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In Vitro Evaluation Of Mercury Tolerance By Endophytic Bacteria
Título Breve: Endófitas Tolerancia A Mercúrio

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ABSTRACT

The objective was to isolate endophytic bacteria from plant species present in soils contaminated with mercury in southern Bolivar, Colombia, and to evaluate in vitro their tolerance capacity to different concentrations of mercury. From each sampling site, roots, stems, leaves and flowers of the plants present were collected. The concentration of mercury in soil and plant tissues was determined using the instrumental technique of cold vapor atomic absorption spectrophotometry. The amount of endophytic bacteria per tissue was determined as CFU/g of soil. The tolerance of endophyte bacterial isolates to different concentrations of mercury was performed in liquid Tris-low phosphate buffer (TLP) medium. The average mercury concentration in soil was 5.9 ± 5.2 and in tissues 4.1 ± 2.2 mg/kg. The amount of endophytic bacteria found ranged from 5.0 107 ± 3.0 104 CFU/ g of tissues. The isolates identified as Bacillus cereus strain ML259; Bacillus mycoides O-1 and Bacillus cereus strain LB1016, showed in vitro tolerance capacity up to 500 and 400 mg/mL of HgCl2. The predominant plant species found in the soil corresponded to Melochia parvifolia, accumulating mercury in concentrations of 4.06 in the root, 3.83 in the stem, 3.6 in the leaf and 2.33 mg/kg in the flower. This plant species becomes a mercury indicator and accumulator plant and a possible alternative to remediate mercury-contaminated soils using the bioremediation technique assisted by endophytic bacteria for the management of soils contaminated with this metal.

Key words: Bacteria, soil, plant tissue, mercury.

INTRODUCTION

In Colombia there are many sources of contamination of water bodies and soil (Reyes et al., 2016, Kobielska et al., 2018). However, due to its repercussions, persistence and the difficulty of treatment mercury is a pollutant that should be considered with special attention. The main cause of mercury contamination in Colombia has to do with illegal mining practices, especially gold mining. Despite representing such activities, an important source of employment and survival for many communities, it also represents a complex source of environmental impacts that generate harmful effects on water bodies, generating negative consequences on aquatic fauna and the environment in general (Gafner, 2018).

In Colombia, every day generates excessive applications of chemical substances for the exploitation of minerals such as gold, inadequate practices of industrial and agricultural wastewater disposal, in water bodies that are fundamental for the population dynamics of animals, plants and humans (Vargas and Marrugo, 2019). Mercury (Hg) in vegetables can absorb and accumulate in their tissues (Singh et al., 2010) and when consumed causes serious health problems, including reduced growth and development, cancer, organ damage, nervous system damage and, in extreme cases, death (Barakat, 2011). Hg in the environment has increased considerably, reaching concentrations that affect ecosystems and human health. In its pure form it is known as elemental mercury (Hg0), which easily volatilizes forming colorless and odorless vapors (Gaiolí, et al., 2012). This metal, when it reaches nature and some time has elapsed, is transformed to methylmercury (CH_3Hg^+). Methylmercury, like other organometallic compounds, is liposoluble and, consequently, has a high toxicity, since it can easily pass through biological membranes, particularly the skin, and from here the incorporation of the metal into the trophic chain continues (Posada and Arroyave 2006).

According to a study conducted by Pérez et al. (2015), the concentration of mercury present in the soil near the Santa Cruz Mine, corresponds to values of 4.7 mg-kg-1, placing these soils within the toxic category, because the values reported by the Ministry of Environment and Sustainable Development for the particular case of mercury is 0.02 mg/L, and the value reported by Pérez et al, (2016), is above (MADS, 2015) the values allowed (Hg 0.00003 mg/L) by the United States Environmental Protection Agency (USEPA) (Nguyen et al., 2013).

The effects of the presence of mercury in soils and water bodies are dire. In addition to the contamination of the water environment, already regrettable in itself, there is a chain of negative consequences both in aquatic fauna and in the health of people of

dimensions that cannot be ignored, has led to the use and application of numerous remediation strategies and the choice of one technique or another will be subject to the cost vs. benefit analysis, so it is necessary and urgent to search for alternatives to remove mercury from soil and bodies of water (Zhao et al., 2016), under the criteria of low cost and environmentally friendly.

The interaction between microorganisms-plant-soil has aroused interest due to the complex interaction between metal-accumulating plants and their associated microorganisms, which has led to the use of new technologies such as microorganism-assisted phytoremediation. Phytoremediation is an emerging technology that uses plants and their associated microbes to clean up contaminants in soil, water and air. In recent years, endophytic bacteria-assisted phytoremediation has been highly recommended for the cleanup of metal-contaminated soils, as bacteria can alleviate metal toxicity in plants through their own metal resistance system and facilitate plant growth under metal stress (Sheng et al., 2008).

