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Identification Of Plant Growth-Promoting Rhizobacteria Associated With Persea Americana Plantations

Lina Chamorro Anaya[1], Alexander Pérez Cordero[2], Donicer E. Montes Vergara[3]

[1]*Universidad de Sucre, Facultad de Educación y Ciencias, Departamento de Biología, Colombia.*
linachamorro15@gmail.com https://orcid.org/0000-0003-2931-5471
[2] *Universidad de Sucre Facultad de Ciencias Agropecuarias, Colombia*
alexander.perez@unisucre.edu.co(Corresponding Author) https://orcid.org/0000-0003-3989-1747
[3] *Universidad de Sucre Facultad de Ciencias Agropecuarias, Colombia*
donicer.montes@unisucre.edu.co https://orcid.org/0000-0002-2860-0505

Abstract

Rhizobacteria have been studied for carrying out important processes in plants generating benefits, among which are activities such as biological nitrogen fixation, phosphate solubilization, production of indoleacetic acid, production of ACC deaminase and siderophores promoting plant growth. The aim of this study was to identify in vitro plant growth promoting rhizobacteria associated with avocado crops. For this purpose, rhizobacteria were isolated from soils from Salitral, Ovejas (Mountain of the María), department of Sucre, Colombia. In vitro phosphate solubilization, nitrogen fixation, siderophore production and ACC deaminase tests were carried out to finally identify them molecularly by amplification and subsequent analysis of 16S rDNA sequences. The highest population densities were 2.95 x 10^7, 2.19 x 10^7, 1.91 x 10^7, 1.64 x 10^7, 1.50 x 10^7, 1.39 x10^7 and 1. 30 x 10^7 CFU/ g of soil for the farms Florida S, Sector 2, El Ojito, Sector 1R, Sector 1D, Florida N and Villa Esperanza respectively, a total of 35 morphotypes of rhizosphere bacteria were isolated, only five isolates showed the ability to promote plant growth identifying them as Burkholderia cepacia, Pseudomona aureginosa, Burkholderia cepacea, Bacillus cereus and Bacillus megaterium. These results show the importance of microorganisms as agents of biotechnological value in the search for alternatives to agricultural and environmental problems in crops of agricultural interest.

Keywords: Rhizosphere bacteria, soil, growth, plant.

Introduction

The mountain of the María is a sub-region of the Colombian Caribbean, the main producer of avocado (Persea americana). Avocado crops in the Montes de María have for years constituted the economy of many families in the region, however the area planted with these crops has decreased considerably due to the presence of armed conflict in the area, which has led to the abandonment of the producing farms, limiting their renewal (de Bolívar et al., 2012; de Colombia, 2003). Another limiting aspect of the crops are the diseases caused by phytopathogens, implementing as control the use of synthetic fungicides indiscriminately, generating alterations in the agroecosystems, loss of the physicochemical properties of the soil, development of strains resistant to fungicides, which increases the severity of the problem and production costs, therefore it is necessary to propose strategies that counteract the problem and allow the recovery and conservation of crops so that the principle of sustainability and development is maintained.

In the search for alternatives for the improvement of plant growth, production and health, the use of plant growth promoting bacteria (PGPB) are considered highly efficient and contribute to the improvement of plants and are environmentally friendly (Gupta et al., 2015). These bacteria are able to improve plant growth through direct mechanisms such as biological nitrogen fixation, phytohormones production, solubilization of inorganic phosphates and mineralization of organic phosphate, reduction of heavy metal toxicity, the activity of the enzyme ACC deaminase and increased root permeability and indirect mechanisms such as the elimination of phytopathogens through the production of lytic enzymes, production of antimicrobial substances, antibiotics, nutrient competitions, space, induction of systemic resistance (IRS) against a wide spectrum of pathogens and production of siderophores as a sequestrator of available Fe, limiting the development of pathogens (Esquivel-Cote et al. , 2013).

