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Biotechnological Applications For Environmental Use With Endophytic Bacteria Associated With Plant Species

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ABSTRACT

Currently, there is an important demand for the acquisition of microbial genetic resources that would benefit industrial, agricultural and pharmaceutical sectors, but there are limitations of economic support from the government and the private sector in order to offer biotechnological packages with endophytic bacteria that offer products such as medicines, biofertilizers and biological control agents that would help the development of healthy crops free of residues, agrochemicals and toxic and dangerous pollutants for health and the environment. The objective of this research was to show the results of the evidence on the diversity of endophytic bacteria associated with agricultural plants and medicinal and aromatic plants, there in vitro evaluation on growth promotion, antimicrobial activity against phytopathogens and the ability to assist and help plants to reduce the effect of heavy metals. There is a genomic bank of endophytic bacteria with the ability to remediate heavy metals, provide nutrients to plants and produce antimicrobial substances for the control of phytopathogens.

Key words. *Genetic resource, endophytic bacteria, phytoremediation, biological control, plant growth promotion.*

INTRODUCTION

As stated by Perez and Chamorro, (2013), endophytic bacteria inhabit plant tissues for at least part of their life cycle without causing any harm to the host, establish symbiotic association and produce great benefits for plants. Endophytic bacteria fulfil a wide range of functions as plant growth promoters, biological control over a variety of phytopathogens, improve the efficiency of phytoremediation processes of toxic compounds in the rhizosphere. These microorganisms are inexhaustible sources of more than 20,000 biologically active compounds,

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which directly influence the performance and survival of host plants. Endophytic bacteria are reported to produce a number of metabolites such as antibiotics, secondary metabolites including some anti-tumour compounds, anti-inflammatory agents.

Endophytic bacteria play an important role in the induction of Induced Systemic Resistance (IRS) against plant pathogens, which includes the production of volatile organic compounds with a broad spectrum against fungi, bacteria and nematodes (Sheoran et al., 2015), as well as the production of siderophores, which are iron chelating compounds (Arora et al., 2001).

There is evidence demonstrating the potential use of endophytic bacteria as biocontrol agents of pathogens that produce diseases of economic importance in various crops (Sharma et al., 2009; Maksimov et al., 2011). For example, the genus Bacillus is one of the most studied and widely used groups as a biocontrol agent due to its root colonizing ability and effective sporulation capacity (Hassan et al., 2010; Hu et al., 2010).

Based on the importance of endophytic bacteria, the aim of this study was to present the experimental evidence that has been developed by the research group in Agricultural Bioprospecting on the biotechnological use of these bacteria to remediate heavy metals, the biocontrol of phytopathogens and the effect on the promotion of plant growth to improve the response system of plants against intoxication with heavy metals and substances produced by phytopathogens.

MATERIALS AND METHODS

1. **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.
2. **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).
3. **Experiments**

The protocols proposed by Pérez et al., (2023) and adjusted according to technology requirements were followed for the heavy metal tolerance and growth-promoting activity tests.

3.1. **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)Cl_2. 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.

3.2. 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.

4. **DNA extraction, amplification and 16S rDNA sequence endophytic bacteria tolerant heavy metals and antimicrobial activity.** It was carried out using the following techniques proposed by the research group:

