown that these rice-associated endophytic microorganisms belong to the Agrobacterium, Bacillus, Chryseobacterium, Flavobacterium, and Pseudomonas strains (Moronta, 2015), which present different mechanisms to protect the plant against any pathogens. The production of bioinoculants from the aforementioned genera has become an alternative to reduce production costs and replace agrochemicals that significantly affect soil microbiota (Ortiz et al., 2018; Valdez et al., 2020). Taking into account the above-mentioned benefits of endophytic bacteria, the present study aimed to evaluate in vitro the growth-promoting capacity of endophytic bacteria isolated from different tissues of rice varieties.

MATERIALS AND METHODS

Study area

The research was carried out at the experimental farm La Victoria of the Fondo Nacional del Arroz, located in the municipality of Mocarí-Córdoba-Colombia with coordinates 08°47′25″ North Longitude 75°51′38″ West Longitude with respect to the Greenwich Meridian, with an average temperature of 29 °C, relative humidity of 80%, average annual rainfall of 1200 mm and altitude of 20 m.

Sampling of plant material

Ten complete plants (root, stem, leaves, inflorescence) of the rice varieties identified as 2000, Tana, 473 and 67 were collected randomly in a zigzag pattern at the experimental farm La Victoria of the National Rice Fund, located in the municipality of Mocarí-Córdoba-Colombia with coordinates 08°47′25″ North Longitude 75°51′38″ West longitude with respect to the Greenwich Meridian, with an average temperature of 29 °C, relative humidity of 80%, average annual rainfall of 1200 mm and altitude of 20 masl. n.m. The samples were labelled with their respective variety and date of collection. The samples were stored and preserved in plastic boxes at 4 °C for transport to the microbiological research laboratory of the University of Sucre and processed within 24 hours after collection.

Isolation of endophytic bacteria

The plant material was separated into its different tissues in order to carry out the disinfection process described by Perez et al. After this process, each tissue was macerated in a porcelain mortar until a homogeneous mixture was obtained. Serial dilutions of each homogenate were made and sown by diffusion technique on the surface of R2A agar and incubated at 28 °C for 72 hours. The population density of endophytic bacteria per tissue, CFU/g tissue, was estimated by direct plate counting. During counting, colonies distinguishable in shape, surface appearance, color and size were selected. The selected isolates were purified and maintained on R2A agar (Pérez, Pérez & Chamorro, 2013).

Growth-promoting activity of endophytic bacteria in vitro

- **Biological nitrogen fixation.** Bacteria were placed with a bacterial loop on the selective medium Burk agar lacking a nitrogen source as an evaluator of nitrogen-fixing activity, which uses a combined carbon source that allows the recovery of a greater amount of microorganisms with possible nitrogen-fixing activity, selecting only those that present the enzymatic system that allows them to reduce atmospheric nitrogen and use it in their metabolism. The results were observed according to the growth of the bacteria in the medium (Park et al., 2005; Tejera et al., 2005).
- **Phosphate solubilization.** Phosphate solubilization was determined by inoculating colonies on NBRID solid culture medium. The appearance of clear halos around the colonies is considered a positive indicator for phosphate solubilization (Dawwam et al., 2013).
- **Siderophore production.** Siderophore production was determined using the chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987). Bacteria were placed in the medium with the aid of a bacterial loop. The ability of the bacteria to produce siderophores was evidenced by the formation of a transparent halo around the colonies.

Molecular identification of endophytic bacteria with plant growth-promoting activity

DNA extraction of endophytic bacteria with positive activity for nitrogen fixation, phosphate solubilization and siderophore production was performed using the protocol proposed by Oliveira et al. (2013). For the amplification of the 16S rRNA gene, 5 sets of specific oligonucleotides were used: FBLS342 and R1392, for the firmicutes class; F948β and R1492 for the beta-proteobacteria class; FD2 and RP1 for the gamma-proteobacteria class; F243 and R1492 for the actinobacteria class; F203 and R1492 for the alpha-proteobacteria class. For the amplification conditions of the first three primers, the protocol proposed by Oliveira et al. (2013) was used; while for the last two primers, the protocol of Gelsomino and Cacco (2005) was used. The amplified products were purified and sent for sequencing to Macrogen. The sequences obtained were compared with those stored in the Genbank. Base alignment was performed in the Clustal W program; phylogenetic inferences were obtained by the Neighbor Joining method based on the kimura-2-parameter model with bootstrap test 1,000 replicates with the MEGA X program.