This research aimed to identify plant growth promoting rhizobacteria (PGPRs) associated with avocado plantations and them in vitro ability to promote plant growth.

Materials and methods

Location. Sampling was carried out in Salitral (09 ° 31 '48 "N - 75 ° 14' 01" W), belonging to the Montes de María, department of Sucre, Colombia. Random zig-zag sampling was carried out to collect soil samples associated with avocado and banana crops. The soil samples were labelled with georeferencing of the sampling site and taken to the Microbiological Research Laboratory of the University of Sucre for microbiological analysis.

Isolation of rhizospheric bacteria. Root soils were taken from associated avocado and banana crops, removing the soil adhering to the roots and washed with sterile distilled water and shaken for 30 minutes. Once sediment, an aliquot of the suspension was taken and serial dilutions from 10-3 to 10-7 were prepared and inoculated on nutrient agar and incubated at 30±1°C for 72 hours (Salgado-Bernal et al., 2012). The population density of rhizosphere bacteria per gram of soil was determined by direct colony counting on the surface of the agar plates. Morphotypes were selected according to their morphological characteristics, color, shape, texture and size (Pérez-Cordero et al., 2014).

Biological nitrogen fixation. Qualitative assessment of nitrogen fixation of rhizosphere bacteria was performed using ASHBY selective medium and the methodology described by Pérez-Cordero et al. (2014). Strains were seeded by composite streak on the surface and incubated at 28°C for 72 hours. The growth of isolates on such media indicated the fixation of atmospheric nitrogen.

Phosphate solubilization. Phosphate solubilization capacity was determined in NBrip and SRS media, strains were incubated at 28°C for 72 hours using the methodology proposed by Pérez-Cordero et al. (2014).

Siderophore production. Qualitative assessment of siderophore production was carried out by direct seeding of morphotypes on the surface of chromium azurol-S (CAS) medium (Schwyn & Neilands, 1987). They were incubated for 7 days at 30°C. Sideropore production was determined by the presence of orange halos around the colonies.

ACC Deaminase. Streak seeding was performed on sterile Dworkin and Foster (DF) salts medium (Belimov et al., 2001; El-Tarabily, 2008), supplemented with 0.3 g/L 1-aminocyclopropane carboxylic acid (ACC) as the sole nitrogen source. Incubation was carried out for 5 days at 30°C. Colonies that grew on the surface were considered ACC deaminase-producing (Andy et al., 2020).

Molecular identification. Rhizospheric bacteria with in vitro plant growth-promoting activity were selected and identified based on morphological characteristics and by Gram staining. DNA extraction was performed according to the protocol described by (Oliveira et al., 2013). Amplification of rDNA fragments was carried out with the use of specific oligonucleotides for eubacterial groups and the products obtained from the amplification were quantified and sent for sequencing, using the service provided by Macrogen (Seoul, South Korea). From the sequences of the amplified products and the homologous sequences obtained at NCBI, analyzed by Clustal W and Mega 10, a phylogenetic identification was obtained by the distance and maximum parsimony method of Neighbor-joining with bootstrap test.

Soil samples and analysis. For the determination of physico-chemical properties, soil samples were collected from the farms established for the sampling. Several small sub-samples were taken from different parts of the farms, selected to represent the total area. The samples were taken in a zigzag pattern over the whole area of the land. Once the subsamples were taken, they were mixed and homogenized to obtain a soil sample of approximately one kilogram, which was labelled and sent to the laboratory of agricultural soils and waters of the University of Sucre for its study (Methods of analysis of the Soil Laboratory - Instituto Geográfico Agustín Codazzi, n.d.).

Statistical analysis. The data were analyzed by Tukey mean comparisons ($\alpha=0.05$) to establish significant differences between rhizosphere bacteria communities (CFU/g soil) in relation to the sampling site, using PSPP and R software version 3.2.3.

