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Endophytic Bacteria A Biological Alternative For Phytoremediation Of Nickel-Contaminated Soil

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ABSTRACT

The objective of this study was to evaluate in vitro the tolerance of endophytic bacteria to different concentrations of nickel. Sampling was carried out in rice soils cultivated in Cordoba, Colombia, during which tissue samples were collected from commercial rice varieties at the grain filling stage. Each tissue was subjected to a surface disinfection process. Endophytic bacteria were isolated on R_2A agar medium; their tolerance to different concentrations of nickel and their ability to produce siderophores were evaluated in vitro. A total of 25 strains of endophytic bacteria were isolated from different tissues, with the highest number of bacteria found in roots and tillers of rice plants. Aeromonas hydrophila, Bacillus cereus and Serratia macescens were isolated as endophytic bacteria from commercial rice plants in the department of Cordoba with the ability to resist different nickel concentrations and the capacity to produce siderophores.

Keywords. *Rice cultivation, bacteria, tolerance, nickel.*

INTRODUCTION

Heavy metals Mercury-Hg, Cadmium-Cd, lead-Pb, Nickel-Ni among others, are a growing problem of environmental pollution worldwide, because unlike organic compounds, they cannot be biodegraded, which is why concentrations in environmental compartments are continuously increasing (Islam, et al., 2007). Heavy metals are attributed with certain environmental pollution and toxicity effects, even causing death or the development of lethal diseases such as cancer.

Nickel (Ni), as a metal in small amounts, is a necessary element for the human body, plants and animals; however, in excessive amounts, it can become highly toxic. Short-term overexposure

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to Ni does not cause health problems, but long-term exposure can cause loss of body weight, anaemia, heart and liver damage and skin irritation (Pérez, 2011). There are six Ni deposits in Colombia, three of which are located in the Caribbean region, in the department of Córdoba: Cerro Matoso, Planeta Rica and Uré; the remaining three are in the department of Antioquia: Ituango, Morro Pelón and Medellín (UPME, 2009).

The use of tolerant endophytic bacteria associated with plant species adapted to environments contaminated with heavy metals has become very important because of their ability to take up these metals and use them as a carbon source (Sheng, et al., 2008; Sun, et al., 2010). Although heavy metals such as Ni and Pb in high concentrations have been reported to be toxic to plants and their associated microorganisms, metal-resistant endophytic bacteria have been shown to promote plant growth (Barzanti, et al., 2007; Idris, et al., 2004; Lodewyckx, et al., 2001).

Nickel is released into the environment from a variety of natural and anthropogenic sources (Pérez-Rodríguez, et al., 2011). Within industrial sources, a considerable amount derives from the combustion of coal, oil, and other fossil fuels. Nickel alloy (steel) manufacturing, electroplating processes, waste incineration and wastewater also contribute (Ahmad & Ashraf, 2011). Generally, the largest portion of nickel and its compounds released into the environment is adsorbed on sediments or soil particles and thus immobilized, and can be taken up by plants grown there and through foodstuffs reach humans. However, in acidic soils their mobility increases due to higher solubility and they can leach into groundwater.

The accumulation of nickel in plant tissues by absorption or other forms of natural association, gives the possibility to be bioavailable to humans and animals through the consumption of these products (Brun, et al., 2001; Gincchio, et al., 2002; Friesl, et al., 2006).

This supports the hypothesis that endophytic bacteria have acquired the ability to be resistant to high concentrations of heavy metals and that they can confer greater plant tolerance to stress caused by these pollutants. In addition, endophytic bacteria are able to enhance plant growth by several mechanisms including: siderophore production, 1-aminocyclopropane-1-carboxylic deaminase (ACC), indole-3-acetic acid (IAA) and phosphate (P) solubilization (Rajkumar, et al; 2009). It has been reported that certain endophytic bacteria can alter the toxicity and availability of heavy metals in plants by producing siderophores, biosurfactants and organic acids (Sheng, et al., 2008; Saravanan et al., 2007).

At the date of this study, there is no reference in the database of specialized literature on the tolerance of endophytic bacteria to different concentrations of Ni, taking into account the above, the objective was to isolate endophytic bacteria from commercial varieties of rice cultivated in the department of Córdoba, and to evaluate in vitro the tolerance capacity of these bacteria to different concentrations of nickel in the form of $NiCl_2.6H_2O$, as well as to evaluate in vitro the capacity to produce siderophores as an indirect measure to help plants to remediate this metal in contaminated environments.

MATERIALES AND METHODS

1. **Study area.** The present study was carried out in cultivable soil with commercial varieties of rice cultivated in the department of Cordoba - Colombia, located the first one in the southeastern end of the department of Cordoba, on the right margin of the river San Jorge, with a surface of 1.897 km; the Municipal head is to an altitude of 55 msnm, 75º 59" 53" of north latitude and 75º 26" 25" of west longitude, average temperature 32 °C, the relative humidity is of 78 % in time of drought and of 81 % in periods of rain. The sampling site is located on the shores of the large swamp at 9º

11'18" north latitude and 75º 33'23" west of the Greenwich meridian, average temperature 28º C.

