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Burkholderia Cepacia Kj935921 A Biological Alternative To Mitigate The Effect Of Cadmium In Tropical Pasture Soils

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ABSTRACT

Cd concentration was determined in soil and tissues of Bothriochloa pertusa (L), A Camus, endophytes were isolated from different tissues and their Cd tolerance capacity was evaluated in vitro. Cd concentration and presence of endophytic bacteria (CFU/g tissue) were determined for each tissue. The Cd values found correspond to 1.5 ± 3.6 mg/kg. The average ranges per tissue were from 1.05 (inflorescence) to 3.21 mg/kg (root). The highest presence of endophytic bacteria was found in the inflorescence with respect to leaf, stem and root. Burkholderia cepacia KJ935921 (97%) was identified as an endophytic bacterium with the ability to tolerate up to 500 ppm CdCl2 and showed in vitro nitrogen fixation, phosphate solubilisation and siderophore production, becoming a biological resource for the management of heavy metal contamination in the environment.

Key words: Pasture, endophytes, Cadmium, growth promotion.

1. INTRODUCTION

At present, the problem of environmental pollution due to the presence of heavy metals has brought with it the loss of the different components of air, water and soil (Singh et al., 2010; Chen et al., 2013), endangering the health of all organisms present in our biosphere. The presence of heavy metals in the various components is increasing every day as a result of anthropogenic actions in the different ecosystems, triggering harmful effects depending on the type of metal or metalloid, such as conditions ranging from damage to vital organs to carcinogenic developments (Sharma et al., 2015; Ruiz and Méndez et al., 2011). The main source of cadmium contamination in humans is the ingestion of contaminated vegetables and/or animal protein contaminated with this metal (Pelaez et al., 2016; Caraballo et al., 2012; Ruiz, 2011; Rodriguez et al., 2008; Calao and Marrugo, 2013). Cadmium has a strong tendency to bioaccumulate in plants, causing imbalances in nutrition and water transport processes (Sing and Tewari, 2003).

The ability of plants to extract cadmium is determined by the concentration of cadmium in the soil and its bioavailability, which depends on the presence of organic matter, pH, redox potential, temperature and the concentrations of other elements. Cadmium competes with macro and micronutrients such as potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) by competing for the same carrier protein (Di et al., 1999). Heavy metals are important as indicators of the ecological quality of soils due to their toxicity and bioaccumulative capacity in agroecosystems and their direct effects on health in all types of living systems, on soil-water-plant-animal-plant relationships and on humans (Weng et al., 2001).

Nowadays, phytoremediation is an effective, economical and environmentally friendly technology that is receiving much attention worldwide, bringing with it great benefits as opposed to traditional techniques of accumulating heavy metals from the soil. Such advantages are its low cost and negligible impact on humans and ecosystems (Glick, 2010; Sheng et al., 2008). The success of phytoremediation depends on the plant's ability to tolerate high concentrations of metals and produce large amounts of biomass (Ma et al., 2011). The efficiency of heavy metal phytoremediation is dependent on the performance of the plant and its ability to accumulate metal ions, the microorganisms associated with these plant species provide benefits to the plant as they can provide nutrients and reduce the harmful effects caused by heavy metals (Ma et al., 2011; Belimov et al., 2005).

Endophytic bacteria live in the internal tissues of the plant and play an extremely important role, which is to contribute to the adaptation of plant species to contaminated sites, and thus enhance their phytoremediation capacity and tolerance to pollutants present in the soil resource such as heavy metals (Li et al., 2012). Similarly, these bacteria also have positive effects on plant development by promoting plant growth and increasing biomass through the production of phytohormones such as indole acetic acid, in turn improve their nutritional status by fixing nitrogen, solubilising phosphates and producing siderophores for the uptake of essential nutrients in the soil (Sessitsch et al., 2013).

