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Endophytic Bacteria Isolated From Bothriochloa Pertusa As An Ecological Alternative For Soil Cadmium Remediation

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ABSTRACT

Phytoremediation is one of the current approaches for environmental pollution management. The combination of plant absorption of metals and endophyte bacteria which provide with ways to metabolize them is envisioned as a major technology for biorremediation. In this study, we studied Bothriochloa pertusa and their endophites' ability to tolerate cadmium. We collected plants from different localities in Sucre, Colombia. Cadmium concentrations on soil and plant tissues were assesed. The density of endophytic bacteria from samples isolated from root and inflorescences was determined (CFU/g of tissue) and their capacity of tolerance to cadmium was evaluated in vitro. Cadmium was present in the soil at a concentration of 1.5±3.6 mg/Kg and from 1.05 to 3.21 mg/Kg (inflorescence and root respectively). Endophytic bacteria were present mostly in the inflorescence compared to the roots. Burkholderia diffusa KF475808 (95%) Burkholderia ambifaria LN88999 (98%); Deinococcus mumbaiensis DQ003135 (98%); Bacillus cereus GU056811 (93%); Bacillus toyonensis BCT-7112 (T) (99.9%); Pseudomonas fluorescens VI8L1 (98%) and Burkholderia cepacia KJ935921 (97%) tolerated up to 500 ppm of $CdCl_2$ and showed in vitro capacity for nitrogen fixation, phosphate solubilization and the production of siderophores. This study demonstrates that endophytidc bacteria in B. pertusa have the potential to be used for fitoremediation of soil cadmium in the study area.

Keywords: Bacteria, Grass, Soils, Sucre, Mojana, pollution, Colosuana**.**

1. INTRODUCTION

Environmental contamination is one of the major global concerns for the XXI century due to the loss of quality of air, soil and water which endangers the health of all organisms present in the biosphere (Chen et al., 2013;Singh et al., 2010), e.g. the levels of heavy metals accumulated in the environment has triggered in harmful effects in in human health ranging from damage to vital organs to carcinogenic events (Nava-Ruíz and Méndez-Armenta, 2011).

Cadmium (Cd) e.g. is a heavy metal that affects human health through the consumption of contaminated plants and animals (Rodríguez et al., 2008 ;Peláez-Peláez et al., 2016). At the Colombian Caribbean, Calao and Marrugo (2013) found evidence of genotoxic effects in human blood of heavy metals including Cd, that produce DNA damage. Furthermore, in crops (grasses) evidence of heavy metals has been found (Argumedo García et al., 2013).

Phytoremediation is an effective, economical and environmentally friendly technology that is receiving a lot of attention worldwide due to its low costs and low impact on human health and ecosystems (Sheng et al., 2008). The success of phytoremediation depends on the plant's ability to tolerate high concentrations of metals and to produce large amounts of biomass (Ma et al., 2011). Specifically, the efficiency of heavy metal phytoremediation is dependent on the plant's performance, its ability to accumulate metal ions and its associated microorganisms that can provide nutrients and reduce the harmful effects caused by heavy metals (Ma et al., 2011).

The ability of plants to extract Cd from their environment is determined by its concentration in the soil and its bioavailability, which depends on the presence of organic matter, pH, redox potential, temperature and the concentrations of other elements. Cd exhibits a marked tendency to bioaccumulate in plants, causing imbalances in their nutrition and water movement (Singh and Tewari, 2003). Cd competes for protein transporters with macro and micronutrients such as potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn) and nickel (Ni) (Di Toppi and Gabbrielli, 1999;Benavides et al., 2005;Wiszniewska et al., 2017).

Endophytic bacteria live in the internal tissues of plants, contributing to the adaptation of plant species to contaminated sites, and thus potentiating their phytoremediation capacity and tolerance to soil contaminants such as heavy metals (Li et al., 2012). Endophytic bacteria associated with different grass species (Poaceae) have in vitro tolerance up to 400 ppm of $HgCl_2$ (Pérez et al., 2016). The colosuana grass [Bothriochloa pertusa (L), A Camus] for cattle pastures in areas of Colombia has been used, reaching a total of 274,005 ha. cultivated (Pérez et al., 2012). Several species of endophytic bacteria isolated from B. pertussa could potentially be used to carry out phytoremediation processes (Pérez Cordero et al., 2010;Pérez Cordero et al., 2018). However, their tolerance to cadmium is unknown, and therefore its use for remediation processes of this heavy metal in soils in which contamination has been evidenced is limited (Marrugo-Negrete et al., 2018).

B. pertussa is cultivated for cattle intake in regions with records of heavy metal contamination (Argumedo-García et al., 2013), however, it is unknown whether this species can accumulate the heavy metal and transfer it to the cattle used for human consumption and hence, subsequently transferred to humans. In order to offer ecological alternatives to carry out phytoremediation processes, but in addition to determining whether there is a possibility of transfer to bovines, we studied the cadmium tolerance of endophytic bacteria isolated from different tissues of colosuana grass present in cattle farms of four localities of the department of Sucre in Colombia.

