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Endophytic Bacteria Tolerant To High Cadmium Concentrations

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ABSTRACT

To assess the in vitro capacity of endophytic bacteria tolerance isolated from different tissues of the rice variety Fedearroz 2000 (F2000), to different concentrations of cadmium in the form of $CdCl_2$. The bacteria were isolated from root, stem, leaf and panicle F2000. Suspensions each log phase was inoculated into minimal medium tris-MMT with different concentrations of $CdCl_2$. The experiment was incubated with stirring at 150 rpm at 32 °C for seven days; growth isolated in the assay was determined by turbidimetry at 600 nm every hour for four days. Siderophore production was determined by the growth of bacteria in mediumazurol-S (CAS). There is an inverse relationship between number of bacteria and tissue concentration of cadmium, being more bacteria in those tissues where there is lower concentration of cadmium. isolates were identified as Bacillus thuringiensis H1M3F2000LIM F14 and P1M8 F2000LIM Bacillus cereus LB10, able to tolerate up to 400 ppm of and 500 $CdCl_2$, respectively. In the middle CAS, H1M3F2000LIM evidenced siderophore production. The information obtained in this study suggests future use of endophytic bacteriaone biological strategy to recover agricultural soils contaminated with cadmium.

Keywords: endophytic bacteria, tissues, cadmium, recovery

1. INTRODUCTION

As manifested [1, 2] in the problem worldwide due to environmental pollution, brings one of the main concerns of this century. Such as loss of different components air, water and soil placing at risk the health of all organisms in our biosphere. The presence of pollutants in the various components has been increasing, as a result of anthropogenic pressure in ecosystems, such as heavy metals who trigger harmful effects depending on the type of metal or metalloid, conditions from occurring damage to vital organs until cancerígenos effect [3,4].

The effect of cadmium in humans has been associated with several pathologies such as kidney failure, pulmonary emphysema, osteoporosis, hypertension and certain forms of cancer such as prostate, similarly is associated with diseases and disorders in various organs, as dysfunctions liver, emphysema, anemia, osteomalacia, neurological impairment and damage to testes, pancreas and adrenal glands. The main source

of cadmium contamination in humans is the intake of vegetables contaminated with this metal. For this reason, joining the organism is commonly orally, as a result of this phosphate fertilization carried out in Agricola soils [5-8]. Cadmium is a heavy metal that has a marked tendency to bioaccumulate in plants, causing imbalances in the processes of nutrition and water transport [9]. The ability of plants to capture cadmium is influenced by the concentration of metal in the soil and its bioavailability, which depends on the presence of organic matter, pH, redox potential and temperature the concentrations of other elements. In the case of cadmium this creates competition with nutrients such as potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) to compete for the same protein Carrier [10].

In rice plants cadmium absorbed and translocated to different parts of the plant including the panicle, which is affected due to the large reduction in the number of rice grains. But not only cadmium contamination can affect rice production and thus the entire guild of farmers who depend on their harvest, but also human health because its accumulation in the panicle is one of the main means of intake cadmium in humans.

The phytoremediation is considered one effective, cheap and friendly technology environment with much attention worldwide, because it brings great benefits as opposed to the traditional technology of accumulating heavy metals from soil. Such advantages are its low cost and negligible impact for human beings and ecosystems [11, 12]. The phytoremediation success depends on the ability of the plant to tolerate high concentrations of metals and produce many biomass [13].

The endophytic bacteria are found along cough different internal plant tissues play a major role, which is to contribute to the adaptation of plants to contaminated sites, and thus potentiate if fitorremediadora capacity and tolerance the present contaminants in soil resources like heavy metals [14]. Similarly, these bacteria also have effects on plant development promoting plant growth and increasing the biomass through the production of phytohormones such as indoleacetic acid, in turn improve the nutritional status thereof by nitrogen fixation, solubilization of phosphates and production of siderophores for uptake of essential nutrients in its development [15]. Starting from this reality in order to contribute to the planning of new ecological alternatives to carry out processes phytoremediation, and ensure that plants can grow and adapters to contaminators environments cadmium, the need arose to isolate endophytic bacteria from different rice plant tissues and evaluate in vitro the ability of different concentrations of tolerance cadmium.