Cattle ranching in the department of Sucre occupies around 768,600 ha of land cultivated with pasture, representing 13.7% of the livestock area in the Caribbean region. The colosoana grass [Bothriochloa pertusa (L), A Camus], covers a total of 274,005 ha in the department of Sucre (Pérez et al., 2012). The reports on endophytic bacteria associated with colosoana grass, indicate the presence of these bacteria associated with different tissues; tests show that these bacteria have the ability to promote the growth of colosuana grass (Pérez et al., 2018), However, there is only evidence of endophytic bacteria associated with species of Cyperus sp and Paspalum sp with in vitro tolerance up to 400 ppm of $HgCl_2$ (Pérez et al., 2016). B. pertusa grass is widely distributed in the Colombian Caribbean region, covering extensive areas of the tropical dry forest (bs-T) and very dry tropical forest (bms-T) life zones in the department of Sucre, becoming an exclusive source of animal feed.

The genus Burkholderia is diverse and widespread in the environment (Compant et al., 2008). Of particular interest is the Burkholderia cepacia complex (Bcc) due to the potential of its members to function as plant growth promoters, bioremediating disease control agents, as well as their opportunistic role as human pathogens causing lung disease in immunocompromised individuals.

With the purpose of offering ecological alternatives to carry out phytoremediation processes, and to guarantee pastures the possibility of growing and adapting to environments contaminated with cadmium, the present study was carried out with the objective of evaluating the in vitro capacity of tolerance to cadmium of endophytic bacteria isolated from different tissues of colosuana grass present in cattle farms in four municipalities of the department of Sucre.

2. MATERIALS AND METHODS SAMPLING SITE

Sampling was carried out from August to December 2018, in cattle farms in four municipalities (San Marcos, Guaranda, Majagual and Sucre) of the department of Sucre, Colombia, sown only with colosoana grass.

2.1. Sampling

Sampling was carried out randomly in a zig-zag fashion, collecting soil samples up to 1 kg and 10 complete plants (root, stem, leaves and panicle) of colsoana grass at the same time. The samples were stored in icopor coolers for conservation and were identified with the respective variety, date of collection, and municipality. Part of the collected tissues and soil samples were sent to the University of Córdoba for cadmium determination and the other samples were taken to the Microbiological Research Laboratory of the University of Sucre and processed within twenty-four hours after collection, as established by (Pérez et al., 2013).

2.2. Cadmium concentration in soil and plant samples

To determine the total cadmium in colosoana grass tissue, 0.5 g of dry material was taken and an acid mixture of HNO3/H2O2 (5+2 mL) was added. On the other hand, 0.5 g of previously dried soil was taken and 10 mL of 65% HNO3 was added. Both soil and plant samples were processed in a Milestone ETHOS TOUCH 127697 series microwave oven and total cadmium was analysed by cold vapour atomic absorption spectrophotometry according to procedures described by (Marrugo et al., 2015).

2.3. Isolation of endophytic bacteria from colosuoana grass

The different tissues were surface disinfected. The surface disinfection process was performed 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 were taken and placed on the surface of the R2A agar culture medium and incubated at 32 °C for 72 hours. The number of bacteria per tissue (CFU/g tissue) was determined by direct colony counting on the surface of R2A agar.

Colonies that differed according to shape, texture, colour and size were selected (Pérez et al., 2015).

2.4. Evaluación in vitro de tolerancia a cadmio

Tolerance of endophytic bacteria to different concentrations of CdCl2 was performed in tris-MMT minimal medium (Rathnayake et al., 2013). The initial concentration of Cd used in the present study was 10 ppm and from these, concentrations of the metal were prepared up to 500 ppm. Aliquots of log-phase endophytic bacterial suspensions were inoculated onto MMT medium. MMT medium without CdCl2 was used as a control. The experiment was performed in triplicate, which was incubated in 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.