2. MATERIALS AND METHODS

2.1. Study area

In order to establish whether B. pertussa are could take up heavy metals to be used to phytoremediation, samplings were made from the Mojana Sucreña region (department of Sucre, Colombia). Previous studies have demonstrated the geoaccumulation of heavy metals that have influenced the fauna and its inhabitants (Argumedo-García et al., 2013;Marrugo-Negrete et al., 2018). Samples were collected from livestock farms in four municipalities (San Marcos, Guaranda, Majagual and Sucre; Figure 1) adjacent to the Mojana Sucreña, sown only with colosuana grass.

2.2. Plant survey

The sampling was carried out randomly in the form of a zig-zag methods, collecting soil samples at the same time until completing 1 Kg and 10 complete individuals (root, stem, leaves, and panicle) of colosuana grass (B. pertussa). Plants samples were stored and refrigerated for preservation. In addition, collection date, municipality and geographic coordinates were taken.

2.3. Cadmium concentration in soil and plant samples

To determine the total amount of cadmium in colosuana grass tissues, 0.5 g of dry material was taken and an acidic HNO_3 / H_2O_2 mixture (5 + 2 mL) was added. Further 0.5 g were taken from the previously dried soil and 10 mL of 65% HNO_3 were added. Both the soil and plant samples were processed in a Milestone ETHOS TOUCH 127697 series microwave oven and total cadmium was analyzed by cold vapor atomic absorption spectrophotometry according to procedures described in Pérez Cordero et al. (2018).

2.4. Isolation of endophytic bacteria from colosuana grass

Prior to the bacteria isolation process, the plant samples were superficially disinfected according to the methodology recommended by Pérez et al. (2010). Serial dilutions of 10^{-1} to 10^{-8} were prepared, from which 0.1 ml aliquots, which were harvested and seeded in R2A agar culture medium and incubated at 32ºC for 72 hours. The density of bacteria per tissue (CFU / g tissue) was determined by direct colony counting technique on the R2A agar surface. Colonies were selected based on shape, texture, color and size.

2.5. In vitro evaluation of cadmium tolerance

The tolerance of endophytic bacteria to different concentrations of $CdCl_2$ was evaluated in Tris-MMT minimal medium (Rathnayake et al., 2013). The initial concentration of Cd used in the present study was 10 mg/l and metal concentrations up to 500 mg/l were prepared from these. Suspensions of endophytic bacteria aliquots in the logarithmic phase were inoculated on the MMT medium. MMT medium without $CdCl_2$ was used as a control. The experiment was performed in triplicate, incubated with shaking at 150 rpm at 32 °C for 120 hours (Zhang et al., 2011). Bacterial growth was determined by turbidimetry at 600 nm every hour for four days.

2.6. Qualitative evaluation of growth promotion of Cd tolerant endophytic bacteria

To evaluate the tolerance of endophytic bacteria to $CdCl_2$ we test their biological nitrogen fixation, phosphate solubilization, indoletic acid and siderophore production activities in vitro. Nitrogen fixation test was performed in selective ASHBY agar medium using the methodology described by Pérez et al. (2014). Bacteria isolates were seeded and harvested on ASHBY agar medium, incubating at 28 °C for 72 hours. The test was positive for those isolates that grew on the surface of the ASHBY medium. Qualitative phosphate solubilization test was performed in NBRIP medium with Ca3PO4 as a source of insoluble phosphorus at pH 7, fallowing the technique described by Pérez et al. (2014). We inoculated the isolated on NBRIP culture medium and incubated it at 28 ° C for 72 hours. The test was positive when the visible transparent halo formation was observed around and below the colony. The production of siderophore was determined in chromium azurol-S (CAS) medium (Schwyn and Neilands, 1987). Briefly, 60.5 mg of CAS was dissolved in 50 ml of distilled water, the above was combined with 10 ml of an iron (III) solution (1 mM of FeCl2.6 H2O and 10 mM of HCl). With stirring, this solution was mixed with 72.9 mg of HDTMA dissolved in 40 mL of water. The resulting blue liquid was sterilized at 121 °C for 15 min. In another container, a mixture of 750 ml of water, 15 g of agar, 30.24 g of pipes, and 12 g of a 50% (w/w) solution of NaOH was also sterilized to reach a pH of 6.8. In addition, we added 4 g of glucose added as a carbon source and incubate for 7 days at 30 °C.

2.7. Molecular taxonomic identification of Cd tolerant bacteria

Genomic DNA extraction was performed according to the protocol described by Oliveira et al. (2013); and the universal primers of the 16S rDNA region were used to identify the bacteria species. The specific initiators used for each of the classes belonging to the bacterial domain (alpha, beta, proteobacteria range and firmicutes) corresponded to those proposed by Oliveira et al. (2013). The amplification products were sent for purification and sequencing to the Macrogen Korea company. Once the nucleotide sequences were obtained, a search was made for the homologous sequences with the sequences stored in the database of the National Center for Biotechnology Information (NCBI). DNA alignment was performed with Clustal W in MEGA X software (Kumar et al., 2018)

2.8. Statistical analysis

With the results obtained, a block design with a factorial arrangement was carried out, and for statistical differences the Tukey multiple range tests was used. Statistical analyses were performed with the free version InfoStat software.

