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Siderophore Production By Bacillus Cereus Strain BN5 In Different Cadmium Concentrations

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ABSTRACT

To evaluate in-vitro the siderophore production capacity of Bacillus cereus strain BN5 at different cadmium (Cd) concentrations. The bacterium was isolated from the rice variety F2000 grown in the municipality of Achi-Antioquia, Colombia. Aliquots of B. cereus strain BN5 in logarithmic phase were inoculated in tris-MMT minimal medium with different concentrations of CdCl2, the experiment was incubated in agitation at 150 rpm at 32 °C/ 90 hours; the growth of the bacteria in each treatment was evaluated by turbidimetry technique at 600 nm. Siderophore production was determined on chromium azurol-S (CAS) medium. There is an inverse relationship between population density and Cd concentration per tissue, with a higher number of bacteria in the panicle than in the other tissues. B. cereus BN5 tolerated up to 500 mg/L $CdCl_2$ and produced siderophores in the different treatments evaluated. This endophytic bacterium could be used in the future as an alternative for bioremediation and safe rice production in Cd-contaminated soils.

Keywords: Bacteria, rice cultivation, cadmium, siderophores

INTRODUCCIÓN

Iron (Fe) is a cofactor for several enzymes and a vital micronutrient required for many cellular processes. This micronutrient is present in the environment in sufficient quantities, but in different microbial habitats it forms insoluble complexes in the presence of oxygen and water at neutral and basic pH [1]. For the acquisition of Fe, microorganisms have developed mechanisms to solubilize it and make its uptake efficient. One of the mechanisms employed by

bacteria and fungi is the production of low molecular weight compounds, Fe^{3+} chelator, which are synthesized under conditions of deficiency of this micronutrient, called siderophores [2]. There are different classes of siderophores with differences in their structure, which are classified into three categories according to the functional groups they use as ligands for iron ions, including catecholates such as enterobactins and vibriobactins, hydroxamates such as staphyloferrin and mixed-type siderophores such as mycobactin [2].

Recent reports from various studies indicate that microbial siderophores can form stable complexes with other metals present in the environment (aluminium, cadmium, copper, gallium, lead, nickel and others) as well as with uranium [3]. Siderophores are produced by various groups of micro-organisms, plant and animal pathogens, free-living micro-organisms and nitrogen-fixing symbionts. Siderophore production is most common in plant growth-promoting rhizospheric bacterial species, which possess the ability under extreme environmental conditions. Groups of bacteria include: Pseudomonas, Azotobacter, Bacillus, Enterobacter, Serratia, Azospirillum, Rhizobiun and Steptomyces [1,4].

Many other plant-associated bacteria such as endophytes can synthesize siderophores, which gives them a competitive advantage in the colonization of plant tissues, helping them to exclude other microorganisms from their ecological niche [5,6].

There is evidence of siderophore synthesis by bacteria growing in environments where toxic heavy metals are present, linking these chelating agents as responsible for the homeostasis of these metals[7]. Several studies suggest that siderophores form stable complexes with other metals, such as Al, Cd, Cu, Ga, In, Pb and Zn3,7. Work by [8] found that the addition of heavy metals such as Al, Cu, Ga, Mn and Ni induced the production of pioverdin in Pseudomonas aeruginosa.

Heavy metal contamination of soils is becoming widespread and causing serious environmental problems. Heavy metals not only destroy the ecology of soil microbes and decrease agricultural crop production, but also affect human health through the food chain [9]. Excessive use of phosphate fertilizers, landfill sludge dispersal and atmospheric deposition have caused widespread cadmium (Cd) contamination in agricultural soils. Cadmium is a highly toxic metal harmful to living organisms at relatively low concentrations (0.001-0.1 mg/L) [10] and has been classified as a human carcinogen [11,12]. Cadmium is taken up from the soil by rice plants growing in contaminated soils through the root and from there it is translocated to the stem and eventually to the grain. Between 22-24% of the total cadmium present in the rice biomass is concentrated in the grain, consumption of contaminated rice can cause serious risks to human health [13].

Phytoremediation is considered an effective, cheap and environmentally friendly technology with much attention worldwide, because it brings great benefits as opposed to the traditional technology of accumulating heavy metals from the soil. Such advantages are its low cost and negligible impact on humans and ecosystems [14,15]. The success of phytoremediation depends

on the plant's ability to tolerate high concentrations of metals and to produce a large amount of biomass [16].