2.5. Qualitative assessment of the growth promotion of Cd-tolerant endophytic bacteria

$CdCl_2$-tolerant isolates were evaluated for them in vitro biological nitrogen fixation, phosphate solubilisation, indoleacetic acid and siderophore production. Nitrogen fixation was performed on selective ASHBY agar medium using the methodology described by (Pérez et al., 2014). Isolates were seeded on the surface of ASHBY agar medium with incubation at 28 °C for 72 hours. The test was positive for those isolates that grew on the surface of ASHBY medium. For the qualitative phosphate solubilisation test, this was performed on NBRIP medium with Ca3PO4 as the insoluble phosphorus source at pH 7 using the technique described by (Schwyn et al., 1987). The isolates were inoculated onto NBRIP medium incubated at 28 °C for 72 hours. The test was positive when visible transparent halo formation was observed around and below the colony. Finally, siderophore production was determined on chromium azurol-S (CAS) medium. For this, 60.5 mg CAS was dissolved in 50 ml of distilled water and combined with 10 ml of an iron (III) solution (1 mM FeCl2.6 H_2O and 10 mM HCl). Under stirring, this solution was mixed with 72.9 mg of HDTMA dissolved in 40 mLt of water. The resulting blue liquid was sterilised at 121°C for 15 minutes. In another vessel, 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 sterilised to reach a pH of 6.8. To the medium, 4 g of glucose is added as a carbon source. The strains are incubated for 7 days at 30°C. The ability of the bacteria to produce siderophores is evidenced by the formation of a ring.

2.6. Identification of Cd-tolerant bacteria

Genomic DNA extraction was performed according to the protocol described by (Green and Sambrook, 2012; Oliveira et al., 2013). The identification of bacteria with Cd tolerance activity was carried out using universal primers from the 16S rDNA region, which encodes the 16S rRNA small ribosomal subunit molecule. The specific primers used for each of the classes belonging to the bacterial domain (alpha, beta, gamma proteobacteria and firmicutes) corresponded to those proposed by (Oliveira et al., 2013). The amplification products were sent for purification and sequencing to Macrogen Korea. Once the nucleotide sequences were obtained, a search for homologous sequences was performed with the sequences stored in the database of the National Center for Biotechnology Information (NCBI). Base alignment was performed with the Clustal W software and analysis and correction with the Mega 4.0 program (Tamura et al., 2007). Using the same program, the method by which phylogenetic inferences were evaluated was determined.

2.7. Statistical analysis.

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

3. RESULTADOS

3.1. Cd concentrations and number of bacteria

The anova criterion of normality was fulfilled, and the response variable was found to have 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 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 by tissue in colosuana grass, Figure 1B shows significant differences (p-value<0.05), with higher concentrations of cadmium in roots with a value of 3.21 and lower in inflorescence (1.05 mg/kg of tissue). In relation to the quantity of endophytic bacteria per tissue, it is observed that the highest quantity of bacteria is reported for inflorescence (2.0 x 10^7 CFU/g tissue) and the lowest in roots (3.0 x (10^5 CFU/g tissue) (Figure 1C). It is also 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 the panicle tissue where lower values of cadmium were found and lower amounts of these bacteria in roots where higher values of this metal were recorded.