3. RESULTS

3.1. Cd concentrations and density of bacteria

The normality test was met, finding that the response variable has a normal distribution (p-value> 0.05) (Shapiro-Wilks). The results indicate significant statistical differences (p-value> 0.05) between the concentration of cadmium in the soil by each municipality, reporting the highest presence of this metal in Sucre (3.6 mg / kg of soil) and the lowest in the municipality of San Marcos (1.5 mg / kg of soil). Regarding the analysis of cadmium concentration per tissue in colosuana grass, Figure 1B shows significant differences (p-value <0.05) found in higher concentrations of Cadmium in roots with a value

of 3.21 and lower in inflorescence 1.05 mg / Kg of tissue. Regarding the amount of endophytic bacteria per tissue, it is observed that the largest number of bacteria are reported for inflorescence (2.0 x 107 CFU / g of tissue) and least in roots (3.0 x (105 CFU / g of tissue) (Figure 1C). Likewise, it is observed that there is an inverse relationship between the amount of bacteria and the concentration of cadmium per tissue. The highest presence of endophytic bacteria is reported for panicle tissue where lower values of cadmium and lower amounts of these bacteria were found in roots where higher values of this metal were registered.

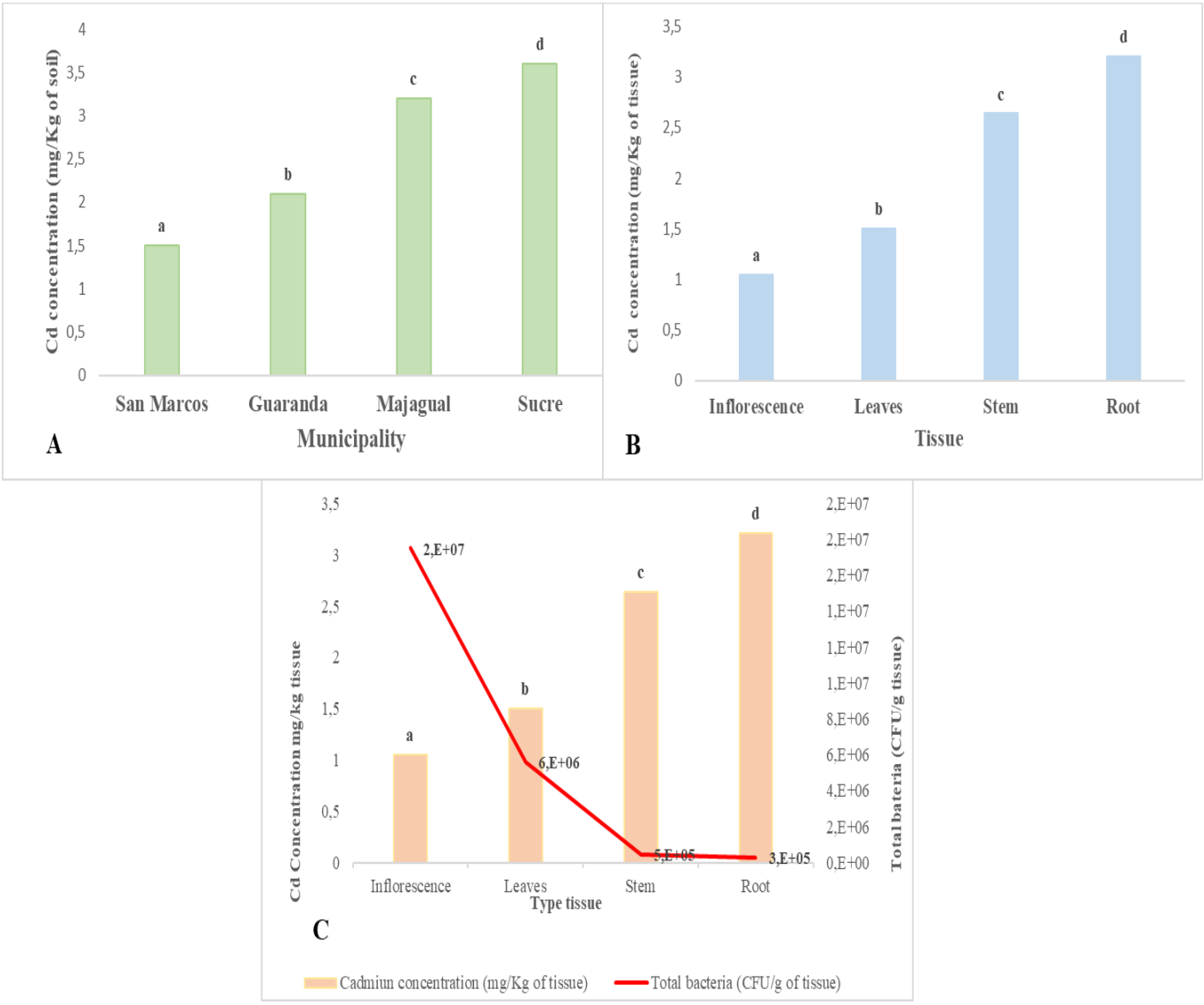

Figure 1. A): Concentration (mg/Kg) of Cadmio by municipalities; B): Concentration (mg/Kg) of Cadmio by tissue of B. pertusa and C): Cadmio in tissue and endophytes.