Endophytic bacteria are found throughout the different internal tissues of the plant and play an extremely important role, which consists of contributing to the adaptation of plant species to contaminated sites, thus enhancing their phytoremediation capacity and tolerance to pollutants present in soil resources, such as heavy metals [17]. Similarly, these bacteria also have effects on plant development, promoting plant growth and increasing biomass through the production of phytohormones such as indoleacetic acid, as well as improving their nutritional status by fixing nitrogen, solubilizing phosphates and producing siderophores for the uptake of essential nutrients in their development [18].

Bacillus cereus has been reported as an endophytic bacterium of plants of the genera Cyperus and Paspalum with the in-vitro capacity to tolerate up to 400 ppm (0.4 mg/L) of mercury in the form of $HgCl_2$ and also of rice plants with the capacity to tolerate up to 400 ppm of Pb in the form of $Pb(NO_3)_2$ and to produce siderophore [19,20]. In order to contribute to the search for new ecological alternatives to carry out phytoremediation processes, and to guarantee that rice crops can grow and adapt to cadmium-contaminated environments, the aim is to isolate endophytic bacteria from different rice plant tissues, evaluate in-vitro their capacity to tolerate different concentrations of cadmium and simultaneously produce siderophores [21].

MATERIALS AND METHODS

Study area. Commercial rice variety Fedearroz 2000 (F2000) was collected from rice farms belonging to the municipality of Nechi located in the Bajo Cauca sub-region of the department of Antioquia, Colombia.

Sampling. Sampling was carried out randomly in a zig-zag pattern collecting complete plants (root, stem, leaves and panicle) of the rice variety Fedearroz 2000 (F2000) and soil samples at a depth of 20 cm. For the collection and selection of the plants, those that showed good phytosanitary status and no symptoms of phytotoxicity were taken into account. The plant samples were stored in Icopor coolers for conservation and were identified with the respective variety, date of collection and municipality. Soil samples and part of the collected tissues were sent to the University of Cordoba for cadmium determination and the other samples were transported to the Microbiological Research Laboratory of the University of Sucre for processing within 24 hours after collection.

From isolates of endophytic bacteria from roots, molecular identification by sequencing was carried out as follows: For DNA extraction, pure bacterial samples were taken and activated on Luria Bertani (LB) agar (Bacto tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, Milli-Qaté water 1000 mL, pH 7, 0) and incubated at 28 oC for 24 hours, after which time pure colonies were again taken and transferred to tubes containing 10 ml LB broth and again incubated for 24 hours at 28 oC with constant agitation at 150 rpm, in an IKA 260 controller. 1

Basic. DNA was extracted according to the protocol proposed by[22]. Amplification of 16S rDNA fragments was carried out with the use of specific oligonucleotides for eubacterial groups [22]. The amplification products were sent for sequencing to Macrogen (Seoul, South Korea) in an automatic sequencer with a 3730XL capillary. The nucleotide sequence entities obtained were compared with those stored in the databases of the National Center for Biotechnology Information (NCBI). Base alignment was performed by cluster W; phylogenetic inferences were obtained by maximum similarity method based on the kimura-2-parameter model with bootstrap test (1,000 replicates) with the MEGA 7 program.

Determination of cadmium concentration in F2000.

Samples of different F2000 fabrics were washed with distilled water to remove adsorbed mineral particles from their surface. Each tissue was then placed separately in paper bags and dried in an oven at 60°C for 24 h. To determine the total cadmium in these samples, 0.5 g of dry material was taken and an acidic HNO3/H2O2 mixture (5+2 mL) was added. On the other hand, 0.5 g of the previously dried soil was taken and 10 mL of 65% HNO3 was added. The samples were processed in a Milestone ETHOS TOUCH 127697 series microwave oven and sent to a specialized laboratory for the determination of cadmium concentration by tissue.

Tolerance of Bacillus cereus strain BN5 to different cadmium concentrations.