Statistical analysis

A block design with factorial arrangement was applied for the differences between the population density (CFU/g tissue) of endophytic bacteria as a function of variety and tissue type. The multiple range test (Tukey) was used to establish significant differences between communities of endophytic bacteria (CFU/g tissue) in relation to variety and type of colonized tissue. Data were analyzed in the free version of InfoStat software.

RESULTS AND DISCUSSION

A total of 107 morphotypes of endophytic bacteria were isolated from four rice varieties identified as Fedearroz 2000 (F2000); Fedearroz Tana (FTana); Fedearroz 437 (F437) and Fedearroz 67 (F67). The analysis of variance between population density of endophytic bacteria by variety and tissue type showed significant differences ($p < 0.05$). The multiple range test for endophytic bacteria populations showed significance in terms of the number of endophytic bacteria per rice variety analyzed. The varieties with the highest densities were F2000 (1.37×10^4 CFU/g tissue) followed by F473 (1.2×10^4 CFU/g tissue); the lowest density of bacteria was for FTana and F67, which had densities of (1.0×10^4 and 9.7×10^3 CFU/g tissue), respectively (figure 1).

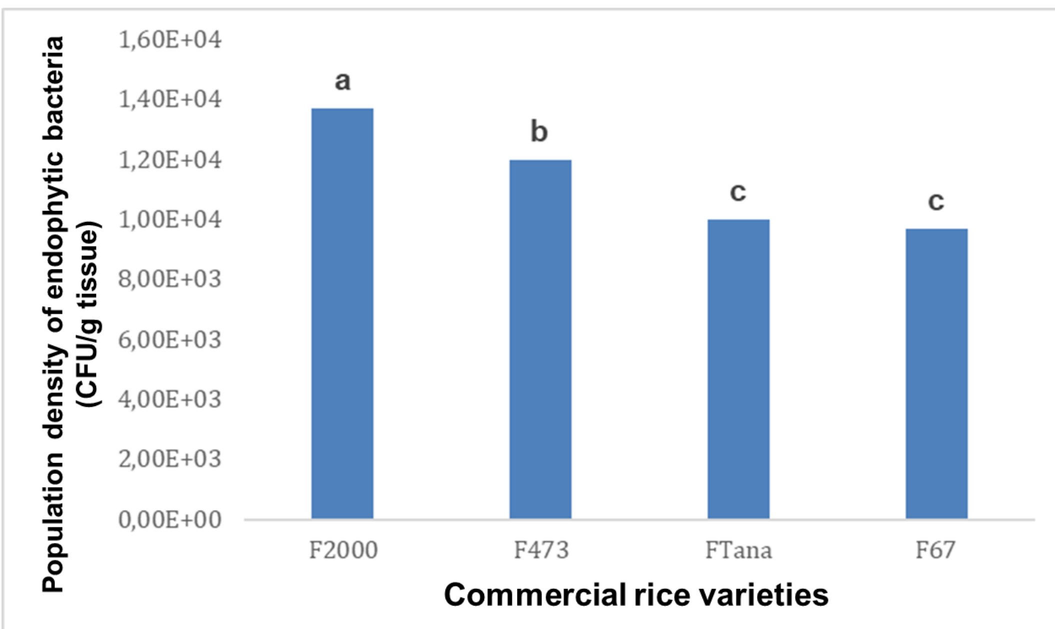

Figure 1. Population density of endophytic bacteria associated with four rice varieties, municipality of Montería, department of Córdoba.

The values found in the present study are higher than those found by Pérez et al. (2013) for the variety Fedearroz 2000 and lower than those reported by Pérez et al. (2015), who recorded values of endophytic bacteria densities of 2.07x1010 CFU/g for the variety Fedearroz 473 and 1.56x1010 CFU/g root for Fedearroz 2000.

Work carried out by Pillay and Norwark (1997) on the population density of endophytic bacteria associated with different plant species concluded that the presence of these bacteria depends on the species of bacteria and the genotype of the host plant, in addition to the stage of development of the plant, the inoculum density, environmental conditions and the time of year.

The multiple Tukey rank test for the density of endophytic bacteria as a function of tissue type showed a higher colonization in roots with $2.2X10^4$ CFU/g tissue and stem with $2.12X10^4$ CFU/g tissue; while the lowest amount of endophytic bacteria were isolated in leaves and panicles with values of $1.1X10^3$ and 7.2X102 CFU/g tissue, respectively (figure 2).