Endophytic bacteria enhance plant growth in metal-contaminated by the following mechanism: a) directly, by producing beneficial substances for plant growth, including solubilization/transformation of mineral nutrients (phosphate, nitrogen and potassium) (Sessitsch et al., 2013), production of phytohormones, siderophores and specific enzymes; and b) indirectly, by controlling plant pathogens or inducing systemic plant resistance against pathogens. In addition, they also alter the metal accumulation capacity of plants by excreting metal-immobilizing extracellular polymeric substances, as well as metal-mobilizing organic acids and biosurfactants (Ma et al., 2016).
The acceptance of endophyte bacteria-assisted phytoremediation as a new biotechnological approach to bioremediation is very welcome due to its potential for sustainability (Emenike et al., 2017), therefore, this research aimed to isolate endophyte bacteria from different plant tissues from areas near the Santa Cruz Mine, Bolivar and evaluate the in vitro tolerance capacity to different concentrations of this metal.

MATERIALS AND METHODS

Collection of the study material. The sampling was carried out in the south of the department of Bolivar in areas near the Santa Cruz Mine; the geographical coordinates of this district correspond to: 08º 46.42´08" North latitude and 74º10.21´,02" East longitude (Figure 1). At the time of sampling was found as a species adapted to the condition of the soil, corresponded to the plant species Melochia parvifolia, registered with voucher code No. 003752 of the herbarium collection of the University of Sucre.

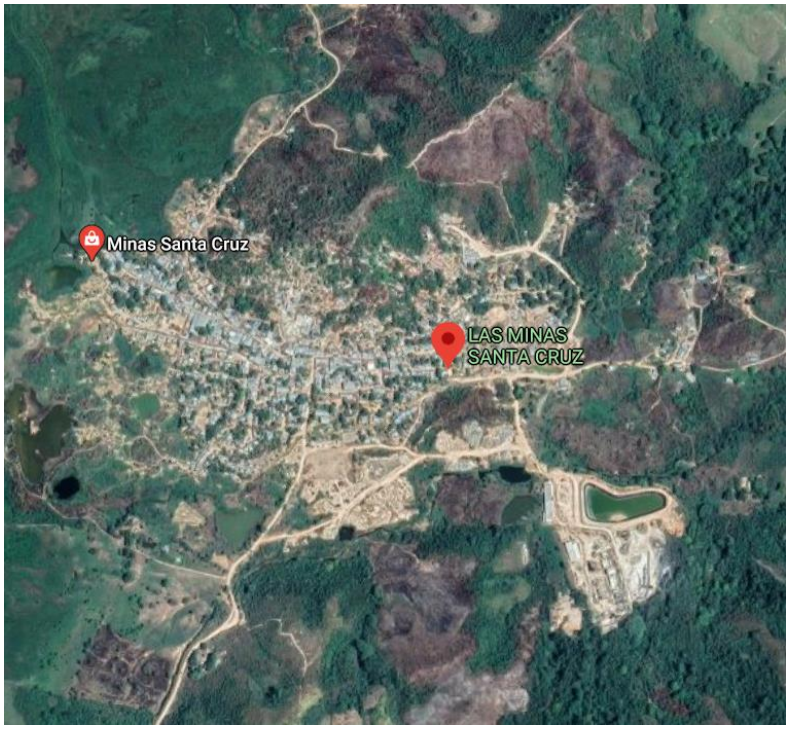

Figure 1. Location of Santa Cruz Mines, South of Bolivar Department, Colombia.

At each selected site, a random zig-zag sampling was carried out, where soil and complete plants (root, stem and leaves) of the species found at the time of sampling were sampled. For the collection and selection of plant material, we selected those species that were found in good phytosanitary condition and without symptoms of mercury phytotoxicity. Soil and plant samples were labeled with georeferencing of the sampling site. Part of these samples were sent to specialized laboratories to determine the mercury concentration and the other part of the samples were taken to the microbiological research laboratory of the University of Sucre for the respective analyses.

Determination of mercury levels in soil and plant tissues. The determination of mercury levels in soil and plant tissue samples was performed in triplicate. The steps for the analysis were as follows: Plant samples were separated by tissues (root, stem, leaves and flower), which were washed with distilled water to remove mineral particles adsorbed on their surface. Then, each tissue was deposited in paper bags and dried in an oven at 60°C for 24 h. To determine the total mercury in these samples, 0.5 g of dry material was taken and an acid mixture HNO_3/H_2O_2 (5+2mL) was added. On the other hand, from the previously dried soil, 0.5 g were taken and 10 mL of 65% HNO3 were added. Both soil and plant samples were processed in a Milestone ETHOS TOUCH

127697 series microwave oven and total mercury was analyzed by cold vapor atomic absorption spectrophotometry, according to procedures described in Marrugo-Negrete et al. (2015).