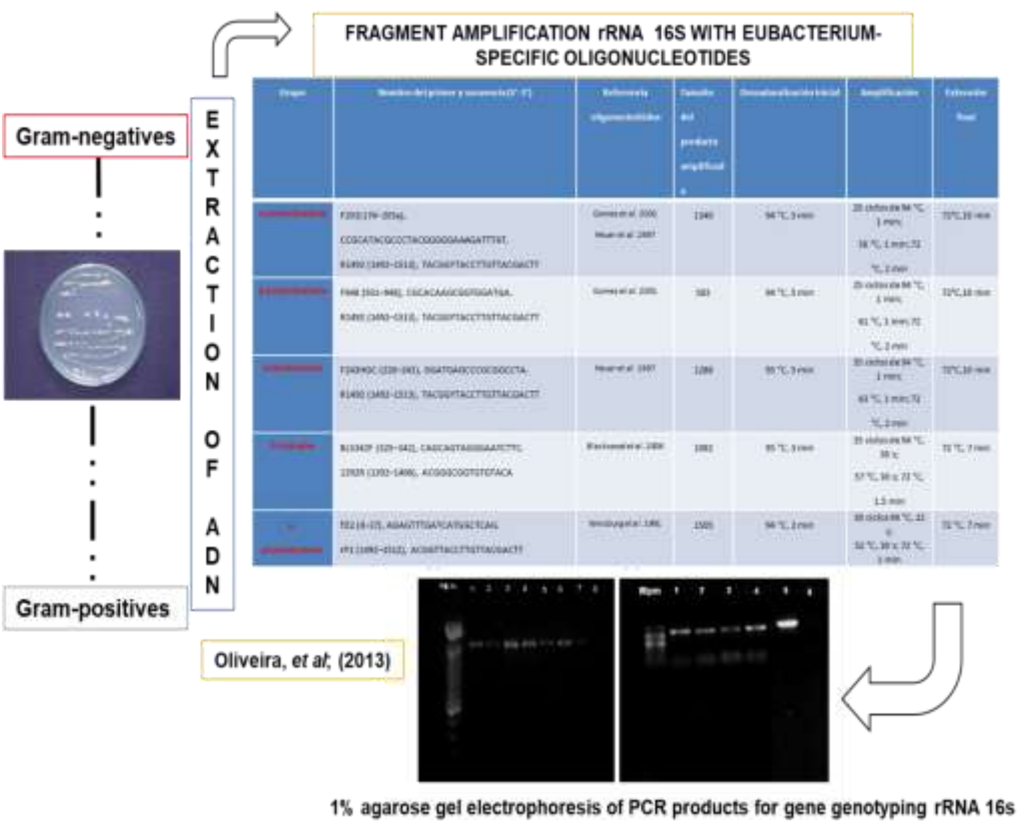

Figure 1. Process of genomic DNA extraction, amplification with bacterial domain-specific oligonucleotides, purification and quantification of PCR products.