Results and discussion

The characterization of the physical-chemical parameters of the soil samples associated with avocado and banana crops, belonging to the village of Salitral-Ovejas, indicate that the soils presented the following physical-chemical characteristics: moderate content of organic matter, slightly basic to slightly acid pH values, potassium and calcium values between abundant to excessive content, potassium with moderate to very poor values and clay loam, loam and clayey loam soil texture.

A total of 35 rhizospheric bacterial morphotypes were isolated from different soils associated with avocado and banana crops. The population density varied in a range of 2.95x107 ± 1.30x10^7 CFU/g soil. The results of the Tukey test of means for population density of rhizosphere bacteria show that there are no significant differences between the different soils analyzed (figure 1), the Florida S farm had the highest population density (2. 95x10^7 CFU/ g of soil), followed by Sector 2 (2.19x10^7 CFU/ g of soil), El Ojito (1.91x10^7 CFU/ g of soil), Sector 1R (1.64 x10^7 CFU/ g of soil), Sector 1D (1.50x10^7 CFU/ g of soil), Florida N (1.39 x10^7 CFU/ g of soil) and Villa Esperanza (1.30x10^7 CFU/ g of soil).

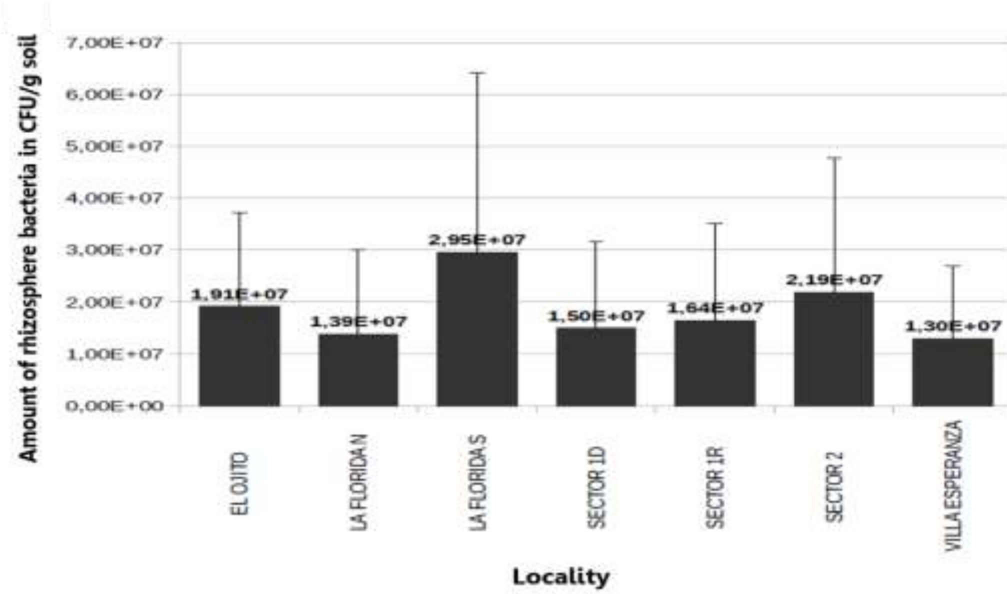

Figure 1. Tukey test of the population density distribution of rhizosphere bacteria by soil, Salitral-Ovejas, department of Sucre, Colombia.

The population density of rhizosphere bacteria in the soils analyzed (figure 1) was variable, which can be attributed to the fact that microbial biomass is related to soil pH and organic matter content, phosphorus availability, soil type, among others (van Eekeren et al., 2010). Bacteria function best at soil pH levels of 7.0, i.e. as p H decreases the ability to decompose organic matter is reduced and they stop providing nutrients to plants affecting their physiological development (Axelrood et al., 2002).

