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In Vitro Evaluation of Rhizosphere Bacteria to Different Mercury Concentrations in Contaminated Agricultural Soils

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ABSTRACT

The objective was to isolate rhizosphere bacteria from plant species present in soils contaminated with mercury in the region San Jorge, department de Sucre, Colombia, and to evaluate in vitro their tolerance capacity to different concentrations of mercury. From each sampling site were collected. The concentration of mercury in soil was determined using the instrumental technique of cold vapor atomic absorption spectrophotometry. The amount of rhizosphere 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 1,086 ± 3,096 mg/kg. The amount of rhizosphere bacteria found ranged from 1.2 X 105 ± 9.1 X 107 CFU/ g of roots. The isolates identified as Bacillus cereus strain ML259; Bacillus mycoides O-1 and Bacillus cereus strain LB1016, showed in vitro tolerance up to 700 and 900 mg/mL of HgCl2. The predominant plant species found in the soil corresponded to Bothriochloa pertusa (L.) A. Camus, accumulating mercury in concentrations of 1,086 ± 3,096 mg/kg in the rhizosphere. 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 rhizosphere bacteria for the management of soils contaminated with this metal.

KEYWORDS: rhizosphere bacteria, soil, mercury, pasture.

1. 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 (CH3Hg+). 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).

rhizosphere 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 rhizosphere 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 rhizosphere bacteria from rhizosphere in livestock farms near the swampy body of Santiago Apostol and evaluate the in vitro tolerance capacity to different concentrations of this metal.

2. Materials and Methods

Sampling site. The sampling was carried out in soils of cattle farms with pasture located in the San Jorge region near the San Benito Abad swamp, department of Sucre, Colombia. At each selected site, a random zig-zag sampling was carried out, where soil and root 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 root 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. The determination of mercury levels in soil samples was performed in triplicate. The steps for the analysis were as follows: Root tissue 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 rhizosphere bacteria. The isolation of rhizosphere bacteria from the different plant root 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 bacteria tolerance to mercury. The isolates of rhizosphere 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.

3. Result and Discussion

Presence of rhizospheric bacteria. The amount of bacteria per gram of root was $1.2 \times 10^5 \pm 9.1 \times 10^7$ CFU/ g of roots. Tukey's test ($p<0.05$) indicates that there is a highly significant difference between the amount of rhizospheric bacteria present associated with Bothriochloa pertusa (L.) A. Camus grass, with the highest values of these bacteria present in soils of cattle farms in sampling sites 12 to 20. It is inferred that these sampling sites were closer to the San Benito Abad marsh, while sampling sites 1 to 11 were distant from the mentioned marsh

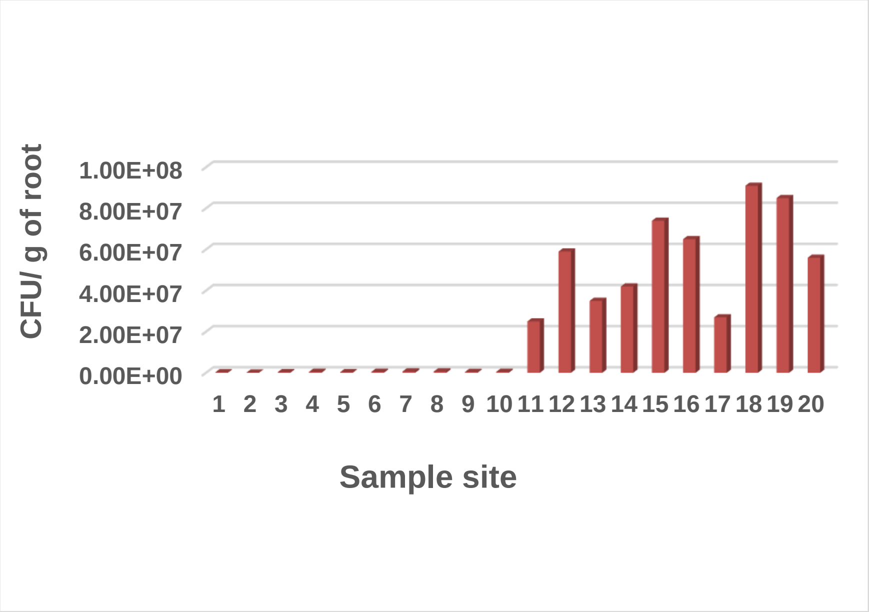

Figure 1. Number of rhizospheric bacteria (CFU/g roots) in soil with Bothriochloa pertusa (L.) A. Camus grassland.

Determination of mercury in soil. The rhizospheric soil mercury content ranged from 1,086 ± 3.096 mg/kg (Figure 2). 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).

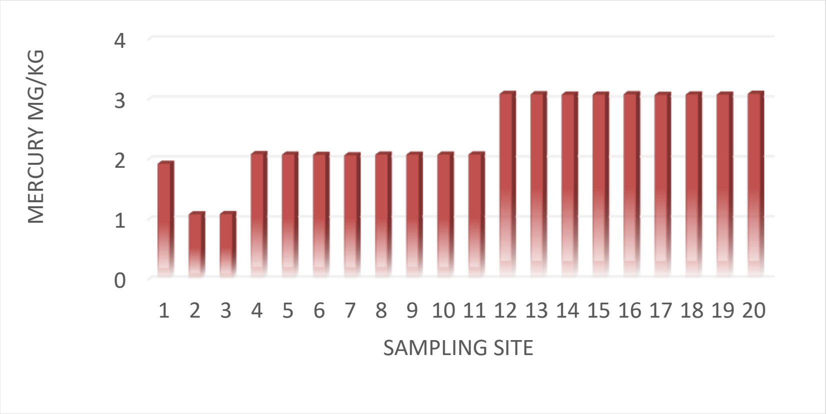

Figure 2. Amount of mercury in mg/kg soil in cattle farms with Bothriochloa pertusa (L.) A. Camus.