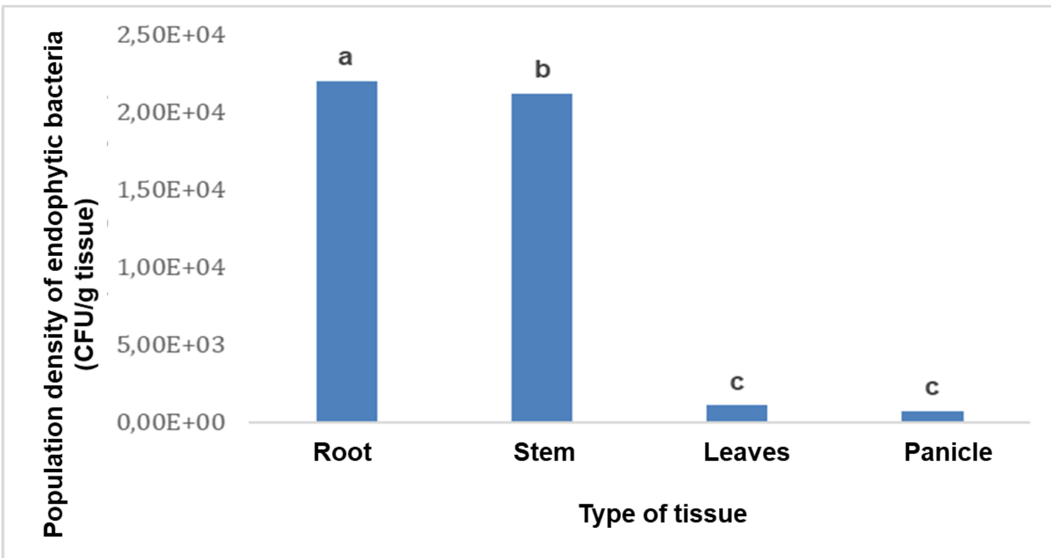

Figure 2. Population density of endophytic bacteria per rice tissue, municipality of Montería, department of Córdoba.

The most colonized tissue was the root in each of the varieties under study. In addition, the presence of endophytes is described for all plant tissues, with a greater number in the root, decreasing as it moves up the stem until it reaches the leaves and finally the fruit or inflorescences (Bacon and Hinton, 2007). The main reason that there is greater colonization in the root compared to other parts of the plant is due to the fact that this area is in direct contact with the soil. Also, the exudates released by the roots attract a great diversity of microorganisms that can colonize the root spaces (Senthilkumar et al., 2011).

Figure 3 shows the results of the in vitro qualitative phosphate solubilization activity assay on NBRID agar; nitrogen fixation on Burk agar and siderophore production on chromium azurol-S (CAS) medium. The results of the assay showed 22 isolates of endophytic bacteria with growth-promoting capacity, 11 of which were qualitatively observed to have the ability to solubilize phosphate; 6 to produce siderophores and 5 to fix nitrogen.