Rhizotrophic bacteria with in vitro plant growth-promoting activity. Of the 35 isolated morphotypes, five showed in vitro plant growth promoting ability (figure 2) and according to the identification based on the 16S rDNA gene, the morphotypes fall into three groups. The first group is Betaproteobacteria, identifying morphotype 5105 as Burkholderia cepacia. The second group corresponds to the class Gammaproteobacteria with morphotype 7102 as Enterobacter cloacae and morphotype 2103 as Pseudomona aeruginosa and finally firmicute identifying morphotypes 2101 as Bacillus cereus and morphotype 5104 as Bacillus megaterium (figure 3).

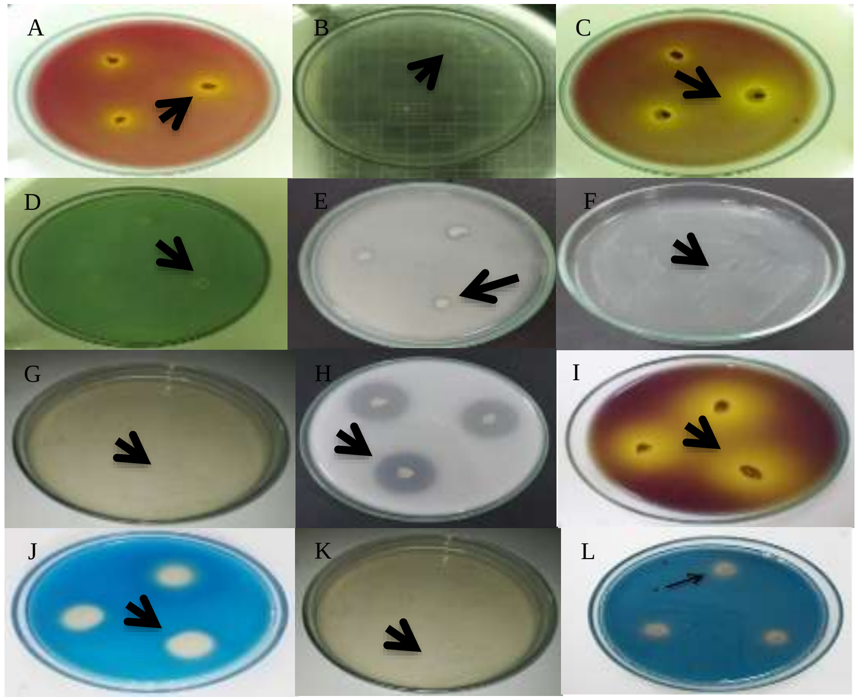

Figure 2. In vitro plant growth promotion activity. Enterobacter cloacae: **A**. Phosphate solubilization in SRS medium. **B**. Nitrogen fixation. Pseudomona aeruginosa: **C**. Phosphate solubilization. Bacillus cereus: **D**. Siderophore production. **E**. Phosphate solubilization in NBRID medium. **F**. Nitrogen fixation. Burkholderia cepacia: **G**. Nitrogen fixation. **H**. Phosphate solubilization on NBRID medium. **I**. Phosphate solubilization on SRS medium. **J**. Production of siderophores. **K**. ACC deaminase production. Bacillus megaterium: **L**. Production of siderophores.