3.2. Results of Cadmium tolerance test of endophytic bacteria.

Total of 57 isolates were obtained from B. pertusa, 23 shows tolerance to different concentrations of Cd (CdCl2). The Tukey test show significant differences (p-value <0.05) between the isolates

tolerance. Samples with the highest tolerance were: SMBpT-1; GBpH-3; MBpI-1; MBpI-6; SBpI-1; SBpR-1 and SBpR-10. When evaluating the bacterium minimum and maximum growths to CdCl2 of the seven isolates with the highest tolerance, it was found that SBpR-1 and SBpR-10 grew up to 500 mg/l of CdCl2, MBpI-6; SBpI-1 up to 400 mg/l (Figures 2 and 3); GBpH-3 up to 350 mg/l and SMBpT-1 and SMBpI-1 up to 300 mg/l (Figure 4, 5, 6).

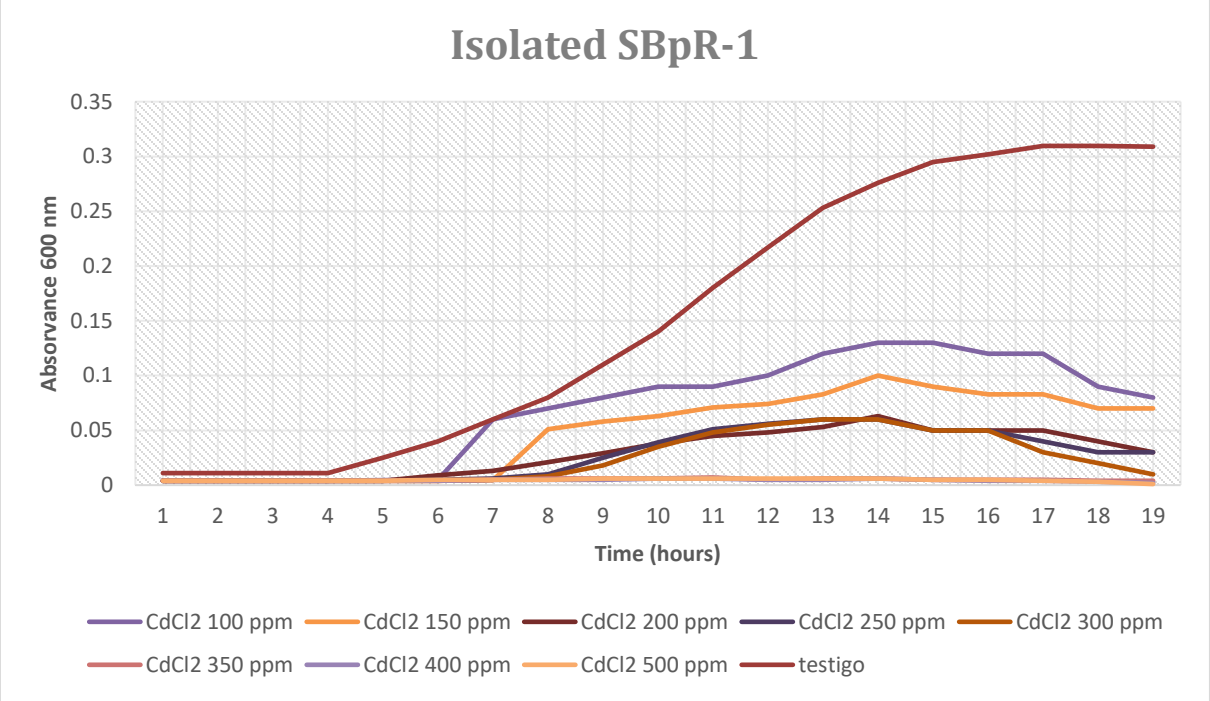

Figure 2. Tolerance test results of SBpR -1 isolates of endophytic bacteria to different concentrations of CdCl2 (ppm).