Isolation of endophytic bacteria. The isolation of endophytic bacteria from the different plant tissues was performed following the protocols described by Pérez et al. (2016) with modifications. The population density of endophytic bacteria was expressed as CFU/g tissue, and was estimated by direct counting of colonies on plates. During counting, colonies were observed and selected for shape, surface appearance, color and size (Perez et al. 2016).

Mercury Sensitivity Assays. Isolates of endophytic bacteria were used to evaluate the sensitivity and tolerance to different concentrations of mercury, for which seeding of these was performed on the surface of R2A agar medium (Pérez et al. 2016), supplemented with increasing concentrations of $HgCl_2$ up to 250mg/L. The assays were incubated at 37°C for 7d. After this time, the colonies that grew were re-isolated and purified on R2A agar, for use in subsequent assays.

Tolerance of bacteria to mercury. From the results of the sensitivity test, the isolates that grew in the highest concentration of mercury were taken and were subjected to tolerance tests of the minimum and maximum growth concentration for which aliquots of suspensions of endophytic bacteria in logarithmic phase were inoculated on Tris-Low Phosphate Buffer (TLP) medium proposed by Rathnayake et al. (2013) at concentrations of 100 to 500mg/L of $HgCl_2$. TLP medium without $HgCl_2$ was used as a control. The experiment was performed in triplicate, which was incubated in shaking at 150rpm at 32°C for 120 hours (Zhang et al. 2011). The growth of endophytic bacteria was determined by turbidimetry at 600nm every hour for four days.

Identification of rhizosphere and endophytic bacteria with tolerance capacity to different mercury concentrations. The isolates of endophytic bacteria that showed greater tolerance to different concentrations of mercury were used for molecular identification. Before starting the DNA extraction process, the isolates were differentiated by Gram staining technique. Genomic DNA extraction was performed according to the protocol described by (Oliveira et al. 2013). The amplification of 16S rDNA of endophytic bacterial communities was performed by PCR technique**.** The amplification of rDNA fragments was carried out with the use of specific oligonucleotides for eubacterial groups (Oliveira et al. 2013). PCR products were sent for sequencing to Macrogen Company (Seoul, South Korea) on an automated sequencer with 3730XL capillary. The nucleotide sequence entities obtained were

compared with those stored in National Center for Biotechnology Information (NCBI) databases. Base alignment was performed by means of the clusteal W program and analysis and correlation with the MEGA 6® program. Phylogenetic inferences were obtained by distance and maximum parsimony Neighbor-joining with bootstrap test (1,000 replicates). The trees for the phylogenetic analysis of the sequences were reconstructed with the MEGA 6.0® program.

Statistical analysis. The results were expressed as the mean ± SD, an analysis of variance was performed, previously determining the normality criterion by means of the Shapiro Wilks test (5%). Significant statistical differences were determined by Tukey's test ($p<0.05$). All data obtained and the statistical analysis were analyzed in the free version of InfoStat software.

RESULT AND DISCUSSION

Determination of mercury in soil and plant tissues. The rhizospheric soil mercury content ranged from 5.9±5.2mg/kg ~~of soil~~ and in Melochia parvifolia tissue was on average 4.1±4.0 in root, 3.8±3.9 in stem, leaf 3.7±3.5 and flower 2.2±3.5mg/kg in flower. The normality criterion was corroborated using the Shapiro-Wilks test (p-value: 0.8480). The ANOVA shows significant statistical differences (p-value<0.05) between mercury levels (mg/kg) per plant tissue. Tukey's test shows significant statistical differences (p-value<0.05) between mercury levels (mg/kg) per plant tissue, finding higher average values in roots of 4.06 and lower in flowers with values of 2.33mg/kg (Figure 2).