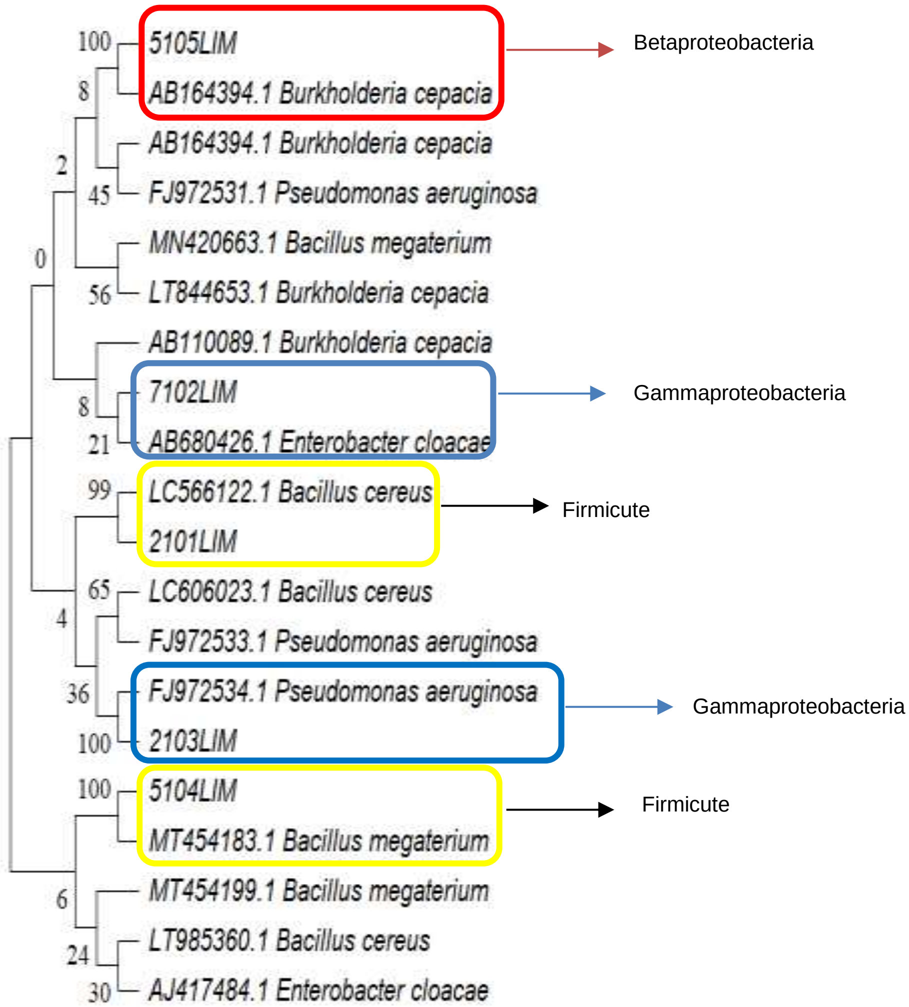

Figure 3. Dendogram derived from sequencing analysis of 16 rDNA related sequences obtained from NCBI.

In this work, two bacteria belonging to the genus Bacillus (Bacillus megaterium and Bacillus cereus) were identified (figure 3), which had the capacity to produce siderophores, solubilize phosphates and fix nitrogen. According to research carried out by Olivares et al. (2021), they isolated, characterized and identified the bacterial isolates associated with the sugar cane rhizosphere, with Bacillus sp being the most abundant bacterium with phosphate solubilizing capacity. These bacteria form complexes with the Ca+2 ion, associated with insoluble phosphorus, transforming into water-soluble di- and mono-basic phosphate, which allows them to be assimilated by plants (Ramírez et al., 2014). These processes occur thanks to the production of enzymes such as phosphatases, C-P lyases, phytases, among others, and the production of organic acids that modify the pH to solubilise the phosphorus in the medium. The production of these organic acids such as gluconic or 2-ketogluconic acid can be variable and depends on root exudates (Ahemad & Kibret, 2014; Molina-Romero et al., 2015; Otieno et al., 2015).

Bacillus megaterium has been shown to increase the percentage of chlorophyll and leaf nitrogen stimulating plant growth (Olivares et al., 2021). Bacillus cereus has also been reported as a rhizospheric bacterium isolated from soils associated with tomato crops with the ability to produce 1-aminocyclopropane-1-carboxylate (ACC-deaminase) and exopolysaccharide under normal and heat stress conditions showing significant increase in shoot length, root and leaf surface area by reducing the adverse effects of heat on tomato plant growth (Mukhtar et al., 2020). It should be noted that Muhammad et al. (2021), found in their research that Bacillus cereus had the ability to enhance the growth of oil palm seedling roots and was used in association with Trichoderma asperellum contributing significantly to the growth of aerial parts, complementing each other's ability to produce indole acetic acid (IAA), phosphate solubilization and siderophores.