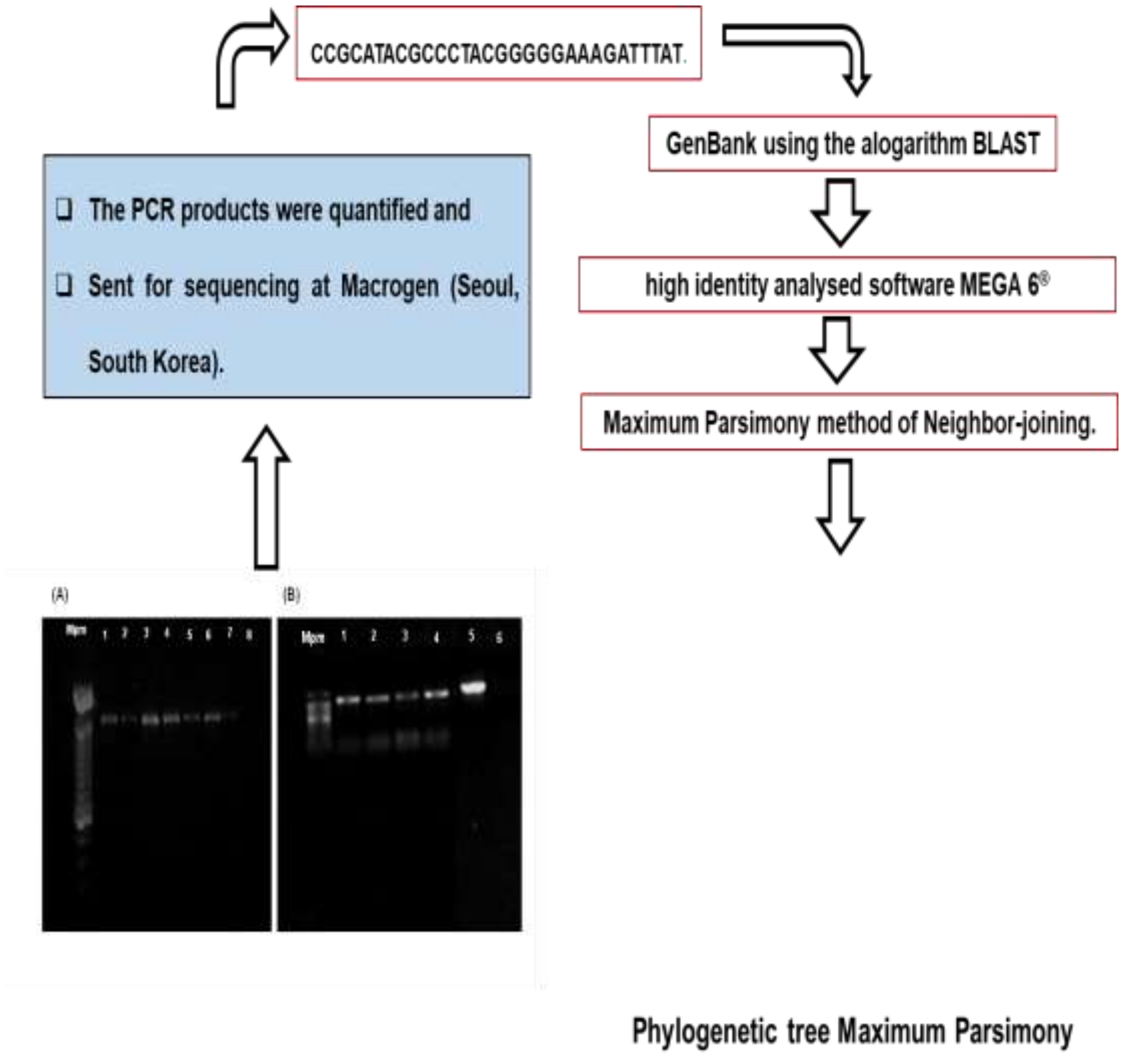

Figure 2. Construction of a phylogenetic tree of endophytic bacteria of biotechnological interest for environmental use.

RESULTDS AND DISCUSSION

The studies carried out on the isolation of endophytic bacteria associated with plant species of commercial interest, medicinal and aromatic plants, tropical pastures, horticulture, and aquatic plant species show the diversity of endophytic bacteria associated with different plant tissues and demonstrate the biotechnological importance of endophytic bacteria for their use in bioremediation and antimicrobial activity against phytopathogens (table 1).

Table 1. Biotechnological applications of endophytic bacteria for environmental use.

Isolated endophytic bacterial strain	Applications
Aeromonas hydrophila	Nickel tolerance, nitrogen fixation, phosphate solubilization and siderophore production (Pérez et al., 2015).
Burkholderia cepacia	Production of siderophores at 50 ppm concentration of mercury chloride and nickel chloride (Montes & Perez, 2022).

	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
	Actividad inhibitoria in vitro contra Burkholderia glumae (Pérez et al., 2022b)
	Lead tolerance, nitrogen fixation, phosphate solubilization and siderophore production (Pérez et al., 2015a).
	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production (Torres et al., 2019)
	Cadmium tolerance, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC) production. Pérez et al. 2023(c)
	Cadmium tolerance, nitrogen fixation, phosphate solubilization and siderophore production Ayubb et al., 2017
	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production Pérez et al., 2016
Burkholderia diazotrophica	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
Burkholderia tropica	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
Herbaspirillum rubrisubalbicans	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
	Antibacterial activity against Burkholderia glumae (Perez et al., (2022b)
	Production of siderophores at 50 ppm concentration of mercury chloride and nickel chloride (Montes & Perez, 2022).
Bacillus cereus	In vitro growth at 100 ppm in medium supplemented with mercury and nickel (Montes & Perez, 2022).
	Production of siderophores at 100 ppm concentration of mercury chloride and nickel chloride (Montes & Perez, 2022).
	Cadmium tolerance, siderophore production (Pérez et al. 2023 b)
	Cadmium tolerance, siderophore production Pérez et al., 2022(a)
	Led tolerance, siderophore production Pérez et al., 2022(c)
	In vitro experiments possess the ability to reduction of N_2 to ammonium (Perez et al.,2022c)