The in-vitro tolerance test of B. cereus strain BN5 was carried out in tris-MMT minimal medium proposed by [23] with eight different treatments (concentrations) of cadmium in the form of $CdCl_2$. The initial concentration of cadmium used was 0.01 mg/ml and from these the different treatments were prepared: T1: 100 (0.1); T2: 150 (0.15); T3: 200 (0.2); T4:250 (0.250); T5: 300 (0.3); T6: 350 (0.35); T 400 (0.4); T7: 450 (0.45) and T8:500 (0.5 mg/mL). Aliquots of B. cereus in log phase were inoculated into 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 [24]. The growth of each bacterium was determined by turbidimetry at 600 nm every hour for four days.

Siderophore production. At the end of the experiment for each treatment, a sample was taken to determine the qualitative production of siderophores. The qualitative analysis was carried out in chromium azurol-S (CAS) medium used by [25]. For this purpose, 60.5 mg CAS was dissolved in 50 ml of distilled water and combined with 10 ml of an iron(III) solution (1 mM FeCl3.6 H2O and 10 mM HCl). Under stirring, this solution was mixed with 72.9 mg of HDTMA dissolved in 40 ml of water. The resulting blue liquid was sterilized 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 sterilized to reach a pH of 6.8. To the medium, 4 g of glucose was added as a carbon source. The bacteria were incubated for 7 days at 30°C. The ability of the bacteria to produce siderophores is evidenced by the formation of a halo.

Data analysis.

The information obtained in the experiment was organized in figures for further interpretation of the results. Analysis of variance and Tukey's multiple range test was used to establish significant differences between the variables analyzed. The trials were conducted in triplicate, with results expressed as means. The data were processed in the statistical program InfoStar.

RESULTS AND DISCUSSION

Samples of the commercial rice variety Fedearroz 2000 (F2000) were collected from rice farms located in the municipality of Nechi-Antioquía, Colombia. The soils of this municipality showed average cadmium values of 5.5 mg/kg, which according to international standards are considered to be of toxic category. With respect to the values of cadmium present per tissue, the average values of this heavy metal per tissue were found as follows: root (2.3); stem (1.92); leaves (1.27) and panicle (0.97 mg/kg of tissue). From these samples, the isolation and determination of the population density of endophytic bacteria per tissue was carried out. From the isolates of endophytic bacteria from root samples where the highest cadmium values were found (2.3 mg/kg), the identification of these endophytes was carried out by sequencing.

Sequencing results showed that the isolate AC22 from the root of the commercial variety F2000 had 97% similarity with sequences stored in Gen Bank with the bacterium Bacillus cereus strain BN5 (Figure 1).