2. MATERIALS AND METHODS

- **Study area.** Commercial rice varieties Fedearroz 2000 (F2000) were collected in the municipality of Mocarí-Córdoba-Colombia with coordinates 8th 47'25 "of North Longitude 75 ° 51'38" west longitude relative to Greenwich Mean Time, with a average temperature of 29 ° C, relative humidity of 80%, average annual rainfall of 1200 mm and height of 20 m.
- **Sampling site.** Sampling was done randomly zigzag, collecting 10 full rice plants per sampling site. Samples were labeled with their respective range and collection date. These were stored and preserved in boxes Polystyrene at 4 °C for transport to the

laboratory microbiological investigations of the University of Sucre and processed within 24 hours after harvest. Isolation of endophytic bacteria. For disinfection and isolation of endophytic bacteria of different plant tissues it methodology was followed proposed by [16]. Which consists: washing the root, stem, leaf and panicle each rice plant with sterile water and cut into segments about 1 cm. Disinfection of each tissue was performed separately in sterilized distilled water, followed by stirring for 15 min in buffer potassium phosphate 0.05 mol L^{-1}, pH 7.0; immersion for 1 min in 70% alcohol; stirring for 5 min in sodium hypochlorite solution 5% and 80% Tween; again immersed for 1 min in 70% alcohol followed by stirring 15 min in phosphate buffer 0.05 mol^{L-1} potassium, pH 7.0 and finally washing four times in distilled water esterilizada16. The population density of tissue bacteria (CFU / g tissue) It was estimated by direct counting on plates. During counting were observed and selected colonies are distinguished in shape, surface appearance, color and size. The selected isolates were purified and maintained on agar R_2A[16].

- **Cadmium concentration.** Samples of rice plants were separated by (root tissues, stem, leaves and panicle), then were washed with distilled water to remove mineral particles adsorbed on its surface. Then each tissue separately deposited in paper bags and dried in oven at 60 ° C for 24 h. To determine the total cadmium in the samples 0.5 g of dry material were taken and thereto is added an acid mixture HNO_3 / $H2O_2$ (5 ± 2 mL). Moreover, the predried 0.5 g soil were taken they were added 10 mL of 65% HNO_3. Samples were processed in a Milestone ETHOS microwave oven TOUCH series 127.697 and sent to specialized for determining the cadmium concentration by laboratory tissues.
- **Tolerance test endophytic bacteria cadmium**. The in vitro assay of endophytic bacteria tolerance at various metal ion concentrations of Cd, was carried out in minimal medium tris-MMT proposed by [17] with eight different concentrations of cadmium in the form of $CdCl_2$. The initial concentration of Cd used was 0.01 mg / ml and from these concentrations of 100 were prepared (0.1 mg / mL), 150 (0.15 mg / mL), 200 (0.2 mg / mL, 250 (0250 mg / mL), 300 (0.3), 350 (0.35), 400 ppm (0.4 mg / mL) and 500 ppm (0.5 mg / mL). Aliquots of bacteria in logarithmic phase was inoculated into the medium MMT. As control means MMT was used without $CdCl_2$. The experiment was performed in triplicate, which was incubated under stirring at 150 rpm at 32 ° C for 120 hours [18]. The growth of each bacterium was determined by turbidimetry method at 600 nm every hour for four days.
- **Siderophore production.** The production capacity of siderophores was carried out in half azurol-S (CAS) used by [19]. For this purpose, 60.5 mg of CAS was dissolved in 50 ml of distilled water, the above was combined with 10 ml of a solution of iron (III) (1 mM $FeCl_3$.6 H2O and 10 mM HCl). Under stirring, this solution

was mixed with 72.9 mg of HDTMA dissolved in 40 ml of water. The resultant blue liquid was sterilized at 121 ° C for 15 minutes. In another container a mixture of 750 ml of water, 15 g agar, 30.24 g of pipes, and 12 g of a 50% solution (w / w) NaOH to reach pH 6.8 was also sterilized. The medium will be added 4 g of glucose as carbon source. Strains are incubated for 7 days at 30 °C. The ability of bacteria to produce siderophores evidenced by the formation of a halo DNA extraction, amplification and 16S rDNA sequence endophytic bacteria tolerant to Cd. They isolate which showed the best results regarding cadmium tolerance and siderophore production were selected. For the extraction of DNA samples pure bacteria were taken and were activated in agar Luria Bertani (LB), (Bacto tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, milli-Qate 1000 mL, pH 7.0) and incubated at 28 ^{o}C/24 hours, after this time were taken again and pure colonies were transferred to tubes containing 10 ml LB broth and incubated again for 24 hours at 28 °C with constant stirring at 150 rpm in a controller IKA 260.1 Basic. The DNA was extracted using proposed protocol by [20]. Amplification of 16S rDNA fragments was carried out using specific oligonucleotide eubacterias groups [20]. The amplification products were sent to sequencing the company Macrogen (Seoul, South Korea) on an automated capillary sequencer 3730XL. Entities of the nucleotide sequences obtained were compared with those stored in databanks of the National Center for Biotechnology Information (NCBI). The alignment of the bases was performed by CLUSTAL W; phylogenetic inferences were obtained by maximum similarity method based on Kimura-2-parameter test bootstrapping (1000 replicates) with 7 MEGA program model.