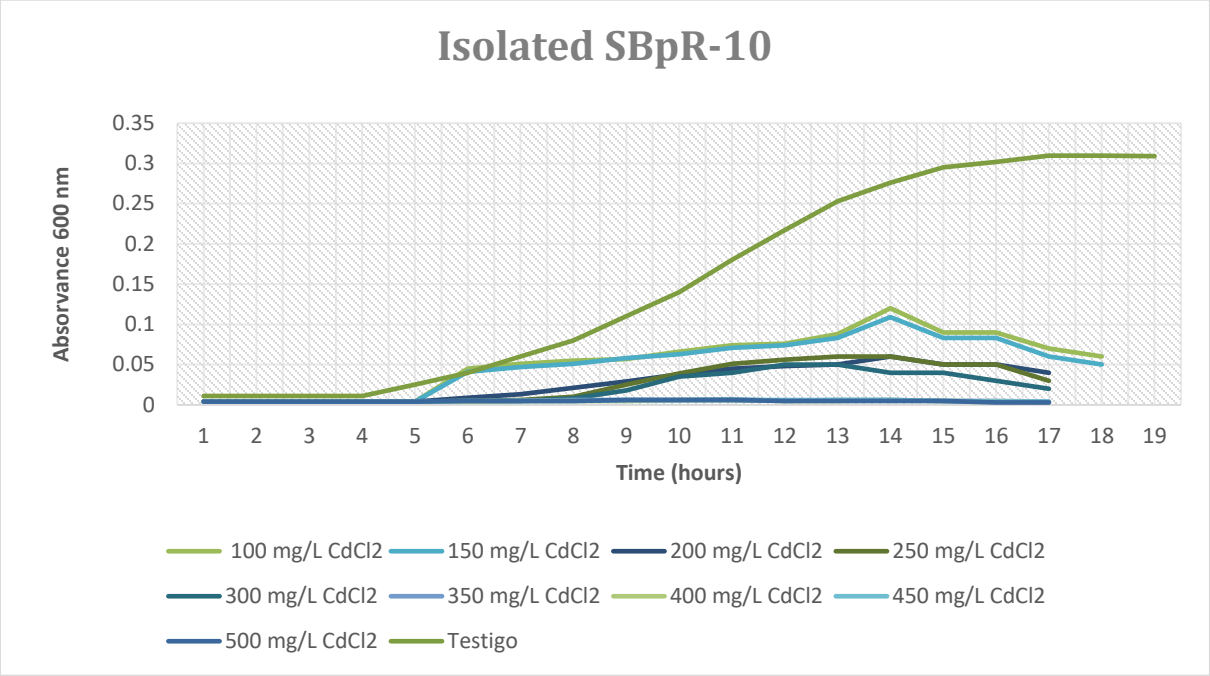

Figure 3. Tolerance test results of SBpR-10 isolates of endophytic bacteria to different concentrations of CdCl2 (ppm).

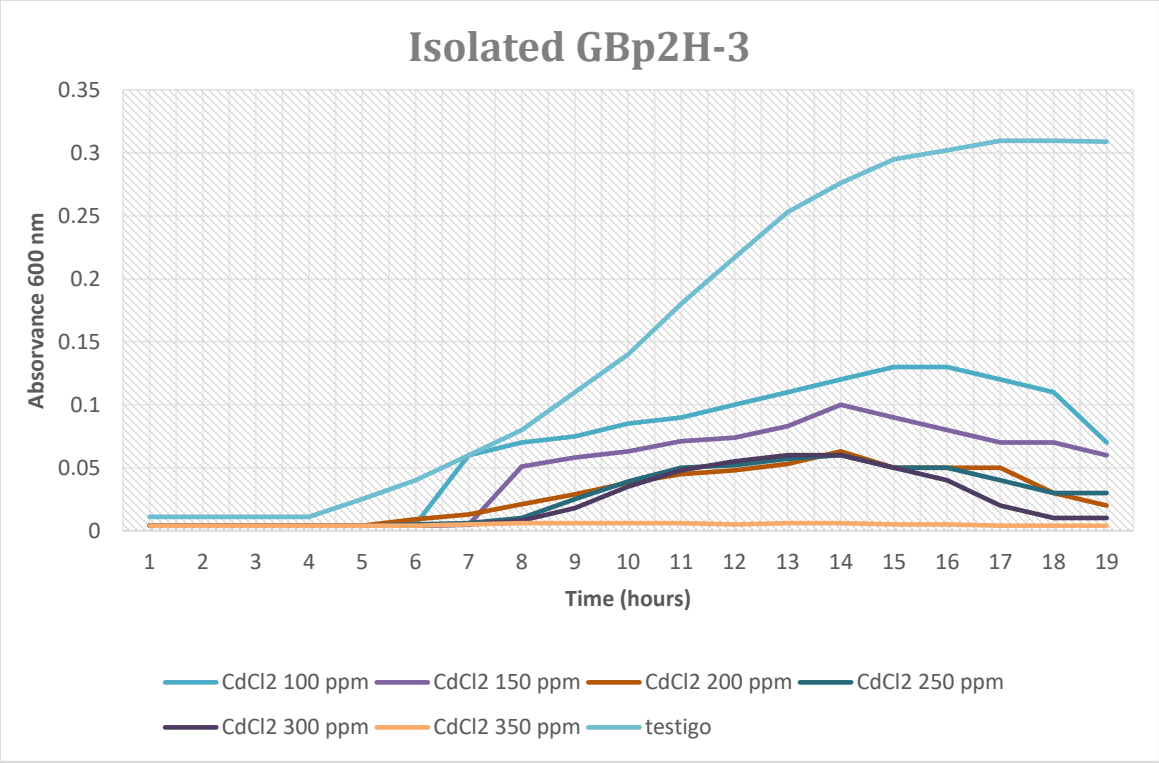

Figure 4. Tolerance test results of GBp2H-3 isolates of endophytic bacteria at different CdCl2 concentrations (ppm).