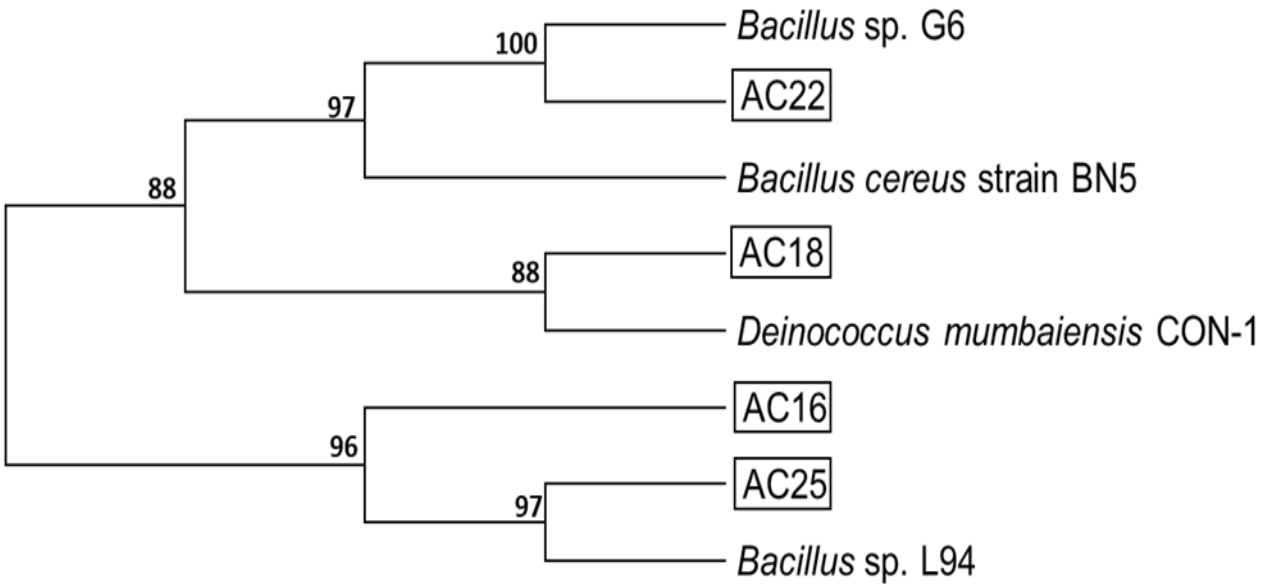

Figure 1. Phylogenetic tree of the Bacillus cereus strain BN5 isolates the variety fice F2000 and their relationships with species of bacteria of the phylum Firmicutes.

When the ANOVA criteria were met, the analysis of variance was performed, which indicated significant statistical differences (p-value<0.05) between the amount of endophytic bacteria per tissue and cadmium concentration. The results of the Tukey test show significant statistical differences (p-value>0.05) between population density and cadmium, with a higher presence in panicle (1.12 x 108 CFU/g tissue) when there are lower values of Cd (0.97 mg/kg) and lower in roots (5.8 x 105 CFU/g tissue) when there are higher values of Cd (2.3 mg/kg). The results obtained indicate that there is an inverse relationship between the concentration of Cd in plant tissues and the density of endophytic bacteria, showing that in tissues such as roots where there are higher values of Cd (2.3 mg/kg), there is a lower quantity of bacteria, while in tissues with a lower concentration of Cd (0.97 mg/kg) there is a high presence of these bacteria (Figure 2).