- **Analysis of data**. The data were organized in figures for better understanding of the results. Analysis of variance and multiple range Tukey test was used to establish significant differences between the variables analyzed. Assays were performed in triplicate and results expressed in half. Data were analyzed in the InfoStar software.

3. RESULTS AND DISCUSSION

By meeting the criteria of ANOVA, we proceeded to the analysis of variance, which indicates significant statistical differences (p-value <0.05) among the population density of endophytic bacteria and tissue concentration of cadmium. The results of the Tukey test show statistically significant difference (p-value> 0.05) among the population density of bacteria tissue, being most densely populated in panicle (1.12 x 10^{10} CFU / g tissue) when there values in that same tissue under Cd (1.6 mg / kg) and lower in roots (9.8 x 10^{8} CFU / g tissue) when found higher values of Cd (3.78 mg / kg). The results indicate an inverse relationship to the concentration of Cd in tissues with respect to the density of endophytic bacteria, showing that in those tissues as the roots where higher value Cd (3.78 mg / kg), there is a fewer bacterium, whereas in tissues with lower concentration of Cd (1.6 mg / kg) there is a high presence of these bacteria (Figure 1).

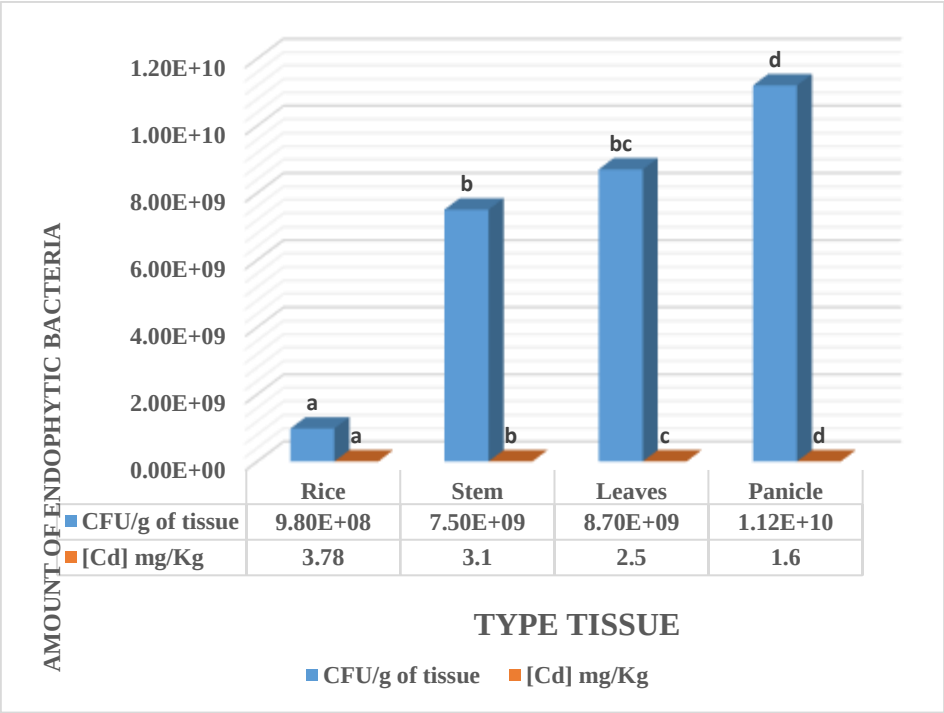


Figure 1. Relationship between population density (CFU / g of tissue) of endophytic bacteria and Cd concentration (mg / kg) in different tissues of the rice variety F2000.

Studies carried out on presence of endophytic bacteria in plant species, they show that the presence of these bacteria is highly variable and depends on conditions such as species of bacteria; the genotype of the host plant, the state of development of the plant, the amount of inoculum, environmental conditions and stress abiotic [21]. According to the manifested by [13], the presence of endophytic bacteria within the plant helps these to grow under conditions and adapt to abiotic stress conditions, because the induced to tolerate high concentrations of heavy metals and promote their growth by nitrogen fixation, phosphate solubilization, siderophore production and ACC deaminase, among others.