Bacillus pumilis	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
Bacillus thuringiensis	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
	Cadmium tolerance, siderophore production (Pérez et al., 2023a)
Lysinibacillus fusifomis	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production (Torres et al., 2019
Pseudomona fluorescens	Showed activity to fix nitrogen, solubilize phosphate and produce IAA (Barbosa-Garcia et al., 2022)
Pseudomona putida	Lead tolerance, nitrogen fixation, phosphate solubilization and siderophore production Pérez et al., 2015
Serratia marscencens	In vitro experiments possess the ability to reduction of N_2 to ammonium (Perez et al.,2022c)
Enterobacter cloacae	In vitro experiments possess the ability to reduction of N_2 to ammonium (Perez et al.,2022c)

Experiments carried out with endophytic bacteria isolated from heavy metal contaminated environments have led to the development of the following technique for the selection and evaluation of endophytic bacterial strains with the ability to tolerate different concentrations of heavy metals (figure 3).

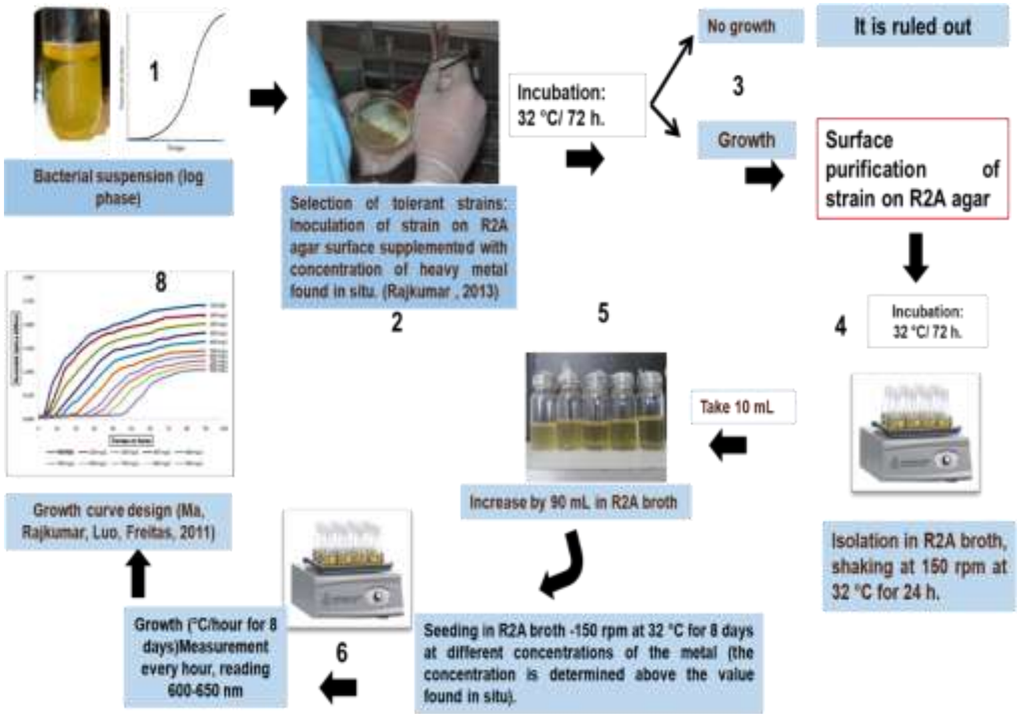

Figure 3. Techniques and process for the selection and evaluation of endophytic bacterial strains with the ability to tolerate different concentrations of heavy metals.