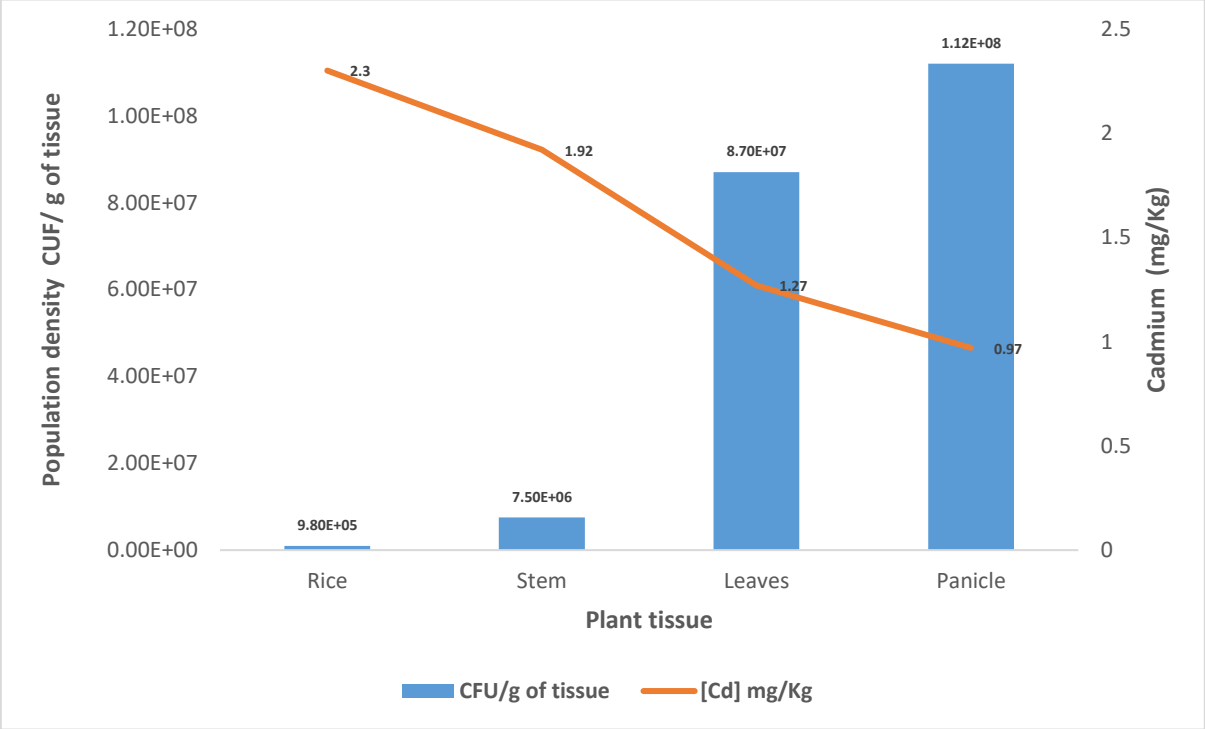

Figure 2. Population density and concentration of cadmium for plant tissue

The average values of cadmium in tissues of the rice variety F2000 found in the present study, root (2.3 mg/kg) and panicle (0.97 mg/kg), classify according to international reference values for plant tissues as excessive for agricultural crops. Rice plants can absorb Cd2+ ions from the soil, studies indicate that there are different processes for uptake from the root, from the root to the stem through the xylem, redirection through the xylem vessels and remobilization from the leaf to the grains [26]. Therefore, perspectives to decrease the cadmium content in rice and other cereal crops is the worldwide concern to avoid contamination in humans and improve human health [27]. Microbial-assisted phytoremediation has been of increasing interest in the

last 10 years, due to its cost-effectiveness and environmental friendliness [28,29]. Microbial remediation of heavy metals involves bio-accumulation, bio-absorption, bio-mineralization and bio-transformation [30].

Figure 3 shows the tolerance curve of Bacillus cereus strain BN5 in different concentrations of $CdCl_2$. Up to 4 hours after the start of the test, the bacterium showed a tolerance percentage (growth) lower than 40% in all the treatments evaluated. After 5 hours, the tolerance behaviour of the bacteria increased until 14 hours, with a tolerance percentage of 76.92% at 100 mg/mL $CdCl_2$ and 69.87% at 150 mg/mL $CdCl_2$. After this time, B. cereus strain BN5 entered a decreasing stage reaching tolerance percentages of 25.85 and 19.41% by 18 hours for 100 and 150 100 mg/mL $CdCl_2$, respectively. In the presence of 200 mg/mL $CdCl_2$, the bacteria tolerated 59.55% up to 12 hours. The lowest percentage of tolerance for the bacteria was observed with 500 mg/mL $CdCl_2$, with 5.71% tolerance up to 10 hours and decreased to 1.42% at 18 hours, compared to the control (Figure 3).

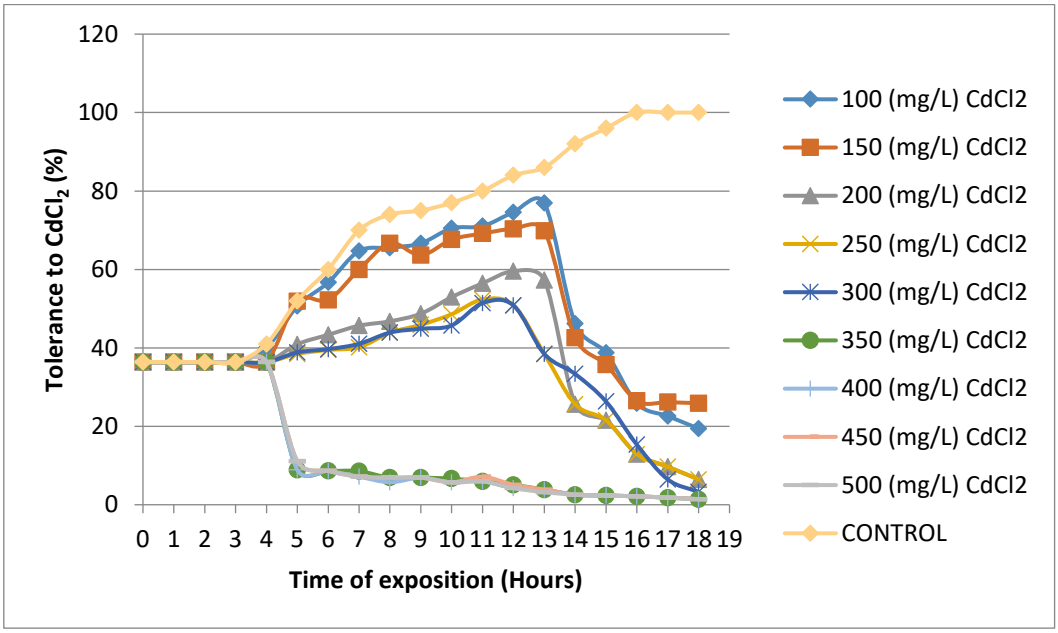

Figure 3. Tolerance curve of Bacillus cereus strain BN5 in different concentrations of $CdCl_2$