In the Figure 2, isolated growth observed H1M3F2000LIM in different concentrations of CdC_{l2}. As shown in Figure, isolated had a growth form nonuniform in different cadmium concentrations evaluated with respect to the control, showing higher growth at concentrations of 100 and 150 ppm. Also it shows that the concentrations of 200 - 350 ppm had an average growth. The lower growth was found at 400 ppm where a long adaptation phase is observed. The isolated as shown in Figure grew in different concentrations until 14 hours after starting the experiment compared this with the witness comprtamiento grew to approximately 19 hours.

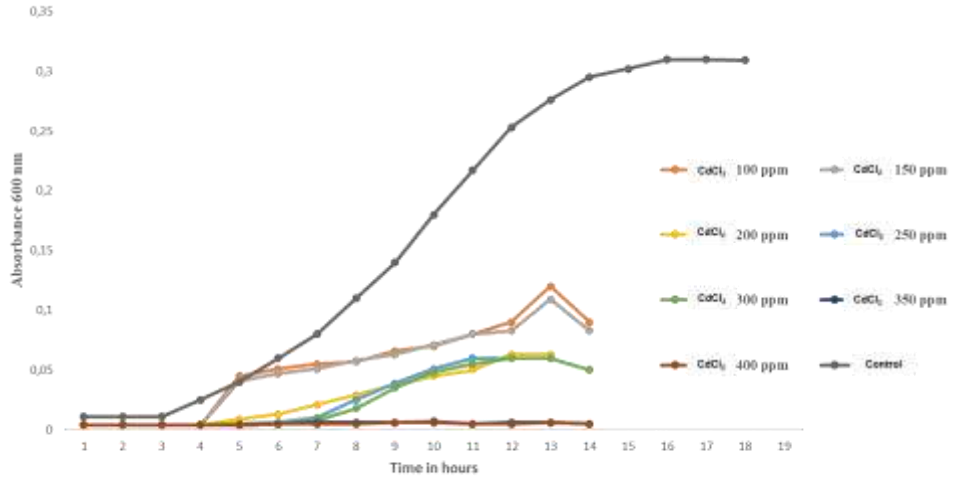


Figure 2. Growth curve of isolated H1M3F2000LIM (Bacillus thuringiensis F14) at different concentrations (ppm) of cadmium in the form of $CdCl_2$.

With respect to isolated P1M8F2000LIM, it appears grew up to 500 ppm, showing a different behavior from the previous isolated, with higher growth 100, 150, 200 and 250 ppm to 16 hours. With respect to the concentrations of 300 and 350 ppm average growth it was observed until 15 hours after which declined (Figure 3). As regards growth at concentrations of 400 and 500 ppm is observed was a slight increase until 15 hours showing a long adaptation phase, compared to the control.

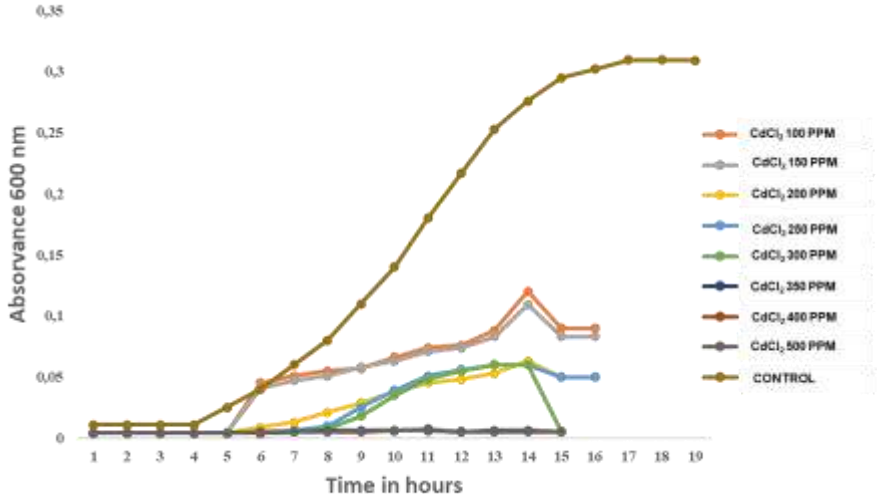


Figure 3. Growth curve of isolated P1M8 F2000LIM (Bacillus cereus LB10) at different concentrations (ppm) of cadmium in the form of $CdCl_2$.

