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ISOLATION OF ENDOPHYTIC BACTERIA AS A BIOLOGICAL MODEL TO CONTRIBUTE TO HEAVY METAL PHYTOREMEDIATION

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ABSTRACT
The aim of this research was to encourage with different processes and schemes for the isolation, in vitro evaluation of the tolerance capacity to different concentrations of heavy metals and plant growth promoting activity. The results of research on endophytic bacteria isolated from different plant species in the Caribbean region of Colombia show the ability of the bacteria to tolerate different heavy metals such as mercury, lead, cadmium, nickel at different concentrations and the ability of these bacteria to fix nitrogen, solubilize phosphates and produce siderophores. The group of researchers have a genomic bank of endophytic bacteria that can be inoculated in plants for the improvement of plant species with the capacity to remediate and improve their nutritional conditions, adaptation and toxicity to environments contaminated with heavy metals.

Keywords. Endophytic bacteria, tolerance, heavy metals, growth promotion.

INTRODUCTION
Endophytic bacteria have an impact on the growth promotion dynamics of plant species. Endophytic bacteria with growth promoting activity such as: phosphorus solubilization, nitrogen fixation, siderophore and indole acetic acid (IAA) production, which can improve the utilization of soil nutrients by plants and promote plants growth. In addition, microorganisms are able to regulate metal resistance systems and promote or limit the accumulation of HMs in plants [10]. Among the plant growth promoting bacteria, siderophore-producing bacteria are of particular interest. Siderophores are small molecules with high affinity for Fe_3^+ produced by bacteria, fungi and plants (Roskova et al., 2022). They not only bind specifically to Fe_3^+ to provide iron nutrition, but also form soluble metal-siderophore chelates with metals such as Al_3^+, Cu_2^+, Cd_2^+, Pb_2^+ and Zn_2^+, reducing their free concentration and toxicity of such a compound (Schalk et al., 2021).

Biological methods including phytoremediation and biological remediation technologies with microorganisms or the combination of both, are the most powerful tools for the remediation of heavy

metal contaminated environments (Genchi et al., 2020). However, the efficiency of phytoremediation has drawbacks due to low biomass production by the heavy metal bioaccumulating plant species and low availability of the metal in the problem environment (Rajkumar et al., 2009). Due to this drawback in the efficiency of phytoremediation, it is necessary to further investigate the promotion of growth and accumulation of the metal in plant tissues.

Endophytic bacteria release a diversity of organic substances that promote plant growth, protect plants in different abiotic and biotic stressful environments and influence the production of resistance genes in plants. Endophytic bacteria are adapted to different environments and host plants and play a key role in detoxification and resistance to heavy metal toxicity such as cadmium, zinc, arsenic, lead, mercury, cadmium and others (Sangsuwan and Prapagdee, 2021).

Several current studies recommend the use of endophytic bacteria to contribute to phytoremediation (Wang et al., 2021). Endophytic bacteria are a broad group of microorganisms that inhabit healthy plant tissues during a certain period of their life cycle on the plant and interact with these to establish complex mutually beneficial symbiotic relationships through physicochemical interactions (Puri et al., 2020; Sturz et al., 2000; Tian et al., 2016). Endophytic bacteria associated with heavy metal tolerant plants proudly produce metal chelation, activate antioxidant enzymes and reduce lipid peroxidation. They eliminate the toxic effects of heavy metals by promoting growth in host plants through the production of growth-stimulating hormones, such as indole-3-acetic acid, to decrease heavy metal contents in plant tissues (Liu et al., 2019; Nagata et al., 2015; Rocha et al., 2016; Ruan et al., 2016). Endophytic bacteria isolated from different plant species possessing the above biological properties can provide nutrition and enhance heavy metal resistance and increase capacity when inoculated and reinoculated into other plants (Adediran et al., 2015).

Based on the above information, the aim of this study is to provide information on the capacity of endophytic bacteria associated with different plant tissues and to enhance the phytoremediation process of these plant species. The objective of the research is to isolate, evaluate and identify strains of endophytic bacteria with the capacity to tolerate heavy metals and the capacity to promote plant growth as a strategy for the adaptability of plant species and the removal of contaminating metals from the soil.