The results of in vitro tests carried out by the microbiological research laboratory on the tolerance capacity of endophytic bacteria show that the species B. cereus, P. aeruginosa and B. cepacia can tolerate up to 100 mg/L mercury chloride ($HgCl_2$) and nickel chloride ($NiCl_2$) despite not having been isolated from contaminated environments and are also iron and heavy metal scavengers with the production of siderophores, this allows us to conclude that these bacteria become a biological alternative in the removal of metals in environments contaminated with them.

Reports from in vitro evaluative studies indicate that mercury- and nickel-resistant Bacillus cereus combat metal stress by expressing proteins with different functions (Matilda et al., 2019), the S-layer protein is one of these proteins known to possess metal-binding properties (Pollmann et al., 2006).

According to Ma et al., (2011), the resistance and/or tolerance to high concentrations of metals shown by endophytes isolated from plants growing in contaminated environments is due to the fact that they are adapted to living under conditions of constant metal stress. Some authors have described the benefits of metal-resistant endophytes on plant growth and development, including: the reduction of high concentrations of ethylene in the plant, which, under stress conditions, causes growth inhibition; the production of IAA, which stimulates plant division.

Specialized metabolites called siderophores excreted into the extracellular space possess a spectrum of functional groups with metal-binding affinity (Koh et al. 2017). The ion exchange capacity of cell walls components, such as peptidoglycans and teichoic acids present in the cell envelope of Gram-positive bacteria, has also been shown to allow metal binding (Kern et al. 2010; Colak et al. 2011). Metal sequestration by siderophores and cell wall components prevents metal entry into the cell. Following metal entry into the cell, polyphosphates, which act as metal chelators, trap the metal inside the cell. Upon hydrolysis, the polyphosphate forms inorganic phosphate, which has an affinity for the metal and helps to export the metal out of the cell (Alvarez and Jerez 2004).

According to the findings by Pérez et al., (2002c), 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 ammonium ions, mechanisms used by microorganisms to promote growth in the analyzed grass species.

The search for endophytic bacteria with growth promoting activity for tropical pastures has found the presence of endophytic bacteria associated with different tissues of Botriochloa pertusa with nitrogen fixing activity, ammonium ion production, phosphate solubilization, phytohormones production and other activities (figure 5).

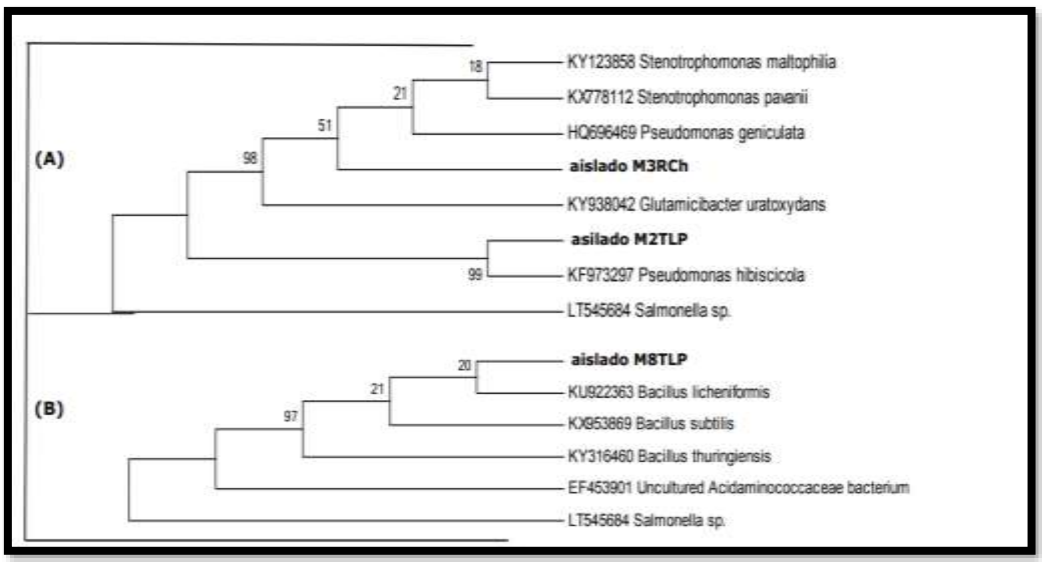

Figure 5. Phylogenetic tree of the M3RCh and M2TLP isolates and their relationships with species of bacteria of the genus Pseudomonas (**A**) and of the isolate M8TLP and its relationship with bacterial species of the genus Bacillus (**B**). M: morphotypes; R: root; T: Stem, Ch: Chapinero and LP: Las Peñas. Source: Perez et al., (2018).