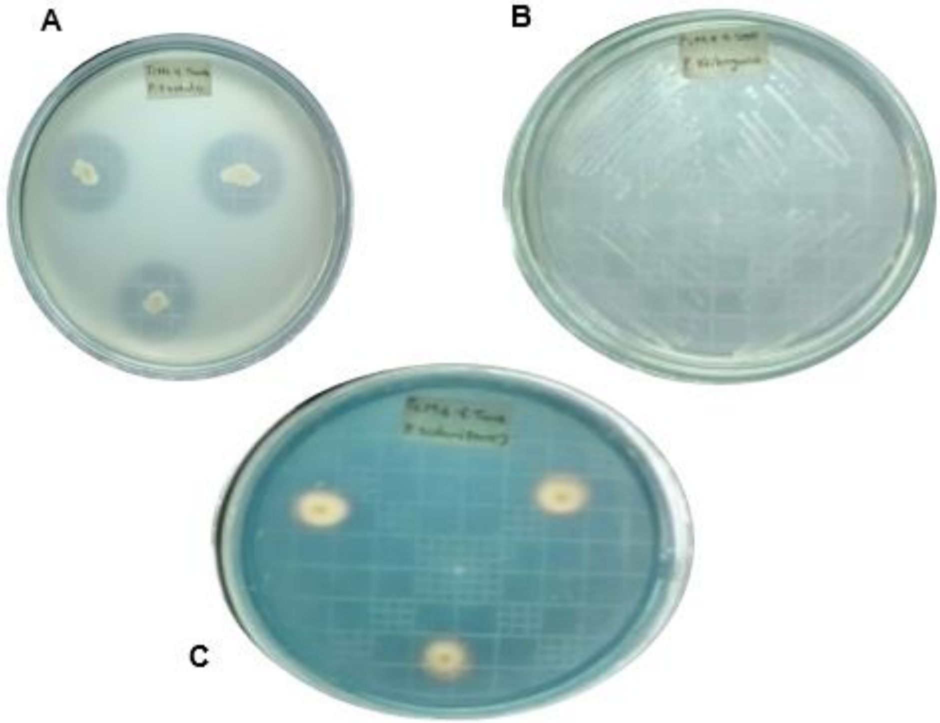

Figure 3. In vitro assay for (A) phosphate solubilization, (B) nitrogen fixation and (C) siderophore production of endophytic bacteria isolated from rice varieties.

The growth of endophytic bacteria on Burk medium suggests that they have the ability to produce the enzyme nitrogenize, an enzyme complex that catalysis the reduction of nitrogen to ammonium (Marreo et al., 2015; Ngamau et al., 2012). The combination of nitrogen-fixing bacteria and mycorrhizae-forming fungi has become a strategy to replace the excessive use of nitrogen fertilizers. Accordingly, a study by Montejo et al. (2018) showed that the combination of diazotrophic microorganisms and mycorrhiza fungi increased leaf length, stalk thickness and root length, which favored better yield and higher production in maize crops.

Likewise, endophytic bacteria have the ability to solubilize phosphate thanks to the production of enzymes such as phosphatases, which have the function of making phosphorus available in the soil and assailable by the plant, favoring its growth (Ramírez et al., 2014). Enzymes that have the ability to solubilize phosphates, such as phosphatases and lipases, have become biotechnological tools for improving yields, which have shown significant results in potato, maize and rice crops (Rodríguez et al., 2006).

The production of siderophores from endophytic bacteria plays an important role in plant protection, as the siderophore captures iron ions and does not make them available to pathogenic organisms (Jin et al., 2010). So far the largest number of studies are based on the application of siderophores to combat diseases affecting crops of economic interest has been related to products obtained from Pseudomonas fluorescents; because the production of siderophores (pioverdins or pyocyanins) by this microorganism has a clear inhibitory effect on the growth of the mycelium of Colletotrichum lindemuthianum, Colletotrichum gloesporioides (Santoyo et al., 2010).

On the other hand, Ortiz et al. (2018) in work carried out on endophytic bacteria associated with blueberry cultivation Vaccinium corymbosum L. (Ericaceae) demonstrated in vitro that the genera

Bacillus, Pantoea, Pseudomonas and Burkholderia have the ability to promote plant growth and can be used as inoculants for blueberry cultivation.

The phylogenetic analysis of the 16S rRNA gene for endophytic bacteria with growth promoting activity (figure 4), shows that 11 bacterial species showed high similarity with sequences stored in the GenBank database, corresponding to Bacillus thuringiensis and B. cereus species belonging to the class Firmicutes. In this analysis, the morphotypes T4M6F2000LIM, P3M6F473LIM, isolated from the stem of the variety Fedearroz 2000 and from the panicle of the variety Fedearroz 473, were identified as B. thuringiensis, showing in vitro phosphate solubilization capacity and siderophore production. The isolate corresponding to P1M3F473LIM, R1M4F2000LIM, R5M5FTANALIM, T2M5F2000LIM, P2M4F473LIM, T1M5F2000LIM, T1M3FTANALIM, H1M3F2000LIM and R2M5F67LIM obtained from panicle, leaf, root and stem of Fedearroz variety 2000, F473, F67 and FTana were identified as B. cereus, showing nitrogen fixation activity, phosphate solubilization and siderophore production.