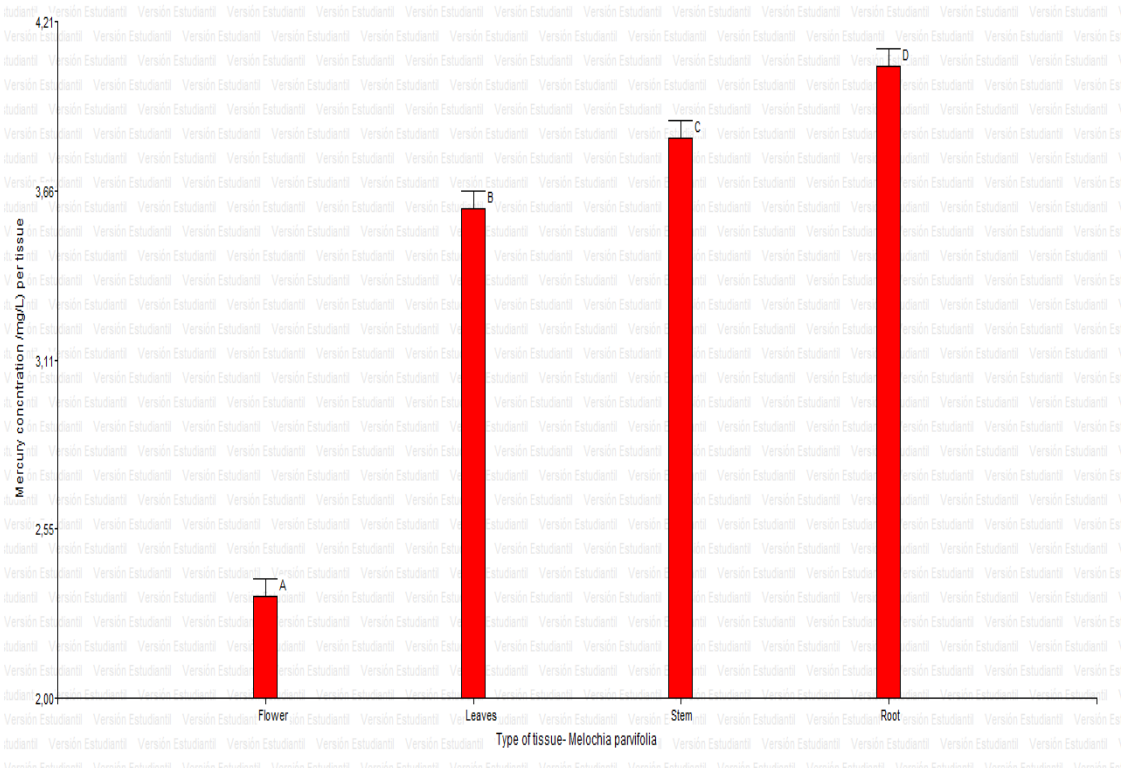

Figure 2. Mercury levels (mg/kg) in different tissues of Melochia parvifolia adapted in Hg-contaminated environment in south of Bolivar, Colombia.

The average mercury levels found in soil taken from an area contaminated with mercury near the Santa Cruz Mine, Sur de Bolívar, Colombia are 5.6mg/kg of soil, which is above the permissible values at which it can cause adverse effects on human health. Investigations of heavy metals in this critical area of the Colombian Caribbean have also shown the presence of heavy metals in sediments with mercury levels of 7.67µg/g, which is above the permissible standard (0.5µg/g). The maximum contamination levels allowed by the Ministry of Environment and Sustainable Development for the particular case of mercury (0.02mg/L) are well above (MADS, 2015) the values allowed for Hg 0.00003mg/L by the United States Environmental Protection Agency (USEPA) (Nguyen et al. 2013). Regarding the average values in plant tissues these ranged from 2.33 to 4.06mg/kg, which are above the normal values (0.005-0.2mg/kg) of international reference for plant tissues (Kabata-Pedias, 2011).

With respect to the physical and chemical parameters of the soil during sampling, the results indicate a soil with an extremely acid pH; organic matter, potassium, magnesium and potassium contents, very poor; very high phosphorus content; calcium and magnesium with null values; abundant sodium content; exchangeable aluminum,

aluminum saturation percentage and sulfur with excessive contents; moderate cation exchange capacity; and silt loam texture. The presence of extremely acidic pH values in the soil of the study area possibly indicates that mercury absorption is high and, as a consequence, easily transported in the soil by runoff to surrounding water bodies, which is in agreement with that reported by Kabata-Pendias (2011) and Terán-Mita et al. (2013). Excessive aluminum content in these soils is closely related to the acid pH of the soil, which could cause inhibition of root growth and, as a result, reduced water and nutrient uptake by plants (Casierra & Aguilar, 2007).

Population density of endophytic bacteria by mercury levels. From the plant species found and reported as Melochia parvifolia voucher N°003752, a total of 35 isolates of endophytic bacteria were isolated. The ANOVA shows significant statistical differences (p-value<0.05) between the amount of endophytic bacteria by tissues evaluated, finding higher average values of these bacteria population of endophytic bacteria in flower with 4.0 x 10^7 CFU/g tissue, while for the root tissue the lowest values were obtained with 5.0 x 10^4CFU/g tissue (Figure 3).