Different assays carried out have shown that the endophytic bacterial species Burkholderia cepacia isolated from different tissues has a broad tolerance activity to different heavy metals as demonstrated by the different findings (table 2).

Table 2. Bukholderia cepacia isolated from different plant species with the capacity to remediate heavy metals and promote plant growth in the Colombian Caribbean. Source: Perez et al., (2023).

Host plant	Endophytic bacterium	Origin	Function	Reference
Rice	*Burkholderia cepacia*	Department of Córdoba, 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
Rice	*Burkholderia cepaceae*	Department of Córdoba, Colombia	Cadmium tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Ayubb et al., 2017
Rice	*Burkholderia cepaceae*	Department of Córdoba	Cadmium tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Ayubb et al., 2017
Aquatic macrophytes	*Burkholderia cepacia*	Department of Sucre, Colombia	Mercury tolerance, nitrogen fixation, phosphate solubilization and siderophore production	Torres et al., 2019
Pasture	*Burkholderia cepacia*	Department of Sucre, Colombia	Cadmium tolerance, siderophore production	Pérez et al., 2022(a)
Pasture	Burkholderia cepacea	Department of Sucre, Colombia	Led tolerance, siderophore production	Pérez et al., 2022(b)
Yam	*Burkholderia Cepacea*	Department of Sucre, Colombia	Cadmium tolerance, siderophore production	Pérez et al. 2023(a)
Yam	*Burkholderia cepacia*	Department of Sucre, Colombia	Cadmium tolerance, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC) production	Pérez et al. 2023(b)

Phylogenetic analysis from the 16S rRNA gene for endophytic bacteria with plant growth promoting activity (figure 6) shows that the 21 morphotypes isolated from the tissues of the different rice varieties showed high similarity with the sequences stored in the GenBank database and identified as follows: Burkholderia cepacia (F7T3, F2T1, F7T1); B. diazotrophica (MOCR1, F4T2, F7R1, F4H2); B. tropica (F2R2, F4T3, MOCH2, F4H4); Pseudomona fluorescens (MOCH3, F4R1, F2R1); Bacillus pumilis (MOCH4, F2H1, F2T2); B. thuringiensis (F7T2, MOCT2, F7H1); Herbaspirillum rubrisubalbicans.