MATERIALS AND METHODS

Sampling site. At each site, random sampling shall be done in a zig-zag fashion, taking samples of soil and whole plants without symptoms of toxicity. Samples shall be labelled, packaged, preserved for transport to the laboratory. Soil samples shall be used for the determination of heavy metal concentration and plant samples for the isolation of endophytic bacteria.

Isolation of endophytic bacteria from plant tissues. Plants collected from each species of interest were subjected to a surface disinfection process. Roots, tillers, leaves, flowers, inflorescence, fruits and seeds of each plant were washed with sterile water and cut into segments of approximately 1 cm. The surface disinfection process for each tissue was carried out according to the methodology recommended by Pérez et al., (2010).

Heavy metal tolerance test. The in vitro assay of endophytic bacteria tolerance at various metal ion concentrations of Cd, Pb, Ar, Pb, Hg was carried out in minimal medium tris-MMT proposed by (Rathnayake, et al., 2013) with eight different concentrations of cadmium in the form of (Cd, Pb, Ar, Pb, Hg)Cl2. The initial concentration of C Cd, Pb, Ar, Pb, Hg used was 0.01 mg / ml and from these concentrations of 100 were prepared (0.1 mg / mL), 150 (0.15 mg / mL), 200 (0.2 mg / mL, 250 (0250 mg / mL), 300 (0.3), 350 (0.35), 400 ppm (0.4 mg / mL) and 500 ppm (0.5 mg / mL). Aliquots of bacteria in logarithmic phase was inoculated into the medium MMT. As control means MMT was used without Cd, Pb, Ar, Pb, Hg. The experiment was performed in triplicate, which was incubated

under stirring at 150 rpm at 32 ° C for 120 hours (Zhang et al, 2011). The growth of each bacterium was determined by turbidimetry method at 600 nm every hour for four days.

Qualitative evaluation of the growth promotion of heavy metal tolerant endophytic bacteria. The strains that showed tolerance to heavy metals were used to qualitatively evaluate in vitro the capacity for biological nitrogen fixation, phosphate solubilization and siderophore production. The qualitative evaluation of the biological fixation of the strains was performed by the methodology proposed by Pérez et al. (2014) on selective ASHBY agar medium.

For the qualitative evaluation of phosphate solubilization of the heavy metal tolerant strains, it was performed, following the methodology proposed by Pérez et al., (2014), on NBRIP medium with Ca3 PO4 as insoluble phosphorus source at pH 7. Each strain was inoculated on the surface of the medium and incubated at 28 °C for 72 hours. The qualitative observation of the strains was determined by observing the formation of a visible transparent halo around and below the colony.

The siderophore production capacity was carried out on chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987). The strains were incubated for 7 days at 30°C. The ability of the bacteria to produce siderophores is evidenced by the formation of a halo.

DNA extraction, amplification and 16S rDNA sequence endophytic bacteria tolerant heavy metals. They isolate which showed the best results regarding heavy metals tolerance and growth promotion were selected. For the extraction of DNA samples pure bacteria were taken and were activated in agar Luria Bertani (LB), (Bacto tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, milli-Qate 1000 mL, pH 7.0) and incubated at 28 oC/24 hours, after this time were taken again and pure colonies were transferred to tubes containing 10 ml LB broth and incubated again for 24 hours at 28 °C with constant stirring at 150 rpm in a controller IKA 260.1 Basic. The DNA was extracted using proposed protocol by (Oliveira et al., 2013). Amplification of 16S rDNA fragments was carried out using specific oligonucleotide eubacterias groups (Oliveira et al., 2013). The amplification products were sent to sequencing the company Macrogen (Seoul, South Korea) on an automated capillary sequencer 3730XL. Entities of the nucleotide sequences obtained were compared with those stored in databanks of the National Center for Biotechnology Information (NCBI). The alignment of the bases was performed by CLUSTAL W; phylogenetic inferences were obtained by maximum similarity method based on Kimura-2-parameter test bootstrapping (1000 replicates) with 7 MEGA program model.

RESULTS AND DISCUSSION

In order to initiate a study to search for endophytic bacteria in heavy metal accumulating plants, a diagnosis and subsequent sampling must be carried out at the identified site following the following scheme as described in figure 1.