With the results of tolerance to $CdCl_2$, a completely randomized design was carried out. The normality criterion was previously established using the Shapiro-Wilks test. For significant statistical differences, Duncan's multiple range test was performed (p-value≤0.05). ANOVA indicates significant statistical differences (p-value>0.05) between $CdCl_2$ concentrations (figure 4) and exposure times (figure 5). The highest tolerance averages were found at concentrations of 100 and 150 mg/L $CdCl_2$, with no statistical differences between these two concentrations. In the case of the exposure times, the bacteria showed a stage of adaptation to the pollutant, with no growth from 0 to 3 days. After this stage, the bacteria showed growth

until day 12 with a tolerance to the pollutant of 46.3% with respect to the control, after which time the growth of the bacteria in the medium decreased.

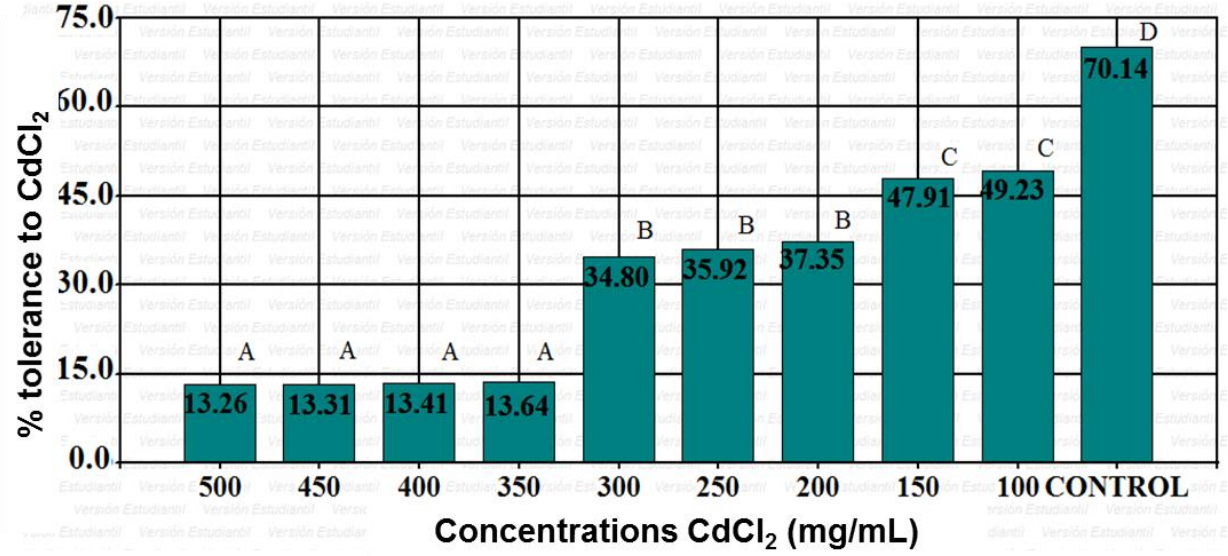

Figure 4. Tolerance(%) of Bacillus cereus strain BN5 in different concentrations of $CdCl_2$.