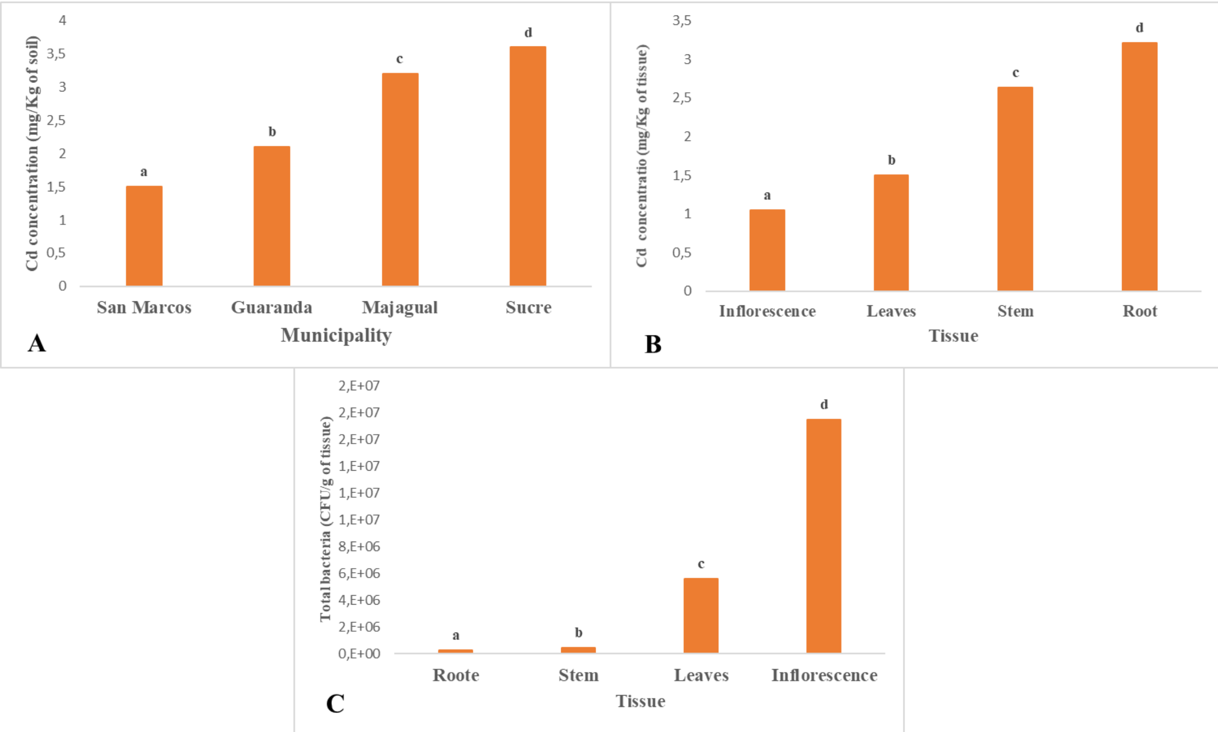

Figure 1. A): Cadmium concentration (mg/kg) per municipality; B): Cadmium concentration (mg/kg) per tissue of B. pertusa and C): endophytic bacteria.

3.2. Cadmium tolerance test results of endophytic bacteria.

From a total of 57 isolates obtained from B. pertusa, 23 showed tolerance to different concentrations of Cd in the form of CdCl2, with significant differences (p-value<0.05) between isolates and Cadmium concentration used. The results of the Tukey test show that the isolate with the highest tolerance was SBpR-10. When evaluating the minimum and maximum growth to CdCl2 with the highest tolerance, it was found that SBpR-10 grew up to 500 ppm CdCl2 (Figure 2).