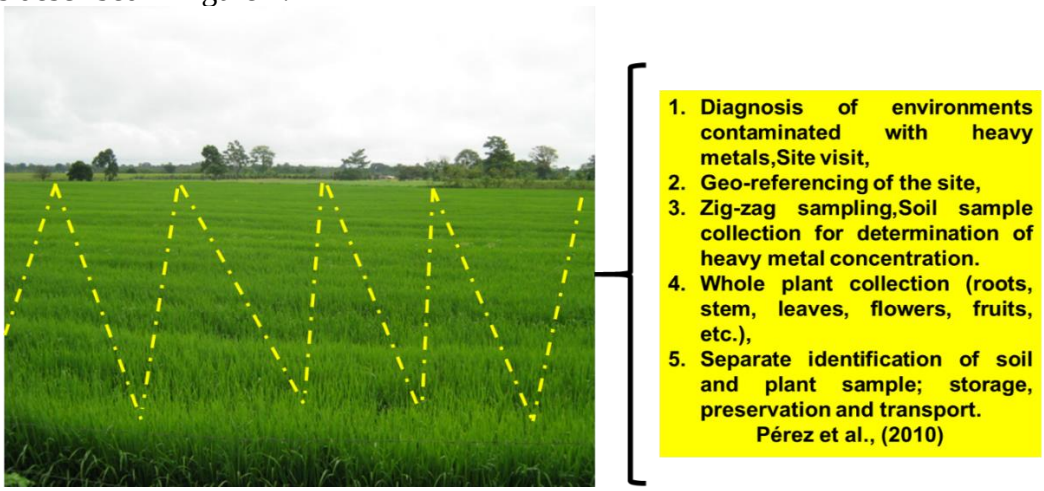


Figure 1. Stages of the diagnostic process and sampling of soil and healthy plants without any symptoms of toxicity for microbiological analysis and heavy metal tolerance tests.

For an isolation process of endophytic bacteria from hyperaccumulator plants in a healthy physiological state without any symptoms of heavy metal toxicity. Once the area to be studied has been identified, the following protocol should be followed once the sampling site has been geo-referenced and the in situ sampling is carried out, the following scheme should be followed (figure 2).

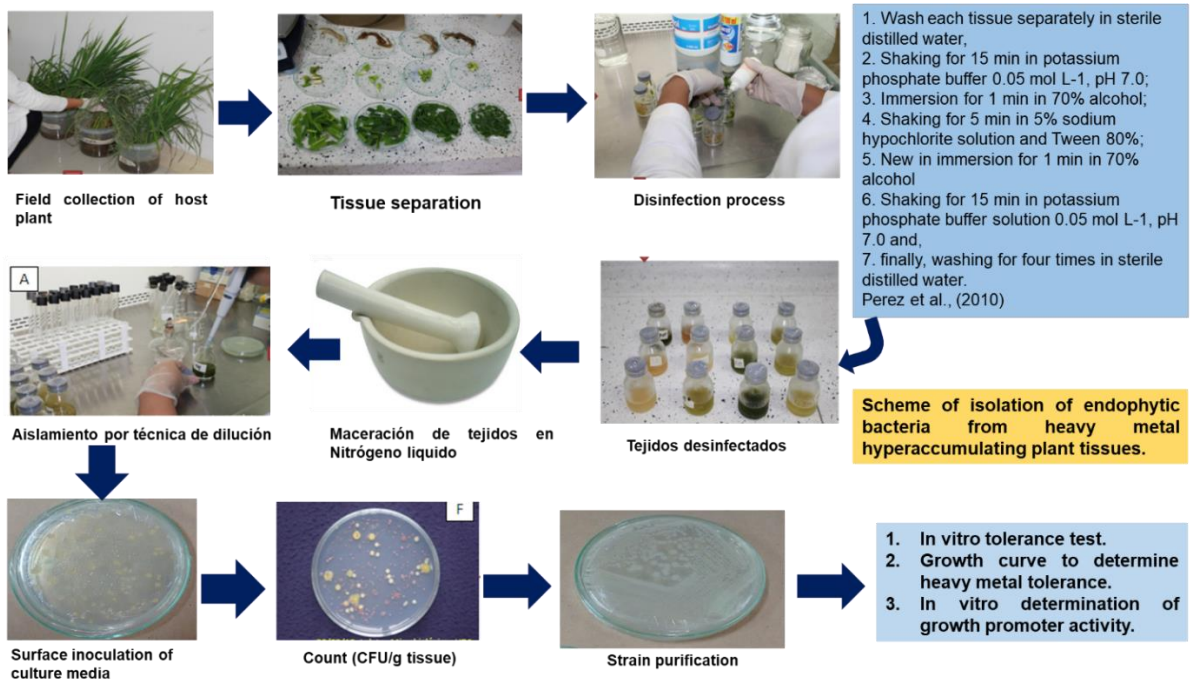


Figure 2. Scheme for isolation of endophytic bacteria from tissues of hyperaccumulating plants from heavy metal contaminated environments.