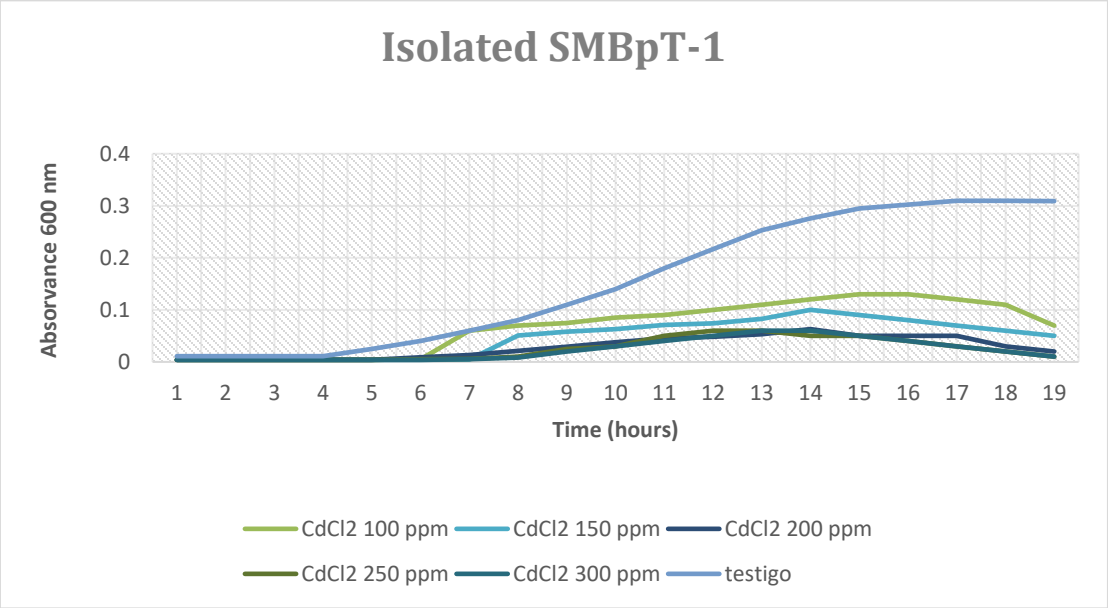

Figure 5. Tolerance test results of SMBpT-1 isolates of endophytic bacteria at different CdCl2 concentrations (ppm).

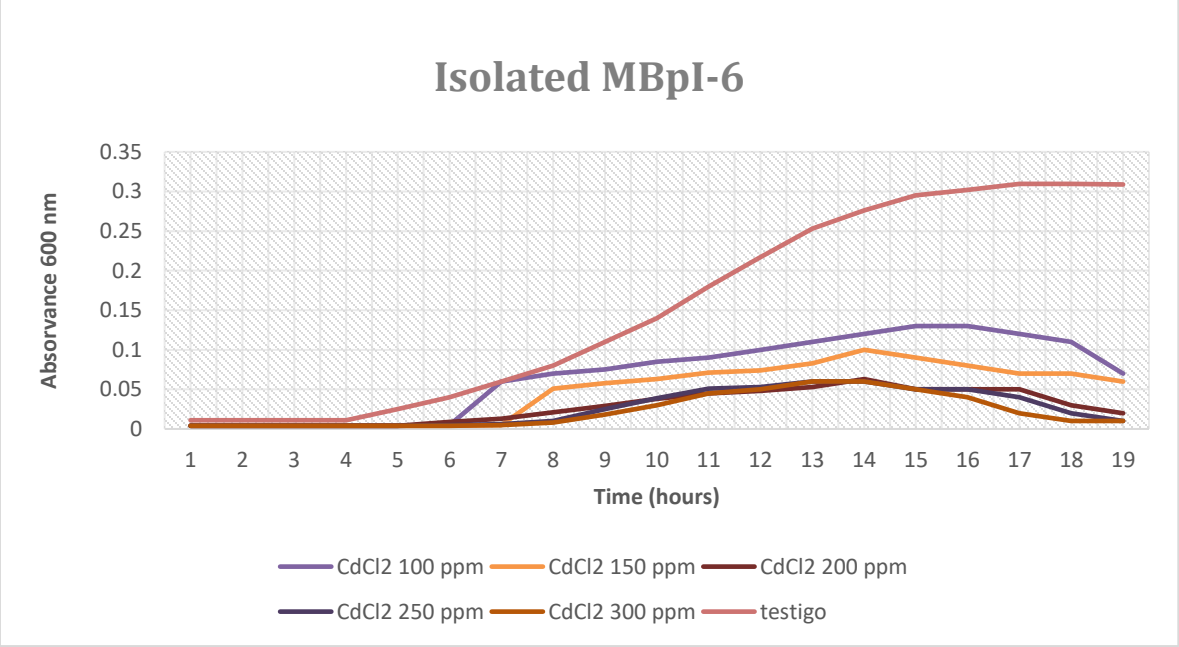

Figure 6. Tolerance test results of MBpI-6 isolates of endophytic bacteria at different CdCl2 concentrations (ppm).

3.3. In vitro evaluation of the ability to promote plant growth of Cd tolerant endophytic bacteria isolates

The ability to fix atmospheric nitrogen was estimated qualitatively throughout the isolates grow capacity in selective ASHBY agar medium. We found six bacterial isolates that shows tolerance to cadmium (Figure 7A), through in vitro capacity to fix nitrogen including: MBpI-6, GBpH-6, SBpI-1, SBp2R-10, MBpI-1 and SBpR-1. The qualitative ability to solubilize phosphate in NBRIP only were found in SBpR-1 and SBp2R-10 isolates (Figure 7B). The qualitative analysis of the production of siderophores was evidenced by the formation of a halo around the colony, in CAS medium, which was observed for the isolates SBpR-1 and SBp2R-10 (Figure 7C).