The results in this study show that with increasing concentration of cadmium in the environment, decreases the growth of the isolates, presenting retarded growth, which leads to a longer adaptation of the bacteria and thus this way to survive the different

concentrations of cadmium used. It is evident according to our opinion that adaptation and growth behavior of both species (isolated) and the tissue type where it was isolated. Note that H1M3F2000LIM It was isolated from leaves of rice variety F2000 and grew to 400 ppm, while the isolated P1M8 F2000LIM, was obtained from panicle and growth reached to 500 ppm.

Regarding the identification of isolates which showed high capacity cadmium tolerance, of the isolated sequences were compared with sequences present in the library NBCI. Phylogenetic analysis of 16S rDNA of endophytic bacteria of the rice variety F2000, showing tolerance activity shown (Figure 4), which states that only two isolates belonging to Firmicutes phylum showed in vitro ability to tolerate up to 500 ppm as $CdCdCl_2$. The H1M3 isolated was identified as Bacillus thuringiensis, one entomopathogenic bacteria that produces a parasporal inclusion, consisting of different protein structures called Cry proteins, these proteins are the major virulence factor of the bacteria, which have a weight ranging from 60 and 140 kDa. These proteins are highly toxic against insects of the orders Lepidoptera, Dipteran, Coleopteran; They have been used as biopesticides and development of transgenic [22] crops. In work on diversity of endophytic bacteria on rice in the department of Cordoba, it was reported Bacillus thuringiensis strain SAU-Ab-1, as an endophytic bacteria associated with rice plants with antimicrobial activity against Burkholderia glumae, which causes bacterial blight in said cultivars [23]. With respect to isolated P1M8 F2000LIM as the result of sequence similarity to keep the bacteria Bacillus cereus LB10. B. cereus has been identified as endophytic bacteria rice plants capable of promoting growth vegetal [24]. Additionally, [25] in his study diversity endophytic bacteria associated with potato cultivation, the bacteria isolated and evaluated the ability to fix nitrogen and indole acetic acid production.

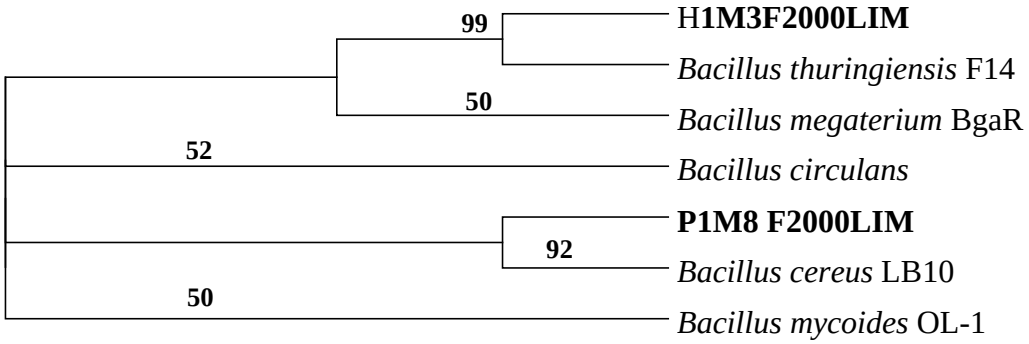


Figure 4. Phylogenetic tree of the H1M3F2000LIM and P1M8 F2000LIM isolates the variety fice F2000 and their relationships with species of bacteria of the phylum Firmicutes H: leaves; M3: morphotypes three; P: Panicle; M8: morphotypes eight; F2000: variety Fedearroz 2000 and LIM: microbiological laboratory research.

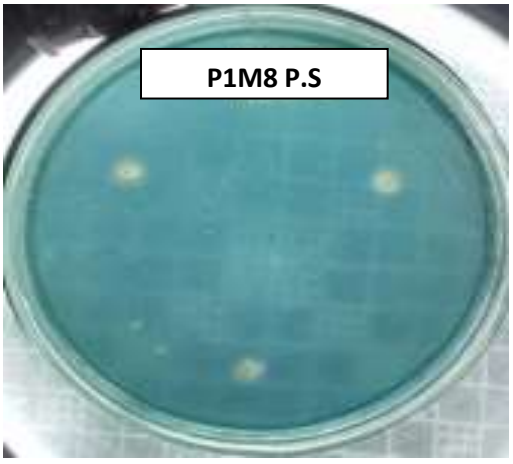
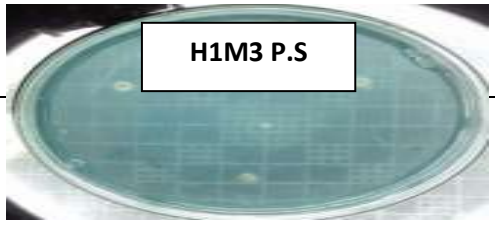
Also study conducted by [26], on isolation endophytic bacteria associated with the genera Paspalum and Cyperus adapted in contaminated soil with mercury in the