Once the heavy metal tolerant strains were selected in the in vitro test, the tolerance capacity of the endophytic bacteria to the heavy metal found in soil samples was tested and the concentrations used were always above the values found in vivo (in soil under natural conditions). The scheme used for the in vitro tolerance capacity test was as described in figure 3.

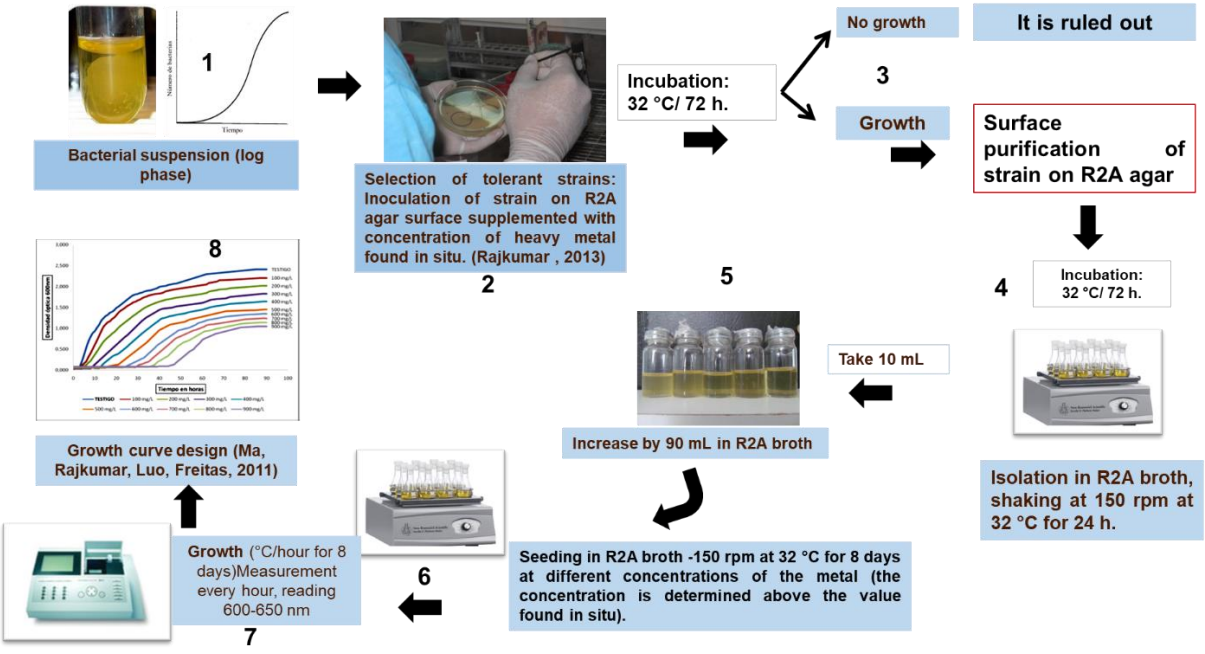


Figure 3. Schematic of tolerance test of endophytic bacterial isolates to different concentrations of the heavy metal, which is to be used in the form of salts.

After testing the in vitro tolerance capacity to heavy metals, those strains of endophytic bacteria with the highest tolerance to the metal were selected, followed by the in vitro evaluation test for growth promotion activity as described in figure 4 below.