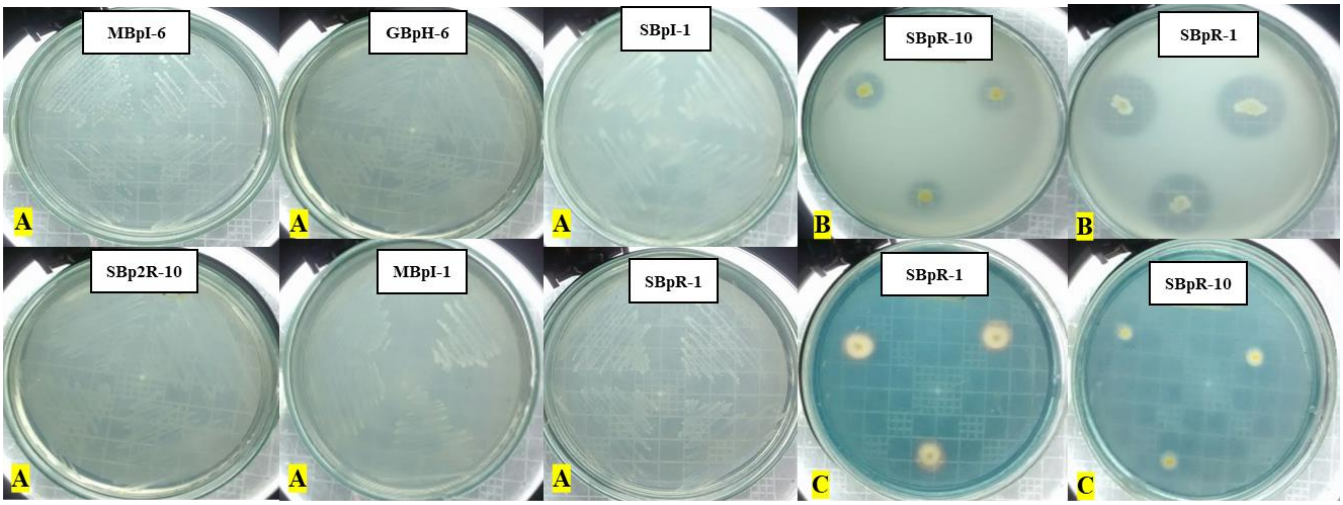

Figure 7. Qualitative assay of nitrogen fixation on ASHBY medium (**A**), phosphate solubilisation on ASHBY medium (**B**) and siderophore production on CAS medium (**C**) of CdCl2-tolerant endophytic bacterial isolates.

3.4. Molecular taxonomic identification of isolates of Cd tolerant endophytic bacteria

The endophytic bacteria identified were: Burkholderia diffusa KF475808 (95%); Burkholderia ambifaria LN88999 (98%); Deinococcus mumbaiensis DQ003135 (98%); Bacillus cereus GU056811 (93%); Bacillus toyonensis BCT-7112 (T) (99.9%); Pseudomonas fluorescens VI8L1 (98%) and Burkholderia cepacia KJ935921 (97%).

The isolate SBpR-10 identified as Burkholderia cepacia KJ935921 and SBpR-1 (Pseudomonas fluorescens VI8L1), tolerated up to 500 mg/L of CdCl2 and showed in vitro capacity for nitrogen fixation, phosphate solubilization and siderophore production. Both isolates were extracted from roots of B. pertusa from cattle farms located in the municipality of Sucre. Isolates referenced as GBpH-3 (Burkholderia ambifaria); MBpI-1 (Deinococcus mumbaiensis); MBpI-6 (Bacillus cereus) and SBpI-

1 (Bacillus toyonensis BCT-7112 (T)), showed nitrogen fixing activity. Finally, the SMBpT-1 isolate identified as Burkholderia diffusa showed no activity and was able to tolerate up to 300 mg/L CdCl2

4. DISCUSSION

4.1. Cadmium in livestock farm soil

The cadmium values in the soil ranged from 1.5 ± 3.6 mg/kg of soil, with a higher amount of cadmium in soil being found in the municipality of Sucre (3.6 mg/kg), followed by Majagual (3.2 mg/kg) and less in San Marcos (1.5 mg/kg). The international reference values for cultivated soils are 0.01-2.0 mg/kg. The data found in the present study are above the reference values. Cadmium enters the soil through the application of fertilizers produced from phosphoric rock (Mendes et al., 2006;Chen et al., 2008). Due to the low fertility levels of rice soils, in Colombia, high doses of fertilizers (nitrogen, phosphorus, potassium, among others) are employed to better improve the soils quality to rice crops (Mendes et al., 2006). However, high amounts of fertilizers also increase the cadmium concentrations in soil, which is also absorbed by grasses like rice o cattle food generating toxic effects on the entire natural cycle, reaching human food directly through the rice or indirectly eating meat from cattle contaminated by cadmium (Castilla et al., 2010).