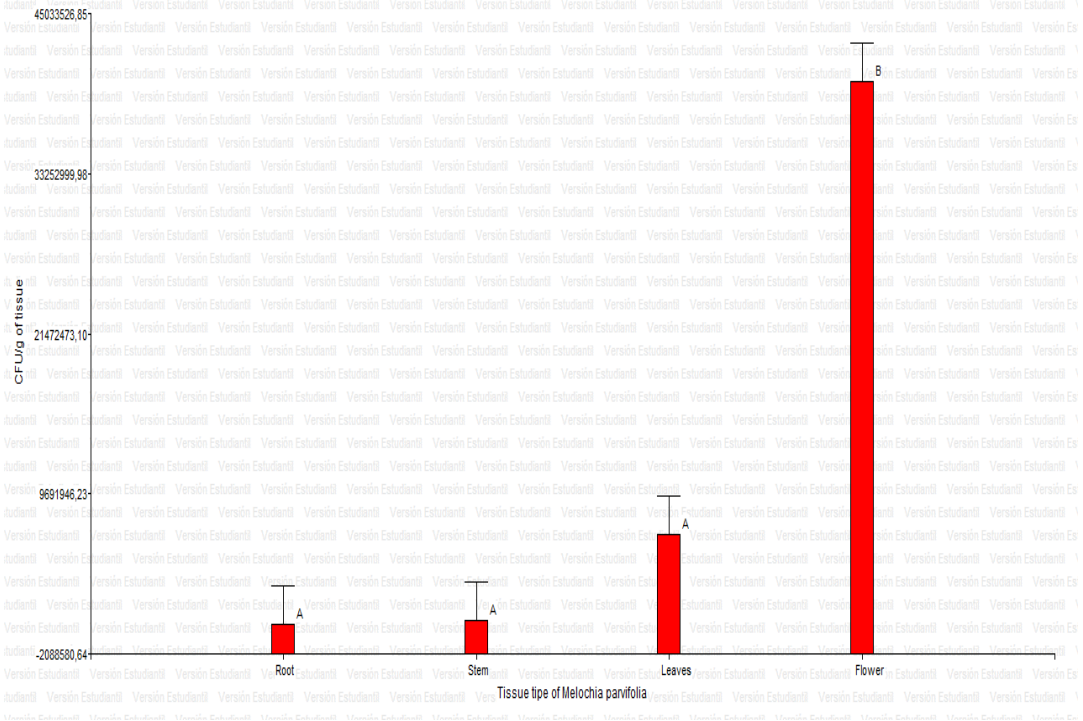

Figure 3. Amount of endophytic bacteria per tissue in the plant species Melochia parvifolia adapted to an environment contaminated with Hg in south of Bolivar, Colombia.

With respect to the levels of mercury and quantity of bacteria per tissue, a higher quantity of these bacteria was observed in the flower (4.0x107CFU/g tissue) where the concentration of mercury accumulated in the tissue was 2.33mg/kg tissue, with respect to the root where lower densities of endophytic bacteria were found (5.0x104CFU/g tissue) with mercury values of 4.06mg/kg tissue.

Evaluation of the sensitivity of endophytic and rhizospheric bacteria to mercury. A total of five isolates showed ability to grow on the surface of R2A culture medium supplemented with 250mg/mL concentrations of HgCl2. The isolates were identified as: MpA6RHgLIM, MpA4THgLIM, MpA10RHgLIM, MpA1RHgLIM, MpA3HHgLIM. The isolates MpA6RHgLIM, MpA10RHgLIM and MpA1RHgLIM were obtained from root; MpA4THgLIM and MpA3HHgLIM from stem and leaf respectively of Melochia parvifolia species.

Growth curve. MpA6RHgLIM, MpA4THgLIM, MpA10RHgLIM, MpA1RHgLIM, MpA3HHgLIM isolates of Melochia parvifolia were re-inoculated on TLP medium at concentrations of 100 to 500mg/L HgCl2. Figure 4 shows the tolerance growth curve of the four endophytic bacterial isolates to different concentrations of mercury.

The isolates MpA4THgLIM and MpA10RHgLIM showed tolerance up to 500mg/L, respectively. Isolate MpA1RHgLIM grew at 400, MpA6RHgLIM at 350 and finally MpA3HHgLIM up to 300mg/L (Figure 5).