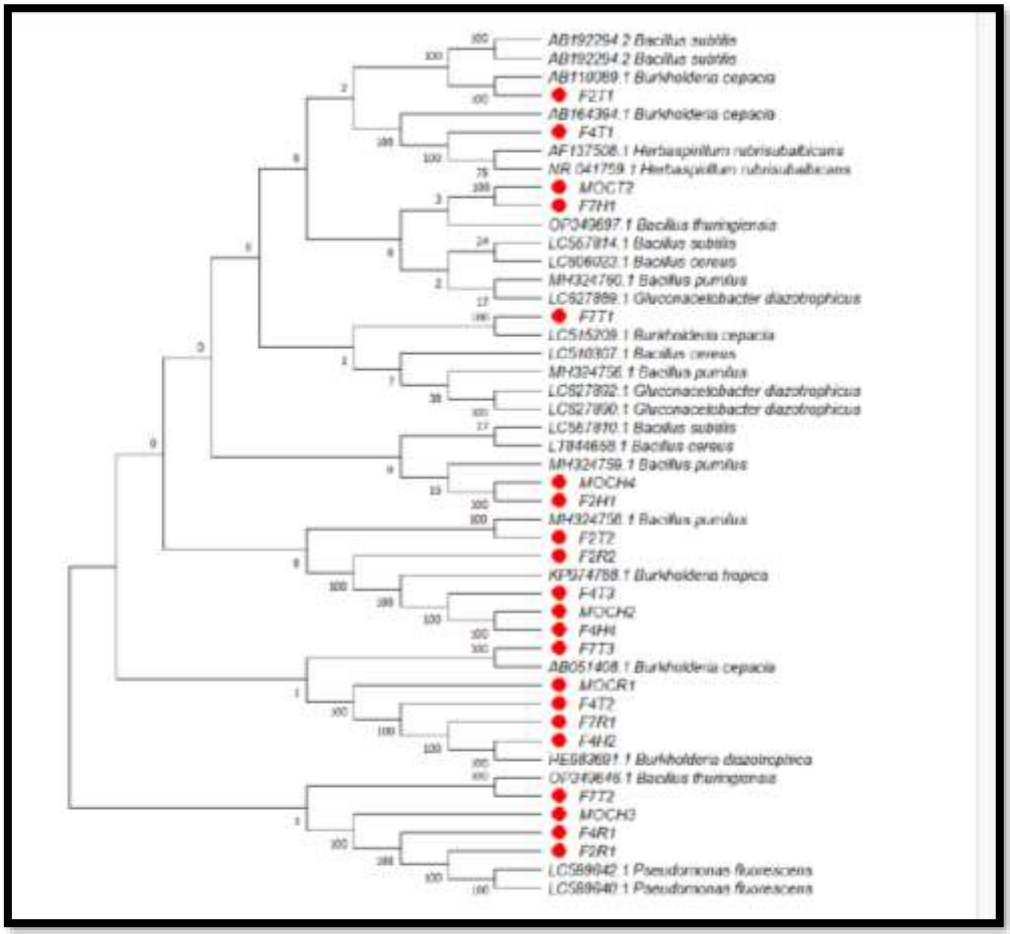

Figure 6. Dendogram using the Neighbour-Joining model from 16S rRNA sequences of plant growth-promoting endophytic bacteria isolated from rice cultivars. Source: Barbosa-Garcia et al., 2023.

Finally, in the search for bioactive compounds such as secondary metabolites with inhibitory activity, endophytic bacteria associated with agricultural and medicinal plants have been isolated for the extraction of these compounds. The results have allowed the development of techniques for obtaining these compounds, as well as the in vitro evaluation of their antimicrobial activity and the selection of species for subsequent use in the management of phytosanitary problems in the region (figure 7). With the publication of the results of the antimicrobial activity of compounds produced by endophytic bacteria, there is scientific evidence of the processes for the extraction of microbial metabolites and the inhibitory activity shown on phytopathogens associated with phytosanitary problems in commercial crops in the region. The results of in vitro evaluations confirm que The results of this study show a high diversity of culturable endophytic bacteria with the ability to inhibit in vitro the growth of the phytobacterium Burkholderia glumae causing bacterial blast in rice (Perez et al., 2023e).