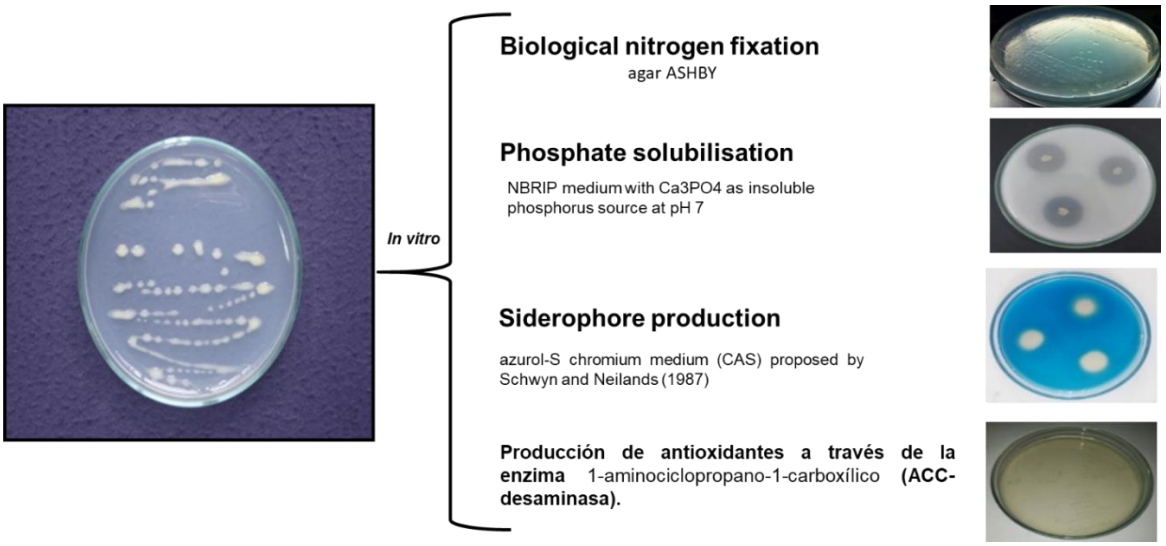
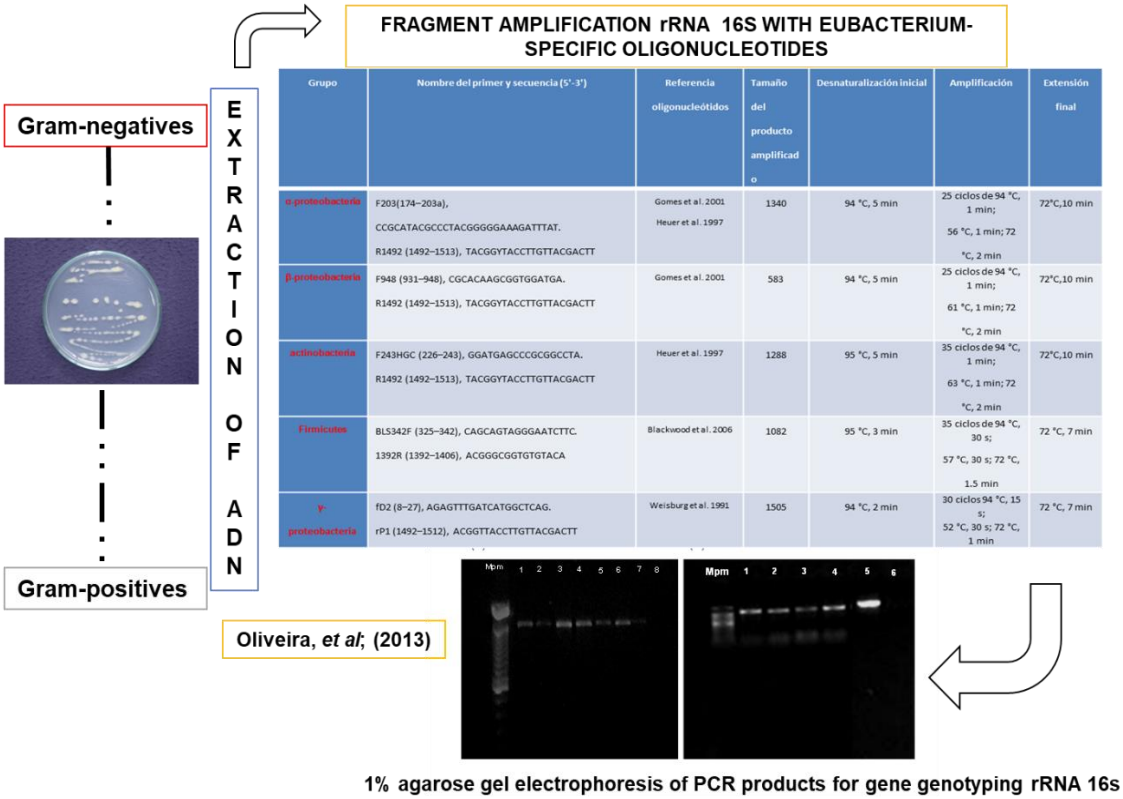


Figure 4. Process for obtaining heavy metal tolerant strains with the ability to promote plant growth in vitro.