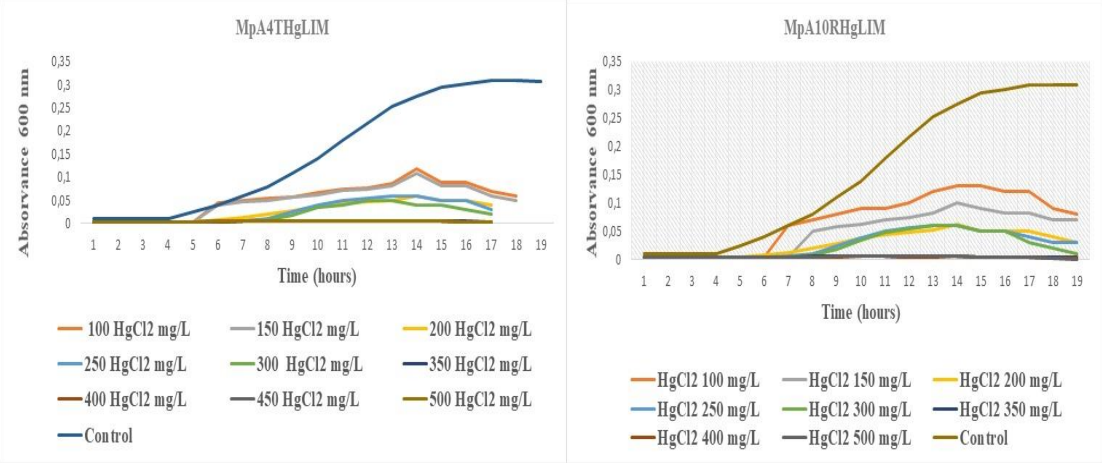

Figure 4. Growth curve of the endophytic bacterial isolates MpA4THgLIM and MpA10RHgLIM at a concentration of 500mg/L of mercury in the form of HgCl2, respectively. Mp: Melochia parvifolia, A: isolate, R: root, T: stem, Hg: mercury; LIM: Microbiological Research Laboratory.

In figure 4, the growth behavior of the isolates MpA4THgLIM and MpA10RHgLIM at a concentration of 500mg/L of mercury in the form of HgCl2 is observed. Comparing the behavior of the control with respect to the two isolates, an adaptation phase of five hours was observed in both, while in the control it lasted 4 hours. For both isolates, maximum growth was observed at concentrations of 100 and 150mg/L, respectively, and moderate growth was observed at concentrations of 200 to 300mg/L. After 19 hours from the beginning of the experiment, the growth of the isolates entered a phase of decay under concentrations of 100 to 300mg/L with respect to the control. With respect to concentrations 350 to 500mg/L, a slight growth was observed with respect to the other concentrations evaluated.

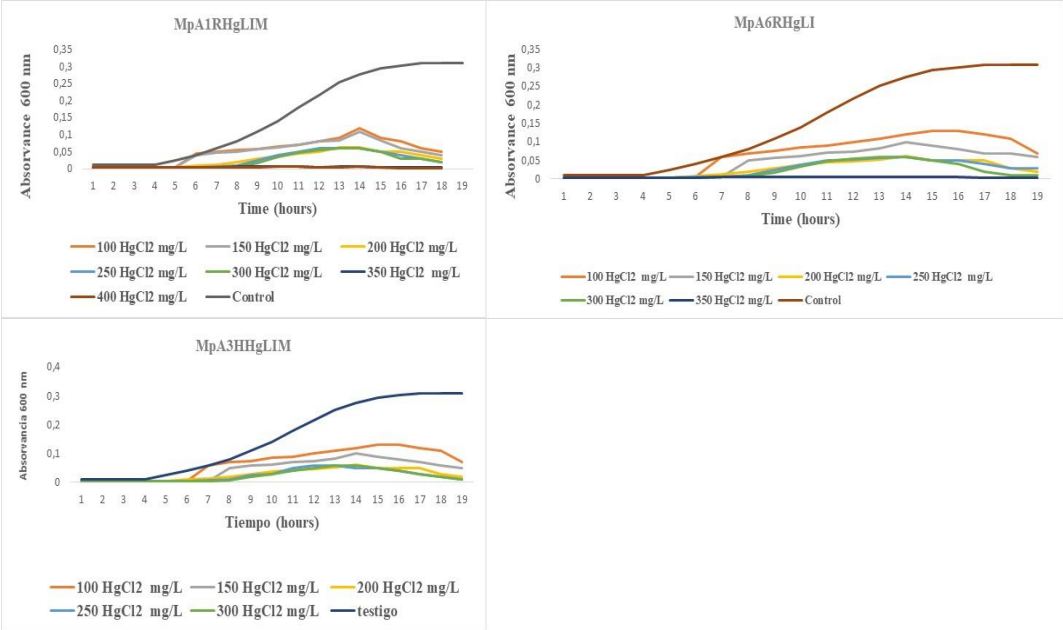

Figure 5. Growth curve of the endophytic bacterial isolates MpA1RHgLIM, MpA6RHgLIM and MpA3HHgLIM at concentrations of 400, 350 and 300mg/L of mercury in the form of HgCl2, respectively. Mp: Melochia parvifolia; A: isolate; R: root; T: stem; H: leaf; Hg: mercury; LIM: Microbiological Research Laboratory.