Studies suggest that cadmium is absorbed and translocate to different plant tissues, including the inflorescence or seeds. However in grasses this metal accumulates preferably in the root (Olsson et al., 2005) sequestered in the vacuole of the cells, and only a small part is transported to the aerial part of the plant, concentrating in decreasing order on stems, leaves, fruits and seeds (Chan and Hale, 2004). Nevertheless Yang et al. (2016) found in rice crops that around 0.73% Cd is transferred to the seed (rice grains) with average values of 1.02 mg/kg. According to European Union for heavy metals, Yang et al. (2016) results are above of the allowed ranks in rice grains. Likewise, Liu et al. (2007) also report trace values of cadmium in rice grains of 0.93 mg / Kg.

Herein we found that Cd values per tissue in B. pertusa are 1.05 ± 3.21 mg/kg. The greater amount was found in roots (3.21) and less in inflorescence (1.05 mg / kg of tissue). According to the international standard code of heavy metals of food, the allowed values of cadmium ranging 0.05 - 0.5 mg/kg in tissue. The data herein found suggest that Colosuana grass tissue is above those allowed by the international code and therefore can produce risk for cattle and finally for human health (KAbata-Pendias, 2011).

4.2. Amount of endophytic bacteria per tissue of B. pertusa

The bacteria quantity values ranged from 3.0 x 105 ± 2.0 x 107 CFU / g of tissue. The highest presence of endophytic bacteria was reported in inflorescence (2.0 x 107) and the lowest values in root (3.0 x 105 CFU / g of tissue). An important outcome between the concentration of cadmium and endophytic bacteria in the plant tissue was found, because of we identified that those tissues with greater amounts these bacteria have lower cadmium values (per mg/kg), e.g. inflorescence has a less cadmium concentration but higher values of bacteria. Inversely cadmium is higher in roots, but batteries are low. Pérez Cordero et al. (2018) found that endophytic bacteria in B. pertusa cadmium free, range 3.18 x108

- 4.48x1010 CFU / g of tissue. Heavy metals in different concentrations decrease the amount of microbial biomass and therefore have an inhibitory effect on the bacteria growth (Pérez et al., 2015;Wu et al., 2015). Heavy metals reduce bacterial growth because of stress produced by great demand for energy to face the toxicity of pollutants (Roane et al., 2009).

4.3. Cadmium tolerance test of endophytic bacteria

The tolerance test shows tolerance up to 500 mg/L of CdCl2 from isolates AF2R-1 and AF2P-10. This metal is considered non-essential trace element and with ecotoxic effects on plants, animals and humans in low concentrations (Rajkumar et al., 2009). The results of the tolerance test suggest that certain species of endophytic bacteria have the capacity to tolerate high concentrations of Cd. According to Rajkumar et al. (2009) Cd tolerance related to the adaptation of the bacteria to live under conditions of constant stress by the metal. Bacteria various tolerance mechanisms to tolerate the harmful effects of toxic metals have developed, including: cellular components that capture ions, neutralizing their toxicity; enzymes that modify the redox state of metals, turning them into less harmful forms, and membrane transporters that expel harmful species from the cell cytoplasm (Rajkumar et al., 2009).

Furthermore, bacteria are capable of resisting heavy metals in contaminated soils through the production of siderophores. Bacterial siderophores contribute to reducing the toxicity caused by heavy metals in plants and also supply the need for iron as an essential element, promoting the development and growth of plants in contaminated environments (Rajkumar et al., 2010).

Burkholderia diffusa, Bacillus cereus and Burkholderia cepacia were found by Pérez et al. (2016) in Cyperus sp and Paspalum plants spadapted in environments contaminated with mercury and also showed in vitro tolerance (500 mg/L of mercury chloride). Lodewyckx et al. (2001) found that B. cepacia has the capacity to tolerate and remediate cadmium. Species of Deinococcus are able to live in extreme environments such as arid deserts, under ionizing conditions, to reactive molecules of oxygen species (ROS) and other oxidative stress including chemical compounds (Slade and Radman, 2011). Deinococcus mumbaiensis was reported by Shashidhar and Bandekar (2006) as a pleomorphic, radiation-resistant bacterium; while Pseudomonas fluorescens producing siderophores such as pyoverines and quinolabactins, which is important for promoting growth and biocontrol in plants (Botelho and Mendonça-Hagler, 2006).

5. CONCLUSION

The cadmium values found in the soil and in the different tissues of B. pertusa highly toxic. We reported for first time the presence of Cd in the study area localities on soils and B. pertusa. B. pertusa showed in vitro tolerance up to 500 Mg/L of CdCl2 and therefore allowed us to suggest the ability of these bacteria to remedy said metal directly. Likewise, it was evidenced the growth promoting activity that indirectly would be contributing to reduce the toxic effect of this metal in the soil and in the tissues.

6. CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. AUTHOR CONTRIBUTIONS

The Author Contributions section is mandatory for all articles, including articles by sole authors. If an appropriate statement is not provided on submission, a standard one will be inserted during the production process. The Author Contributions statement must describe the contributions of individual authors referred to by their initials and, in doing so, all authors agree to be accountable for the content of the work. Please see here for full authorship criteria.

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“Implementación de la fitorremediación asistida por bacterias endófitas en ambientes contaminados con metales pesados” by the financial support.

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