Southern Bolivar, Colombia, reported the presence of endophytic bacteria Bacillus cereus GU056811 with in vitro ability to tolerate up to 400 ppm (0.4 mg / L) mercury as $HgCl_2$. Also study conducted by[27] concluded that endophytic bacteria Bacillus cereus 1DH1LIM has the ability to tolerate up to 400 ppm of Pb as $Pb(NO_3)_2$ and of producing siderophore.

Qualitative tests siderophore production in medium azurol-S (CAS), showed that only isolated P1M8F2000LIM identified as Bacillus cereus LB10 after being subjected to growth to 500 ppm of Cd as $CdCl_2$ it showed in vitro siderophores production capacity (Table 1), whose activity possibly directly related bacteria tolerance to mentioned metal. According to the points by[28], the siderophore production by bacteria may possibly contribute to the plants to reduce toxicity caused by the presence of heavy metals and also supply the need of iron as an essential element, promoting the development and growth of plants in contaminated environments.

Studies show that endophytic bacteria having this ability produce siderophores to Fe^{3+} bind with high affinity to solubilize and promote its absorption in an efficient manner. These molecules may also be complexed with other divalent metal ions to be assimilated by the plants [27, 13]. Furthermore, these microbial compound, its production is stimulated by the presence of heavy metals and helps reduce its toxicity to plants, enhancing growth the same in poor environments nutrients [28,14] and also promote the production of indoleacetic acid by reducing the harmful effects of heavy metals by chelation reaction [29].

Table 1. Relationship between isolates of endophytic bacteria and siderophores of production

Isolated	Siderophores of production	In vitro activity
P1M8 F2000LIM (Bacillus cereus LB10)	(+)	
H1M3F2000LIM (Bacillus thuringiensis F14)	(-)	

Furthermore, studies conducted argue that the impact on the quantity and quality in the production of siderophores by the bacterial species Pseudomonas fulva promoter plant growth due to the increased exposure of Cd^{2+} (0. 0.5, 1.0, 2.0 mM), it was evaluated for changes in the production of siderophores. The results demonstrate that in the presence of 2.0 mM Cd^{2+} it is produced the siderophores synthesis the hidroximatos, catecholates and phenolates group occurs with respect to lower levels of Cd^{2+} (0.5 and 1.0) [30]. In our view, possibly mechanism siderophore production around the environment where bacteria grow sequester heavy metal allows them and reduce their accumulation within the cell. Consequently, the bacteria reduce the transport of heavy metals in high concentrations to avoid poisoning cytoplasm of the cell.

4. CONCLUSION

were identified two isolates of endophytic bacteria associated with the variety of commercial rice F2000, as H1M3F2000LIM (Bacillus thuringiensis F14) and P1M8 F2000LIM (Bacillus cereus LB10) who showed in vitro ability to tolerate up to 400 and 500 ppm of $CdCl_2$, respectively. Meanwhile in the middle CAS, isolated P1M8 F2000LIMHe showed siderophores of production. however, it is reported to Bacillus thuringiensis as entomopathogenic bacteria and as a endophytic bacteria rice plant with activity demonstrated in vitro to inhibit Burkholderia glumae, while Bacillus cereus showed well tolerated mercury and lead in this study were found to tolerate cadmium, becoming a endophytic bacteria ability to tolerate various heavy metals. The results obtained in this studio evidence that this bacterium is able to tolerate up to 500 ppm of $CdCl_2$. Both endophytic bacteria isolated in this work becomes a new biological strategy to remediate contaminated with cadmium commercial rice varieties cultivated soils future.

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6. AUTHORSHIP CONTRIBUTIONS

All authors have jointly and equally contributed to the argumentation and writing of the manuscript.

7. FUNDING.

None.

8. CONFLICT OF INTEREST

We declare no conflict of interest.

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