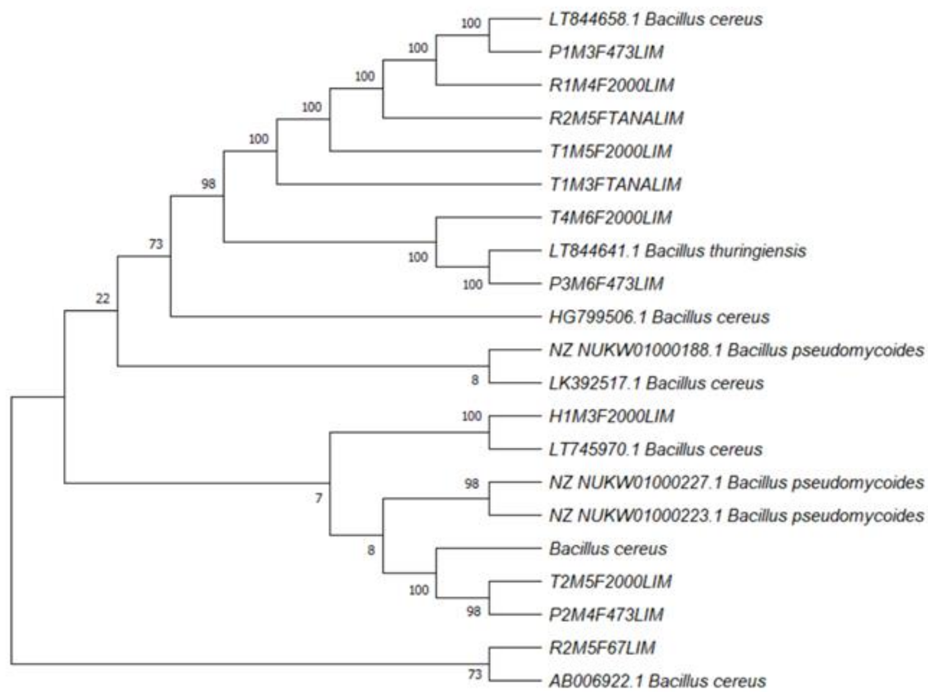

Figure 4. Dendogram based on the Neighbor-Joining method from the 16S rRNA gene sequences of asylated endophytic bacteria of four commercial rice varieties using the kimura-2-parameter model. H: leaf; P: panicle; T: stem; R: root; M: morphotype; F: Fedearroz variety; LIM: Laboratorio de Investigaciones Microbiológicas Universidad de Sucre.

Bacillus thuringiensis has been reported as an endophytic bacterium with the ability to solubilize phosphate and produce siderophores that can improve the quality of production in agricultural sectors (Portela et al., 2013). In addition, this bacterium produces a variety of metabolites, including bacteriocins, antibiotics and extracellular enzymes (such as proteases and chitinases), which are key compounds for pathogen suppression (Barboza et al., 2008). Studies by Wang et al. (2020) showed that different strains of B. thuringiensis have the ability to protect Brassica campestris L. (Brassicaceae) against the disease sclerotiniosis caused by Sclerotinia sclerotiorum. The results obtained indicated that the diversity of B. thuringiensis strains are effective for biological control against S. sclerotiorum. Also, this bacterium is used to control insects and nematodes that cause serious problems in crops of economic interest (Crickmore et al., 2020).

Bacillus cereus is reported as an endophytic bacterium associated with rice plants as a potential growth promoting agent (Angulo et al., 2014; Okunishi et al., 2005); and as reported by Dawwam et al. (2013), it is an endophytic bacterium associated with potato crop which has shown according to nitrogen fixation and AIA production assays. According to Wang et al. (2019), B. cereus has the ability to bioremediate rice crops with Cadmium contamination, as well as promote plant growth by producing Indole Acetic Acid (IAA).

The genus Bacillus is characterized by nitrogen fixing activity, and species of this genus have been reported as cadmium resistant endophytes in Solanum nigrum L. (Solanaceae) and nickel in Oryza sativa L. (Poaceae) (Pérez et al., 2011). Generally, members of this genus have been isolated from a large number of wild species of commercial interest such as potato (Calvo & Zúñiga, 2010), wheat, rice and sugarcane (Gopalakrishnan et al., 2012). In turn, isolations of these bacteria are made from rhizospheric or non-rhizospheric soil of the same crop where their potential as a growth promoter is to be evaluated. Their growth-promoting activity is mainly associated with the production of AIA, phosphate solubilization and siderophore production, generating increases in the root and aerial portion of the plants (Beneduzi et al., 2008).

CONCLUSIONS

In this study, a high diversity of endophytic bacteria was found associated with different tissues of four commercial rice varieties which qualitatively demonstrated in vitro phosphate solubilization, nitrogen fixation and siderophore production, and the species Bacillus cereus and B. thuringiensis were molecularly identified as endophytic bacteria, which possibly promote plant growth in these rice varieties in the field.

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