Another in vitro plant growth promoting rhizobacterium identified in this work is Pseudomona aeruginosa exhibiting phosphate solubilizing capacity which is reported by Yasmin et al. (2017) with the ability to solubilize different nutrients in the soil thanks to its conversion of insoluble nutrients into soluble ones by releasing extracellular enzymes, which increase the availability of nutrients in the soil, production of IAA and ACC acting in promoting plant growth of the strain and was reported as an antagonist against various plant pathogens including Fusarium sp and Xanthomonas oryzae pv. oryzae (Xoo), this biocontrol is due to the production of hydroxamate-type siderophores, such as ferribactin, pseudobactin and other catechol-type siderophores that sequester Fe III from the environment when it is deficient and limit the growth of pathogens becoming antagonistic mechanisms (Aguado-Santacruz, 2012). The reported ACC deaminase activity in P. aeruginosa may help to reduce the level of ACC caused by ethylene under biotic and abiotic stress conditions, protect the host plant and regulate plant growth (R. P. Singh & Jha, 2016).

The rhizobacterium Enterobacter cloacae isolated and identified in this study presented nitrogen-fixing and phosphate solubilization activity, research carried out by Peñaloza Atuesta et al. (2020) confirmed that rhizobacteria belonging to the genus Enterobacter present in vitro inorganic phosphate solubilization capacity and presented antagonistic activity against Burkholderia glumae, with 3-phenylpropanoic acid (3-PPA) being the main compound present in the extracts. Strain K2 identified as Enterobacter sp was reported as a rhizobacterium capable of resisting heavy metals (cadmium, lead, arsenic, nickel and mercury) by promoting plant growth through phosphate solubilization, IAA production, ACC deaminase and nitrogen fixation (Pramanik et al., 2018). Nitrogen-fixing bacteria use atmospheric nitrogen as a nutrient and in root nodules there is catalysis of the nitrogenize enzyme complex, which reduces the triple covalent bond of the gaseous nitrogen molecule being able to combine it with oxygen or hydrogen to form ammonium ($NH4+$) and nitrate ($NO3+$), these chemical forms can be used by plants and by the microorganisms themselves (Dos Santos et al., 2012).

Burkholderia cepacia was the species with the highest in vitro plant growth promotion (nitrogen fixation, phosphate solubilization, siderophore production and ACC deaminase production) (figure 2) and according to the work of Ghosh et al. (2016) Burkholderia tropica, Burkholderia unamae and Burkholderia cepacia strains possess the ability of siderophore production on CAS agar medium, tryptophan-dependent IAA production, antifungal activity against pathogenic fungi and increased total phosphate content in Lycopodium plants after soil treatment with these isolates. Likewise, Moreno-Conn et al. (2021) identified the presence of two strains identified within the genus Burkholderia with plant growth promotion in rice plants under greenhouse conditions favoring the availability of phosphorus in the soil.

Plant growth promoting rhizobacteria (PGPRs) have been reported not only to promote plant growth but also to increase soil fertility by solubilizing nutrients through different direct and indirect mechanisms which is suggested as an ecological approach to ensure sustainable agriculture (Bishnoi, 2015; Romão-Dumaresq et al., 2017; N. Singh et al., 2018).

Conclusion

In this study, five rhizosphere bacteria Burkholderia cepacia, Enterobacter cloacae, Pseudomona aeruginosa, Bacillus cereus and Bacillus megaterium were identified, each executing distinct plant growth promoting activities for each other, which is based on the fact that they develop metabolic processes for the plant according to the needs required by the plant. It is suggested that these can be used in microbial consortia for the improvement of avocado and banana plantations.

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