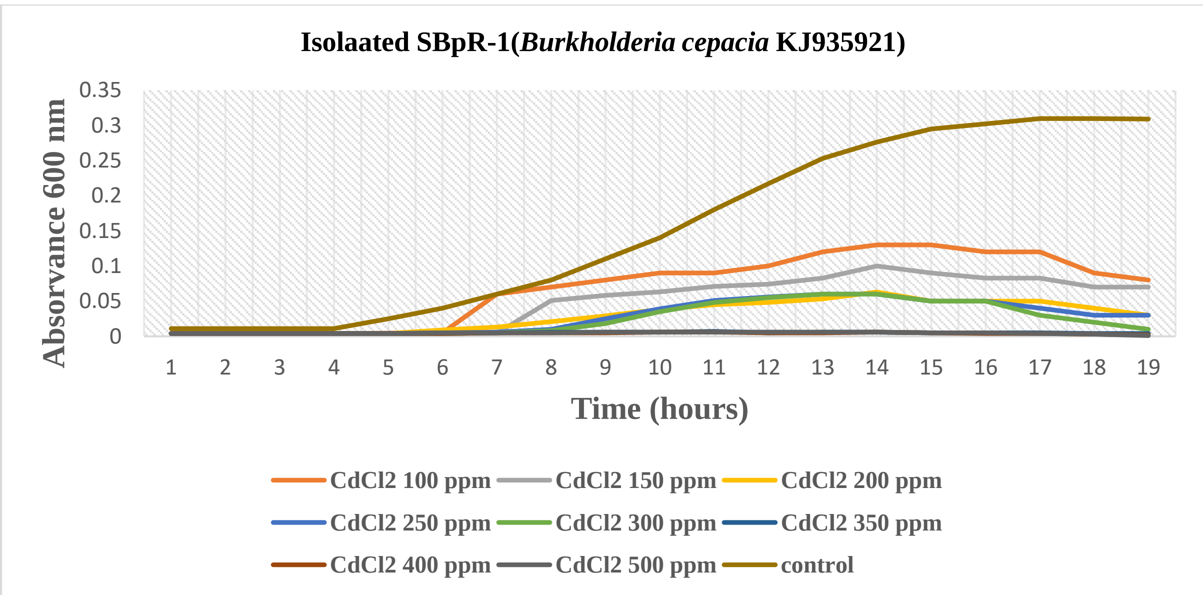

Figure 2. Tolerance test result of isolate SBpR-10 (Burkholderia cepacia KJ935921) to different concentrations of CdCl2 (ppm). Bp: Bothriochloa pertusa; R: root.

3.3. In vitro assessment of the plant growth-promoting capacity of Cd-tolerant endophytic bacterial isolates

The ability to fix atmospheric nitrogen was qualitatively estimated by the ability of the isolates to grow on selective ASHBY agar medium. Of the seven isolates with maximum cadmium tolerance, all showed significant growth on this medium (figure 4A). The endophytic bacterial isolate identified as SBp2R-10 showed in vitro ability to fix nitrogen; phosphate solubilisation was determined on NBRIP medium and ability to produce siderophoresimultaneously (Figure 4B). Qualitative analysis of siderophore production was evidenced by the formation of a halo around the colony on CAS medium, which was observed for the isolate SBp2R-10 (Figure 4C).

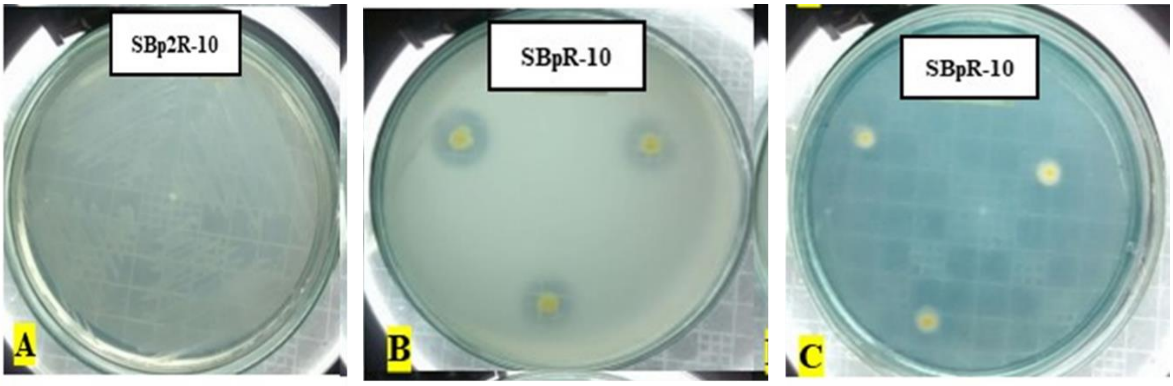

Figure 4. Qualitative assay of nitrogen fixation in ASHBY medium (A), phosphate solubilisation in ASHBY medium (B) and siderophore production in CAS medium (C) of CdCl2-tolerant endophytic bacterial isolates. Bp: Bothriochloa pertusa; R: Root.

3.4. Identification of Cd-tolerant endophytic bacteria isolates

All isolates were morphologically characterised and identified using 16S rRNA gene PCR amplification (Table 1). The identified endophytic bacterium was reported with a homology percentage of 97% with the bacterium Burkholderia cepacia KJ935921 (97%).

Table 1. Identification of endophytic bacteria isolated from different tissues of Cd-tolerant colosoana grass, tolerant to Cd in the form of $CdCl_2$, using NCBI BLAST-N 16S rRNA gene sequences.

Isolote	Closest match / species identity	Group	Activity		
			N	P	S
SBpR-10	Burkholderia cepacia KJ935921 (97%)	Beta Proteobacteria	+	+	+

N: nitrogen fixation; P: phosphate solubilisation; S: siderophore production; +: positive activity; -: negative activity; Bp: Bothriochloa pertusa; R: root.