Finally, once the in vitro plant growth promoting activity test was performed, strains of endophytic bacteria isolated from heavy metal contaminated environments were identified and evaluated in vitro for their ability to tolerate different concentrations of the metal and their qualitative ability to promote plant growth. The strains with these activities were used for their molecular identification by sequencing technique, as described in figure 5A and 5B.



Grupo	Nombre del primer y secuencia (5'-3')	Referencia oligonucleótidos	Tamaño del producto amplificado	Desnaturalización inicial	Amplificación	Extensión final
α-proteobacteria	F203(174–203a), CCGCATACGCCCTACGGGGGAAAGATTTAT. R1492 (1492–1513), TACGGYTACCTTGTTACGACTT	Gomes et al. 2001 Heuer et al. 1997	1340	94 °C, 5 min	25 ciclos de 94 °C, 1 min; 56 °C, 1 min; 72 °C, 2 min	72°C,10 min
β-proteobacteria	F948 (931–948), CGCACAAGCGGTGGATGA. R1492 (1492–1513), TACGGYTACCTTGTTACGACTT	Gomes et al. 2001	583	94 °C, 5 min	25 ciclos de 94 °C, 1 min; 61 °C, 1 min; 72 °C, 2 min	72°C,10 min
actinobacteria	F243HGC (226–243), GGATGAGCCCGCGGCCTA. R1492 (1492–1513), TACGGYTACCTTGTTACGACTT	Heuer et al. 1997	1288	95 °C, 5 min	35 ciclos de 94 °C, 1 min; 63 °C, 1 min; 72 °C, 2 min	72°C,10 min
Firmicutes	BLS342F (325–342), CAGCAGTAGGGAATCTTC. 1392R (1392–1406), ACGGGCGGTGTGTACA	Blackwood et al. 2006	1082	95 °C, 3 min	35 ciclos de 94 °C, 30 s; 57 °C, 30 s; 72 °C, 1.5 min	72 °C, 7 min
γ-proteobacteria	fD2 (8–27), AGAGTTTGATCATGGCTCAG. rP1 (1492–1512), ACGGTTACCTTGTTACGACTT	Weisburg et al. 1991	1505	94 °C, 2 min	30 ciclos 94 °C, 15 s; 52 °C, 30 s; 72 °C, 1 min	72 °C, 7 min

Figure 5A. Process steps of rDNA extraction, amplification of 16S rRNA sequences using specific oligonucleotides for heavy metal tolerant eubacteria and growth promotion.