The figure 5 shows the growth behavior of the isolates MpA1RHgLIM, MpA6RHgLIM and MpA3HHgLIM at concentrations of 400, 350 and 300mg/L of mercury in the form of HgCl2, respectively. The growth behavior of the control in relation to the three isolates, an adaptation phase of 5 hours was observed in the three isolates, while in the control it lasted 4 hours. Maximum growth was observed at concentrations of 100 and 150mg/L, respectively, and moderate growth at concentrations of 200 to 300mg/L. For MpA1RHgLIM, which reached growth up to 400mg/L, it was observed that at concentrations of 200 to 300mg/L, the isolate showed

similar growth behavior for this concentration. Meanwhile, the isolate MpA6RHgLIM, which grew up to 350mg/L, showed a slight growth compared to the other concentrations evaluated. Finally, the isolate MpA3HHgLIM, which tolerated up to 300mg/L, showed a slight growth compared to the other concentrations evaluated. The same figure 5 shows that at concentrations of 250 and 300mg/L up to 15 hours, the growth behavior was similar. After 19 hours from the beginning of the experiment, the growth of the isolates entered a phase of decay with respect to the control. With respect to the concentrations 350 to 500mg/L, a slight growth was observed with respect to the other concentrations evaluated.

The mercury is a highly reactive metal when it is in cationic form or bound to other compounds; biochemically it has an affinity for functional groups present in enzymes that catalyze critical reactions in an organism; it has been found that metal ions interact with cellular components such as DNA and proteins, causing damage and conformational changes that can alter the cell cycle (Tchounwou et al. 2012).

Molecular identification of mercury-tolerant endophytic bacteria. The sequences of the isolates obtained were compared with sequences present in the NBCI library. The phylogenetic analysis of 16S rDNA gene of endophytic bacteria shows that the isolate MpA6RHgLIM has high homology with the sequences of the bacterium Bacillus thuringiensis strain F14; MpA4THgLIM with Bacillus cereus strain ML259 and Bacillus mycoides O-1; MpA10RHgLIM with Bacillus cereus strain LB1016; MpA10RHgLIM with the genus Bacillus sp and MpA3HHgLIM with the genus Pseudomonas sp (Figure 6).