The isolate SBpR-10 identified as Burkholderia cepacia KJ935921 and SBpR-1 (Pseudomonas fluorescens VI8L1), tolerated up to 500 ppm CdCl2 and showed in vitro nitrogen fixation, phosphate solubilisation and siderophore production. Both isolates were isolated from roots of B. pertusa from cattle farms located in the municipality of Sucre.

4. DISCUSSION

4.1. Cadmium in soil on livestock farms

Soil cadmium values ranged between 1.5 ± 3.6 mg/kg soil, with the highest amount of cadmium in soil found in the municipality of Sucre (3.6), followed by Majagual (3.2) and the lowest in San Marcos (1.5 mg/kg soil). International reference values for cultivated soils are 0.01-2.0 mg/kg (Kabata-Pendias, 2011). The values found in the present studies are above the reference values. Cadmium enters the soil through the application of fertilisers produced from phosphate rock (Monteiro et al., 2006; Chen et al., 2008; Subero et al., 2016). Due to the low fertility levels of rice soils, in Colombia high doses of fertilisers (nitrogen, phosphorus, potassium, among others) are applied to the soil and to the crop (Monteiro et al., 2006), increasing the levels of cadmium in the soil and its availability in the food chain, being easily absorbed by the different tissues of the grass, generating toxic effects on the entire natural cycle (Castilla et al., 2010).

The values of cadmium per tissue of B. pertusa were 1.05 ± 3.21 mg/kg of tissue, with the highest amount of this metal found in the root (3.21) and lower in the inflorescence (1.05 mg/kg of tissue). At international level for plants, the permitted values correspond to 0.05 - 0.5 mg/kg of tissue, indicating that the values found in the different tissues of Colossean grass are above those permitted at international level (Kabata-Pendias, A. 2011).

Studies by (Insuasty et al., 2008) suggest that cadmium is taken up and translocated to different plant tissues, including the inflorescence or seeds. Work carried out on accumulation in other grasses such as rice indicates that approximately 0.73% of the total Cd present in rice crops is transferred to the grains (Insuasty et al., 2008), accumulating to average values of 1.02 mg/kg, which represent concentrations above the European Union guidelines for the concentration of this metal in rice grains (Yang et al., 2016). On the other hand, (Liu et al., 2007), report trace values of cadmium in rice grains of 0.93 mg/kg. Other studies suggest that this metal accumulates preferentially 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 in stems, leaves, fruits and seeds (Méndez et al., 2007; Wu et al., 2015).

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

The values of bacterial numbers ranged between 3.0 x 105 ± 2.0 x 107 CFU/g 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 tissue). The results also show that there is an inverse relationship between the presence of endophytic bacteria and cadmium values in the tissues, with higher amounts of these bacteria being found in those tissues where lower cadmium values per mg/kg were recorded. Under normal growth conditions of B. pertusa pasture, in the department of Sucre, the presence of endophytic bacteria is reported in a range of 3.18 x108 ± 4.48x1010 CFU/g of tissue (Perez et al., 2018). These reported values are above those found in the present study, indicating that heavy metals, at different concentrations, have an inhibitory effect on bacterial growth, in general, heavy metals (such as Al, As, Cd, Hg, Pb, etc.) decrease the amount of microbial biomass (Wu et al., 2015; Perez et al., 2015). Bacterial growth in the presence of metals may be slower, as microorganisms may be stressed. This is a consequence of diverting energy from growth to the maintenance of other functions, as there is a high energy demand to cope with the toxicity of the pollutants (Timberley et al., 2009).