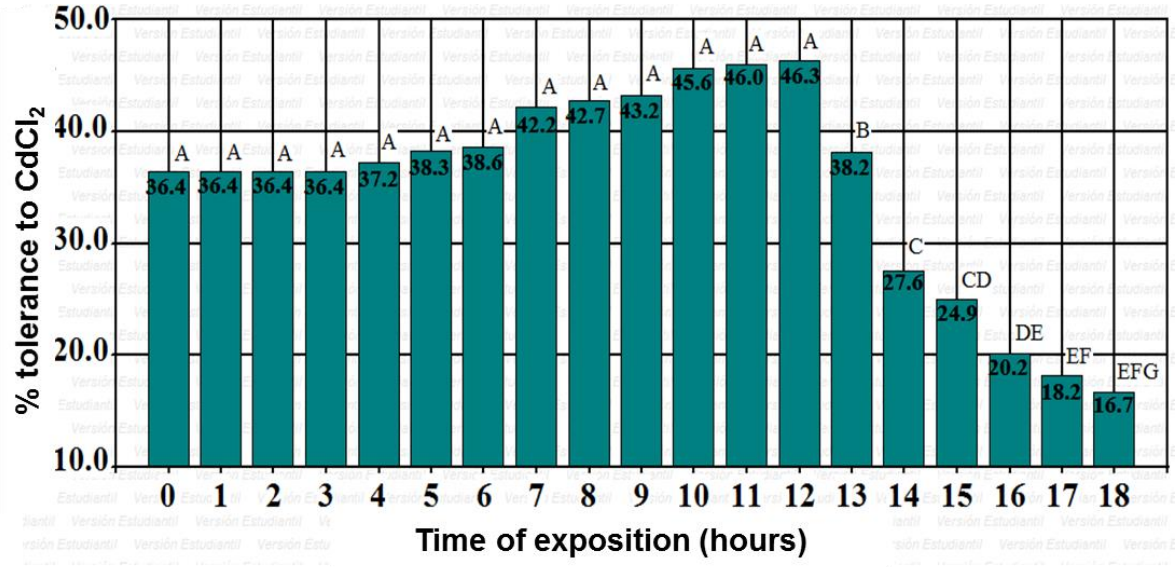

Figure 5. Tolerance (%) according to exhibition time of Bacillus cereus strain BN5 in different concentrations of $CdCl_2$.

The results found in the present study show that as the concentration of cadmium in the medium increases, the percentage of tolerance of Bacillus cereus strain BN5 decreases, presenting a

slight growth, up to 4 hours of the experiment, showing a longer adaptation time of the bacteria when in contact with the metal and developing physiological mechanisms to survive the different concentrations of cadmium evaluated.

According to our opinion the adaptation and growth behaviour of both bacteria and accompanying this hypothesis by the conclusions of other studies. Cd^{2+} causes toxicity in the bacterial cell in different ways: by interacting with nucleic acids, by binding to proteins essential for respiration producing reactive oxygen formers that produce oxidative damage, and by displacement of Zn^{2+} by Cd^{2+} on proteins. The Cd2+ ion enters the bacterial cell through the transport system used by essential divalent cations [31]. To avoid toxicity with Cd^{2+} it must be rapidly and efficiently removed from the bacterial cell or converted into a biologically inactive form. In general, there are two possible basic mechanisms of Cd^{2+} resistance, intracellular or extracellular complexation of toxic metal ions and reduced accumulation based on active cation flux. The latter is the main mechanism developed in prokaryotes. However, enzymatic. However, enzymatic transformations of metal ions (oxidation, reduction, methylation and demethylation) are also defense mechanisms in bacteria [32,33]. Bacteria achieve resistance to Cd by biosorption, bioaccumulation, precipitation, complexation and metal outflow.

The siderophore production capacity of Bacillus cereus BN5, as a function of Cd concentration, was evaluated in chromium azurol-S (CAS) medium of the bacterium, showing that at concentrations of 100, 150, 200, 300, 400 and 500 mg/L of $CdCl_2$, it produced siderophores (Figure 6). The production of siderophores by bacteria can possibly help 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 [34]. In addition to iron chelation, siderophores serve as a mechanism for bioremediation, where the role of siderophores in reducing cadmium and lead toxicity has been observed. These studies by [35] suggest a possible application of microbial siderophores in bioremediation for the reduction of metal toxicity and the oxidative stress they induce.