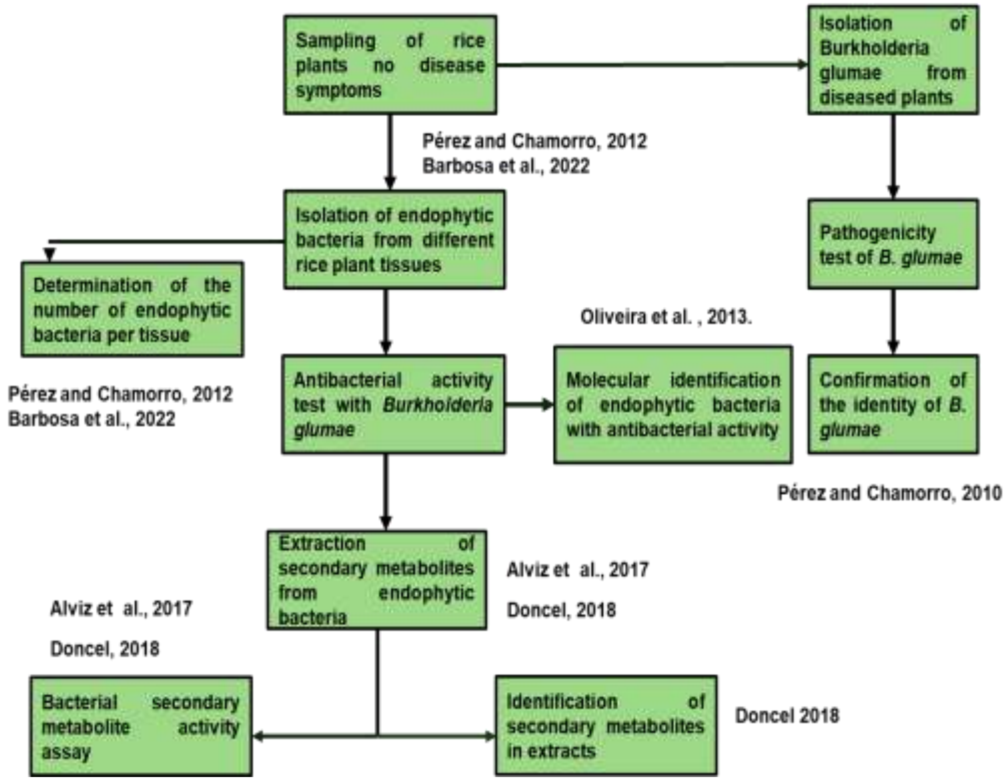

Figure 7. Stages in the process of evaluation of secondary metabolite-like compounds produced by endophytic bacteria isolated from plants against phytopathogens. Source: Pérez et al., (2023e).

CONCLUSION

There is a high diversity of endophytic bacteria associated with different plant tissues under different environmental conditions. Despite the existence of experimental and scientific evidence on the benefits of these bacteria for assisting heavy metal remediation processes, the bio-control of phytopathogens and the capacity to induce resistance mediated by the release of compounds or substances to promote plant growth, the benefit and environmental use of these bacteria, as well as their use as a biotechnological package for agro-sustainable production and environmental management of pollution problems, is still unknown.

As stated by (Knief et al.; 2011), with the use of techniques such as metaproteomics, metaproteogenomics and metatranscriptomics, the genomic potential and functionality of endophytic bacteria can be better deciphered and a deeper insight into the benefits of these interactions with plants can be gained. These tools will provide insight into the overall expression of proteins (metaproteomics) or mRNAs (metatranscriptomics) from microbial communities. Metaproteogenomics will link the proteome and genome of the environmental sample, which will allow the identification of various proteins. Recently metaproteogenomics techniques have been used to study endophytic bacterial communities in the phyllosphere and rhizosphere of important commercial food crops worldwide.

Additionally, endophytic bacteria can protect crops from pathogenic pests and diseases in what is known as biological control. Therefore, bioremediation, biological control, biostimulation and bionutrition are applications in the agricultural industry that promote more productive, nutritious and healthy crops with less negative effect on the environment.

Another important aspect that needs to be addressed through research is global warming and the possible shortage of food as a result of human activities. It is no secret that due to the contamination of soils by the use of agrochemicals, an imbalance has been generated in the physical (erosion), chemical (nutrient deficit, acidity, salinity, etc.) and biological aspects of the soil (deficiency of organic matter) and microorganisms, which have reduced the production capacity of the soil and therefore the reduction of harvests.

This is why it is necessary to carry out research aimed at identifying the potential of bioprospecting with the use of endophytic bacteria for the development and application of pathogen biocontrollers, biofertilizers and bioremediators to contribute to the sustainability of agricultural activities. Knowing all these alternatives that exist to carry out activities that provide solutions to the environmental situation, which is becoming increasingly worrying, can guarantee a sustainable future with a better quality of life, as it will be possible to control the release of polluting compounds into bodies of water, soil and air.

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