4.3. Cadmium tolerance test results of endophytic bacteria.

The tolerance test shows tolerance up to 500 ppm CdCl2 of the isolate SBpR-10. This metal is considered a non-essential trace element with ecotoxic effects on plants, animals and humans at low concentrations (Rajkumar et al., 2009). Tolerance test results suggest that certain species of endophytic bacteria are able to tolerate high concentrations of Cd. According to the reports of (Rajkumar et al., 2009) this tolerance is possibly related to the adaptation of the bacteria to live under conditions of constant metal stress. These bacteria have evolved several tolerance mechanisms to tolerate the harmful effects of toxic metals, including: cellular components that capture the ions, neutralising their toxicity; enzymes that modify the redox state of the metals, converting them into less harmful forms; and membrane transporters that expel the harmful species from the cell cytoplasm (Rajkumar et al., 2009).

Although the tolerance pathways were not evaluated in the present study, possibly one of the possible explanations is the production of siderophores in these two isolates (Table 1). Bacteria with the ability to resist heavy metals in contaminated soils through siderophore production. According to (Rajkumar et al., 2009) bacterial siderophores contribute in plants to reduce the toxicity caused by the presence of heavy metals and also supply the need for iron as an essential element, promoting the development and growth of plants in contaminated environments.

4.4. Identification of endophytic bacteria

A total of seven species of endophytic bacteria isolated from B. pertusa with the ability to tolerate cadmium were identified through 16S rDNA sequences. Burkholderia difusa, Bacillus cereus and Burkholderia cepacia studies carried out by (Pérez et al., 2016), identified these species as endophytic bacteria isolated from Cyperus sp and Paspalum spadapted in mercury-contaminated environments and showed in vitro tolerance of up to 500 pp of mercury chloride. Other work by (Lodewyckx, et al., 2001) reports B. cepacia as an endophytic bacterium with the ability to tolerate and remediate cadmium. Burkholderia ambifaria was reported as an endophytic bacterium of yam plants with antimicrobial activity (Okon, 2022). Other reports record B. ambifaria as a soil and rhizosphere bacterium of various crops (Coenye et al., 2003; Coenye et al., 2001). The role of endophytic Burkholderia cepacea in plant growth promotion is evidenced by several studies (Zhao et al., 2016; Baghel et al., 2020). The bacterial employ several mechanisms through which they affect plant growth and development under a given set of conditions. The mechanisms of PGP involve: production of phytohormones, including indole-3-acetic acid (IAA), cytokinins, gibberellins, etc.; enhanced acquisition of nutrients through the process of fixation, solubilization, or release of low molecular weight organic acids; production of ACC (1-aminocyclopropane-1- carboxylate) deaminase enzyme, which inhibits plant senescence through the regulation of ethylene hormone.

5. CONCLUSION

The cadmium values found in the soil and in the different tissues of B. pertusa are in the highly toxic category. The soils evaluated belong to cattle farms in the department of Sucre, located in the municipalities of Guaranda, Majagual, San Marcos and Sucre. At the time of this study, there was no knowledge of the presence of this metal in the soil and in the different tissues of B. pertusa, nor was there any monitoring of this problem or measures to reduce and mitigate the presence of cadmium in these soils. There is the presence of endophytic bacteria associated with B. pertusa, coexisting with this metal in the different tissues analysed. These bacteria showed in vitro tolerance up to 500 ppm of $CdCl_2$, which indicates the capacity of these bacteria to directly remediate this metal. Likewise, growth-promoting activity was demonstrated, which indirectly contributes to reducing the toxic effect of this metal in the soil and in the tissues.

It has been experimentally demonstrated that plant-associated Burkholderia spp. contribute to nutrient uptake, especially under stress conditions. Endophytic Burkholderia spp. enhance plant survival against deadly pathogens through mechanisms such as competition, induced systemic resistance and antibiosis. At the same time, they show a broad plant tolerance to multiple abiotic stresses especially drought, salinity and cold. Several attempts have been made to decipher the potential of Burkholderia spp. by genome extraction, and these bacteria have been found to harbour genes for plant symbiosis and to provide multiple benefits to host plants. Specific traits for host recognition and nutrient acquisition were confirmed in the Burkholderia endophyte by genomics and proteomics-based studies. This could pave the way to exploit Burkholderia spp. for biotechnological applications such as biotransformation, phytoremediation, insecticidal, antimicrobial, etc. activity. All this makes Burkholderia spp. a promising microbial agent to improve plant performance under multiple adversities.

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