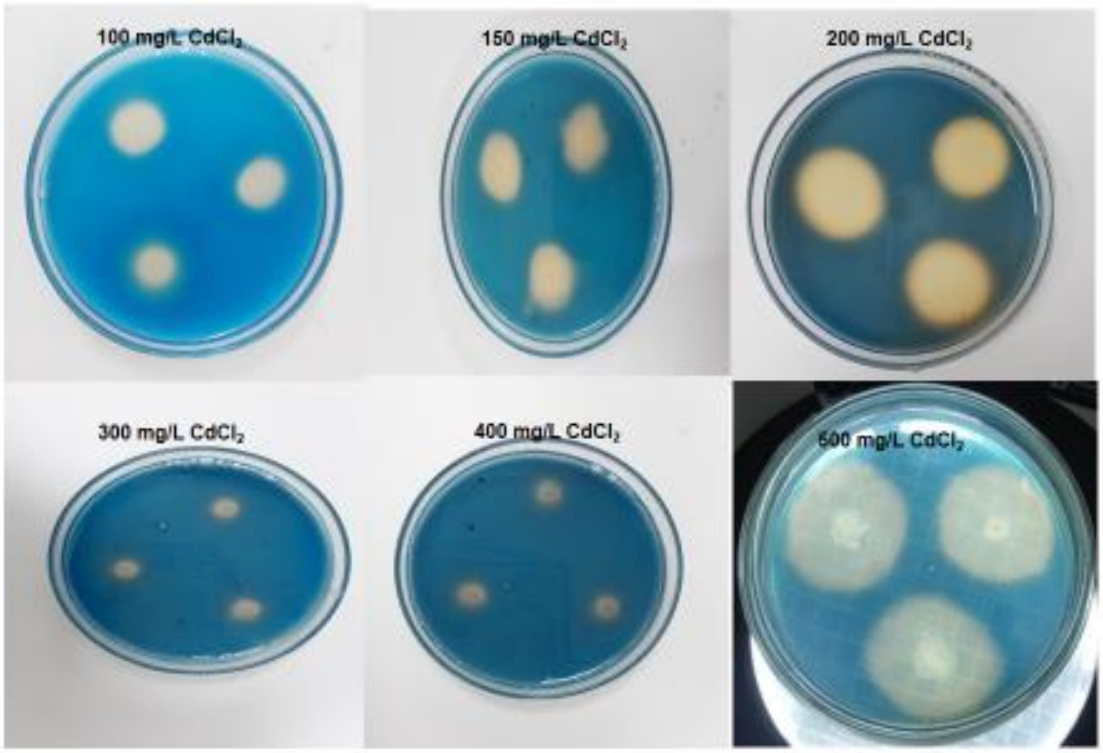

Figure 6. Qualitative siderophores production by Bacillus cereus strain BN5, in different concentrations of $CdCl_2$.

Studies carried out by [36] concluded that the quantity and quality of siderophore production by the plant growth-promoting bacterial species Pseudomonas fulva was due to increased exposure to Cd2+ (0, 0.5, 1.0, 2.0 mM). The study aimed to determine the changes in siderophore production. The results obtained show that in the presence of 2.0 mM Cd^{2+}, the synthesis of siderophores from the hydroxymate, catecholate and phenolate groups occurs with respect to lower concentrations of Cd^{2+} (0.5 and 1.0). From our point of view, possibly the mechanism of siderophore production around the environment where the bacteria grow allows them to sequester heavy metals and decrease their accumulation inside the cell. Consequently, the bacteria reduce the transport of heavy metals in high concentrations into the cytoplasm and prevent cell intoxication.

B. cereus is an endophytic bacterium found in rice plants with the ability to promote plant growth [37]. Likewise, a study carried out by[19] on the isolation of endophytic bacteria associated with the genera Cyperus and Paspalum in mercury-contaminated soils in southern Bolivar, Colombia, reported the presence of the endophytic bacterium Bacillus cereus GU056811 with the in-vitro capacity to tolerate up to 400 ppm (0.4 mg/L) of mercury in the form of $HgCl_2$. Similarly, a study carried out by [20] concluded that the endophytic bacterium

Bacillus cereus 1DH1LIM has the ability to tolerate up to 400 ppm Pb in the form of $Pb(NO_3)^2$ and to produce siderophore.

CONCLUSION

Bacillus cereus strain BN5, a Gram-positive, cadmium-tolerant endophytic bacterium was isolated from the root of a rice variety grown in soils with high cadmium content. In-vitro assays indicate that it is able to tolerate up to 500 mg/L CdCl2, and produces siderophores at different concentrations of this metal. This bacterium coexists in root tissue with cadmium concentrations of 2.3 mg/kg. The results obtained in this study emphasize the possibility of exploring the possibility of obtaining cadmium-tolerant endophytic bacteria for remediation and safe rice production in cadmium-contaminated soils.

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