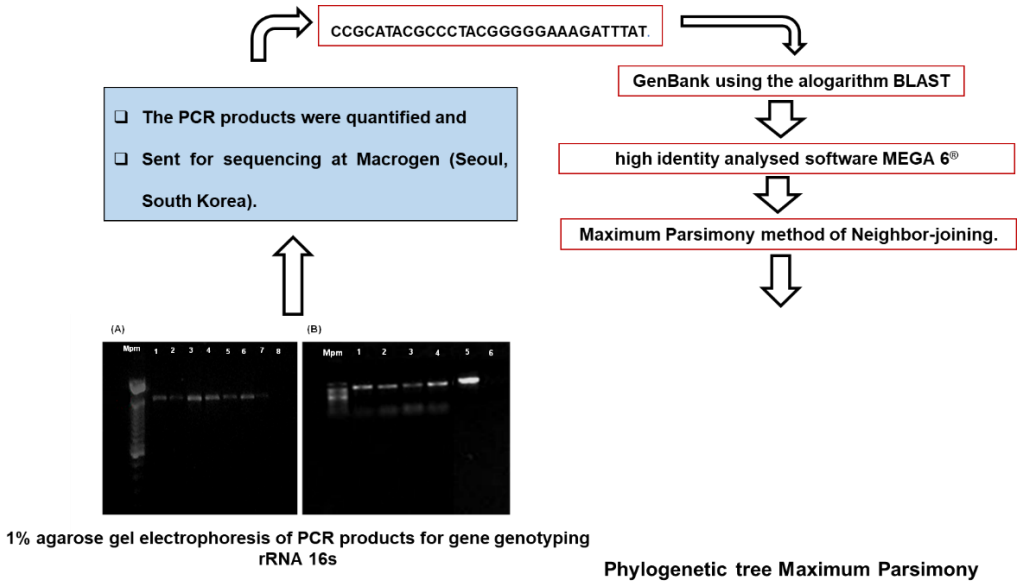


Figure 5B. Phylogenetic tree of maximum similarity of heavy metal-tolerant and growth-promoting endophytic bacteria.

As a result of the search for species of endophytic bacteria from different host plant tissues and their tolerance to different heavy metals, the following information is available in the genomic bank of the microbiological research laboratory attached to the research group in Agricultural Bioprospecting of the University of Sucre.

Table 1. Endophytic bacteria isolated from different plant species with the capacity to remediate heavy metals and promote plant growth in the Colombian Caribbean.

Host plant	Endophytic bacterium	Origin	Function	Reference
Rice	*Aeromonas hydrophila*	Department of Cordoba, Colombia	Nickel tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Pérez et al., 2015
Rice	*Pseudomonas putida*	Department of Cordoba, Colombia	Lead tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Pérez et al., 2015
Rice	*Burkholderia cepacia*	Department of Cordoba, Colombia	Lead tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Pérez et al., 2015
Paspalum arundinaceum	*Burkholderia cepacia*	Department of Bolivar, Colombia	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Pérez et al., 2016
Cyperus luzulae	*Ralstonia solanacearum*	Department of Bolivar, Colombia	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Pérez et al., 2016
Paspalum arundinaceum	*Bacillus cereus*	Department of Bolivar, Colombia	Mercury tolerance siderophore production	Pérez et al., 2018
Rice	*Burkholderia cepaceae*	Department of Cordoba, Colombia	Cadmium tolerance, nitrogen fixation, phosphate solubilisation and siderophore production	Ayubb et al., 2017
Rice	*Burkholderia cepaceae*	Department of Cordoba	Cadmium tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Ayubb et al., 2017
Aquatic macrophytes	*Lysinibacillus fusifomis*	Department of Sucre, Colombia	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Torres et al., 2019

Aquatic macrophytes	*Burkholderia cepacia*	Department of Sucre, Colombia	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Torres et al., 2019
Rice	*Bacillus Cereus*	Department of Cordoba	Cadmium tolerance, siderophore production	Pérez et al., 2022(a)
Pasture	*Burkholderia cepacia*	Department of Sucre, Colombia	Cadmium tolerance, siderophore production	Pérez et al., 2022(b)
Pasture	*Bacillus cereus*	Department of Sucre, Colombia	Led tolerance, siderophore production	Pérez et al., 2022(c)
Pasture	Burkholderia cepacea	Department of Sucre, Colombia	Led tolerance, siderophore production	Pérez et al., 2022(c)
Yam	*Burkholderia Cepacea*	Department of Sucre, Colombia	Cadmium tolerance, siderophore production	Pérez et al. 2023(a)
Rice	*Bacillus thuringiensis*	Department of Sucre, Colombia	Cadmium tolerance, siderophore production	Pérez et al. 2023(a)
Rice	*Bacillus cereus*	Department of Sucre, Colombia	Cadmium tolerance, siderophore production	Pérez et al. 2023 (b)
Yam	*Burkholderia cepacia*	Department of Sucre, Colombia	Cadmium tolerance, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC) production	Pérez et al. 2023(c)

CONCLUSIÓN

Endophytic bacteria are a natural biological resource associated with healthy plant tissues, without any symptoms of toxicity, which can be isolated, evaluated in vitro for tolerance to heavy metals and assessed for their growth-promoting capacity and their use as inoculants in plant species to improve physiological, nutritional and adaptive conditions in environments contaminated with these metals.

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AUTHOR CONTRIBUTION. Alexander Perez Cordero: experiment execution, data analysis. Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. All authors have read and approved the manuscript.

CONFLICT OF INTEREST. All the authors of the manuscript declare that they have no conflict of interest.

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