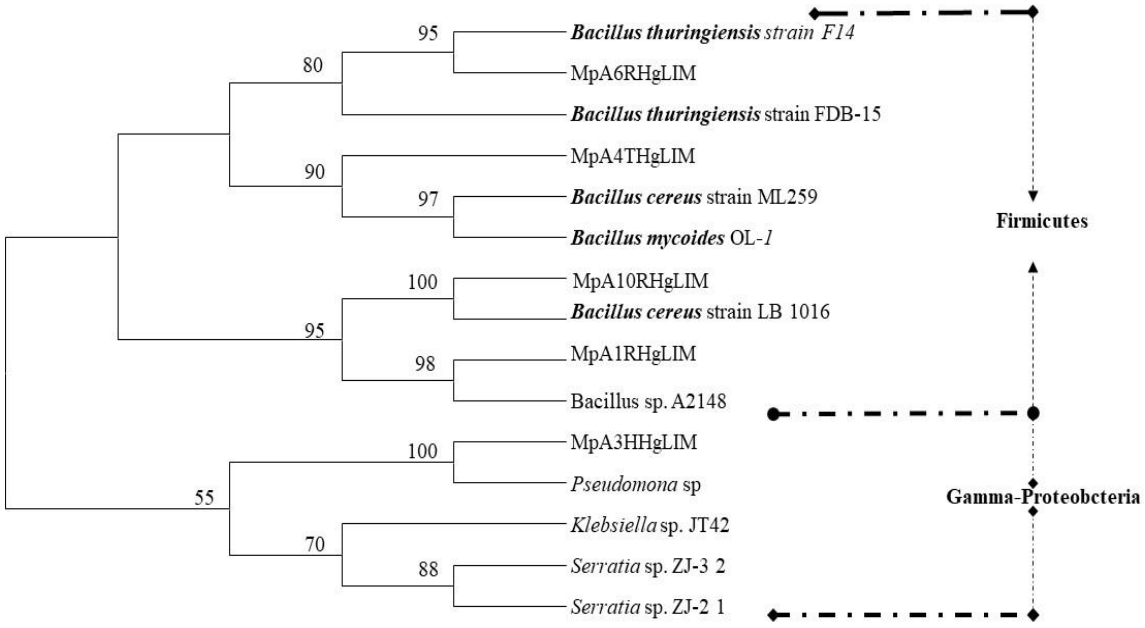

Figure 6. Phylogenetic tree of maximum similarity derived from the analysis of 16S rDNA gene sequences of endophytic bacteria isolated from plant species from mercury-contaminated environments. At the base of each clade, the branch support expressed as the percentage of times the analysis produced the same association between sequences is observed. Mp: Melochia parvifolia; A: isolate; R: rhizosphere; R: root; T: stem; Hg: mercury.

The tolerance to heavy metals in bacteria has been extensively studied worldwide. Several bacterial species have been reported for metal resistance. Most of the bacterial species that claim to be possible candidates for heavy metal bioremediation belong to the genera Bacillus, Pseudomonas and Streptomyces (Uslu & Tanyol, 2006). The genus Bacillus are commonly found in soils and plants where they play an important role in carbon and nitrogen cycling. They are common inhabitants of fresh and stagnant waters, and are particularly active in sediments (Koneman, 2001).

Reports to date on the bacterial species Bacillus thuringiensis, as noted by Ortiz et al. (2018), this species was isolated from tissues of blueberry plants (Vaccinium corymbosum L.) cv. Biloxi with plant growth promoting activity of this bacterium. On the other hand, Dash et al. (2013), isolated B. thuringiensis from Indian coastal marine sediments and in vitro studies showed that this species has the ability to tolerate up to 50ppm of mercury chloride (Dash et al. 2013). Another study with this same species of bacteria, isolated from the Odisha coast of India, showed ability to tolerate up to 50ppm of Hg (Dash et al. 2013). Bacillus cereus, has been identified as an endophytic bacterium isolated from Cyperus and Paspalum in vitro showed ability to tolerate up to

400ppm (400mg/L) of mercury in the form of HgCl2. In another study conducted, they isolated this species of bacteria from tissues of rice varieties indicating the tests carried out show the ability of these to tolerate up to 400ppm of Pb in the form of Pb(NO3)2 and also the production of compound called siderophore (Perez et al. 2016; Perez et al. 2018).

On the other hand, the genus Pseudomonas is the most heterogeneous and ecologically important group of known bacteria. Because the nutritional requirements of species of this genus are very simple, representatives have been detected in virtually all natural habitats and tend to be predominant among bacteria associated with the rhizosphere of plants (Arora, 2015; De Oliveira et al. 2015). The role of Pseudomonads in Bioremediation is a consequence of their environmental importance and their metabolic diversity thanks to their ability to degrade a wide range of organic compounds as demonstrated by many authors in their research where they have shown to be efficient in the bioa¬cumulation of heavy metals (Ramteke, 2000), this process has gained importance in recent years due to its good performance, low cost, specificity and easy reusability (Ahuja et al. 2001). Deng & Wang (2012) reported that a bacterial strain of pseudomonads sp. isolated from marine sediments removed and accumulated more $Hg2^{+}$ on the cell surface. The bio-uptake of mercury was probably through functional groups attached to the bacterial cell wall, such as carboxyl, phosphate, hydroxyl, thio, and pyridine groups that contributed to Hg^{2+} uptake, and the carboxyl groups were the most important in this action.

CONCLUSIONS

To date, this is the first report in Colombia on the presence of the plant species Melochia parvifolia adapted to mercury-contaminated environments. This plant species accumulates in root, stem, leaf and flower high amounts of mercury that it removes and bioaccumulates in its tissues. Likewise, the presence of endophytic bacteria associated with the tissues of this plant species is reported, which could be contributing to its adaptation to the environment and contributing to the removal of mercury, which will allow the future use of M. parvifolia in mercury removal processes due to its capacity to tolerate and accumulate this metal in its tissues and harbor within them a diversity of endophytic bacteria that on the one hand stimulate plant growth and on the other, use various mechanisms to reduce the presence of mercury in the environment.

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CRediT authorship contribution statement: Ramón Paternina-Hernández: Research, Data analysis, Writing - Original draft. Alexander Pérez-Cordero: Conceptualization, Investigation, Methodology, Writing - review & editing. Donicer Montes-Vergara: Data analysis, Writing - review & editing.
Conflict of interest: The manuscript was prepared and revised by all authors, who declare the absence of any conflict which can put the validity of the presented results in risk.

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