Figure 3 shows the results of the tolerance test of the bacterium Bacillus subtilis at a concentration of 700 and 900 mg/L mercury in the form of $HgCl_2$. The graph shows which strain of the bacterium showed mercury tolerance behaviours at the concentration of 700 and 900 mg/L $HgCl_2$. The strain had a similar growth behaviours for both 700 and 900 mg/L, at both concentrations the adaptation stage lasted up to six days compared to the control. The maximum growth behaviours for the strain at both concentrations was up to 17 days after inoculation in the test and from 22 hours onwards the strain started the lysis or cell death stage. These results suggest that between 6 days and 22 days, i.e. the strain requires 16 days to interact with the heavy metal under optimal environmental and nutritional conditions for bacterial growth.

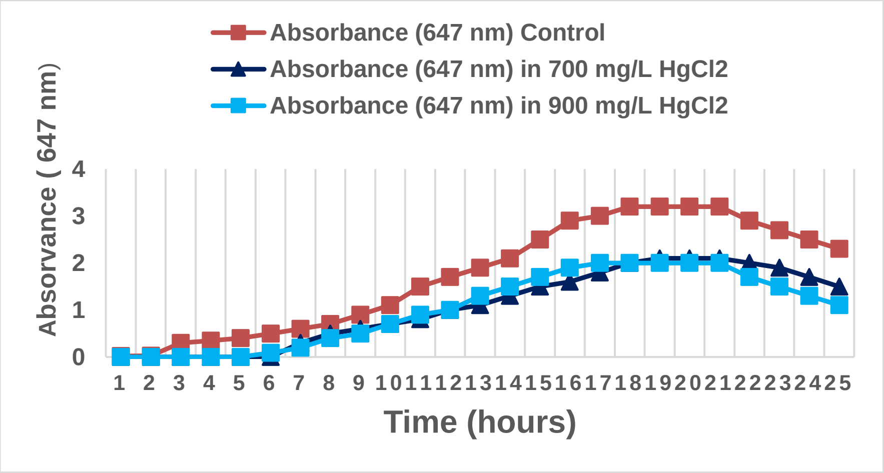

Figure 3. Bacillus cereus (MpA10RHgLIM) growth test at 700 and 900 mg/L $HgCl_2$ concentration.

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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 4).

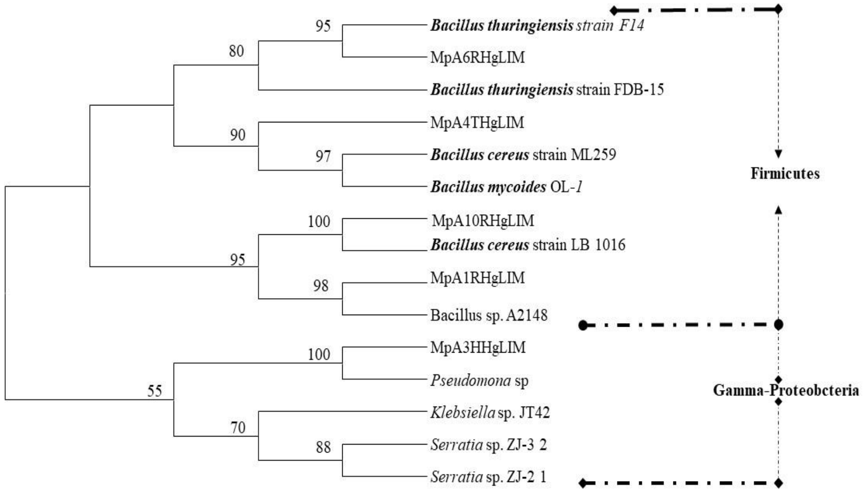

Figure 4. Phylogenetic tree of maximum similarity derived from the analysis of 16S rDNA gene sequences of endophytic bacteria isolated from plant species from mercury-contaminated environments. At the base of each clade, the branch support expressed as the percentage of times the analysis produced the same association between sequences is observed. Mp: Melochia parvifolia; A: isolate; R: rhizosphere; R: root; T: stem; Hg:

mercury; LIM: Laboratorio Investigaciones Microbiológicas. 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 (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).

Bacillus cereus has been identified as rhizosphere and endophytic bacterium isolated from Cyperus and Paspalum in vitro showed ability to tolerate up to 400ppm (400mg/L) of mercury in the form of $HgCl_2$. 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 $Pb(NO_3)_2$ and also the production of compound called siderophore (Perez et al. 2016; Perez et al. 2018).

4. Conclusions

Bacillus cereus was isolated from rhizosphere of Bothriochloa pertusa (L.) A. Camus grass located in cattle farms near the San Benito de Abad swamp complex, in the department of Sucre, Colombia, showed in vitro the ability to grow in 700 and 900 mg/L $HgCl_2$. To date, this is report in Colombia on the presence of Bothriochloa pertusa (L.) A. Camus adapted to mercury-contaminated environments. This plant species accumulates in root 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 Bothriochloa pertusa (L.) A. Camus 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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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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