2. **Sampling.** Sampling was carried out randomly in a zig-zag fashion, collecting at each site ten complete plants (including roots, tillers, leaves and panicle) of commercial rice varieties, established on rice farms at the time of sampling in each municipality. The samples were identified with the respective variety, date of collection, farm and municipality. These were stored and preserved for transport to the Microbiological Research Laboratory of the University of Sucre and processed within 24 hours after collection, according to Pérez et al. (2013).
3. **Isolation of endophytic bacteria from rice plants.** Plants collected from each rice variety were subjected to a surface disinfection process. Roots, tillers, leaves and panicle of each plant were washed with sterile water and cut into approximately 1 cm segments. The surface disinfection process for each tissue was carried out according to the methodology recommended by Perez, et al. After the disinfection process, each tissue was placed in a porcelain dish and macerated with liquid nitrogen until a homogeneous sample was formed. Serial dilutions of each sample were prepared from which aliquots were taken and deposited on R2A agar surface and incubated at 32 °C for 72 hours. The population density of bacteria per tissue (CFU/g tissue) was determined by direct colony counting on the surface of the agar plates. During counting, colonies that differed in shape, texture, color and size were observed and selected (Pérez et al, 2014).
4. **In vitro evaluation of nickel resistance.** Aliquots of log-phase suspensions of endophytic bacteria were inoculated onto the surface of R2A agar supplemented with 50 mg/L Ni in the form of NiCl2.6H2O (Rajkumar et al., 2013). The plates were incubated at 32°C for 72 h. The Ni tolerant colonies were collected and purified on R2A agar containing 50 mg/L Ni and incubated in the same condition as above, after this time the colonies were picked, inoculated in R2A broth on 150 rpm shaking at 32 °C for 24 h. Ten mL of each sample was taken and inoculated in flask with 100 36 mL of R2A broth supplemented with different concentrations of Ni ranging from 50 to 900 mg /L (Rajkumar et al., 2013). The samples were incubated at 150 rpm shaking at 32 °C for 8 days. Two controls were used one of R2A broth inoculated with bacterial suspension without Ni and the other nutrient broth with different concentrations of Ni without bacterial inoculum. The growth of endophytic bacteria in the experiment was determined by the turbidimetry technique, quantified at 600 nm, every hour for seven days, the readings were performed in a spectroquantpharo 300 spectrophotometer (Ma et al., 2011).
5. **Production of siderophores.** The qualitative production of siderophores was carried out on the chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987), which consisted of dissolving 60.5 mg of CAS in 50 ml of distilled water, to this mixture was added 10 ml of iron (III) solution (1 mM FeCl3.6H2O and 10 mM HCl), under stirring. The solution was mixed with 72,9 mg HDTMA dissolved in 40 ml water. The resulting blue liquid was autoclaved at 121°C for 15 minutes. 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 adjusted to pH 6.8 was also autoclaved in another vessel. To the medium 4 g of glucose was added as a carbon source. Isolates were seeded using wooden sticks and incubated for 7 days at 30°C. The ability of the bacteria to produce siderophores was evidenced by the formation of a transparent halo around the colonies.
6. **Identification of Ni-tolerant bacteria.** Endophytic bacterial strains with Ni-tolerant activity and production of growth-promoting siderophores were initially subjected to identification by Gram staining. Genomic DNA extraction, amplification with bacterial

domain-specific oligonucleotides was carried out according to the protocol suggested by Oliviera et al, 2013.

PCR products were quantified and sent for sequencing at Macrogen (Seoul, South Korea) in an automatic sequencer with 3730XL capillary. For the study of evolutionary relationships between the 16 rDNA sequences obtained, the sequences were compared with the nucleotide sequences stored in the GenBank database using the BLAST alogarithm. All sequences with high identity obtained in this study were imported into MEGA 6® software and aligned with other rRNA gene fragment sequences using clusteal W. The alignments were manually cut and the calculation of phylogenetic trees were constructed based on the aligned sequences using the Maximum Parsimony Neighborjoining method. To check the robustness of the obtained trees and the levels of statistical significance within nodes was performed by bootstrap analysis with 1,000 replicates and values higher than 50% were reported.

RESULTADS AND DISCUSION

Figure 1 shows the amount of endophytic bacteria isolated per tissue in commercial rice varieties. The average number of endophytic bacteria per root was: 4.9 x 10^9 CFU/g root, 3.7 x 10^7 CFU/g tillers, 3.7 x 10^6 CFU/g leaves and 6.8 x 10^4 CFU/g panicle.

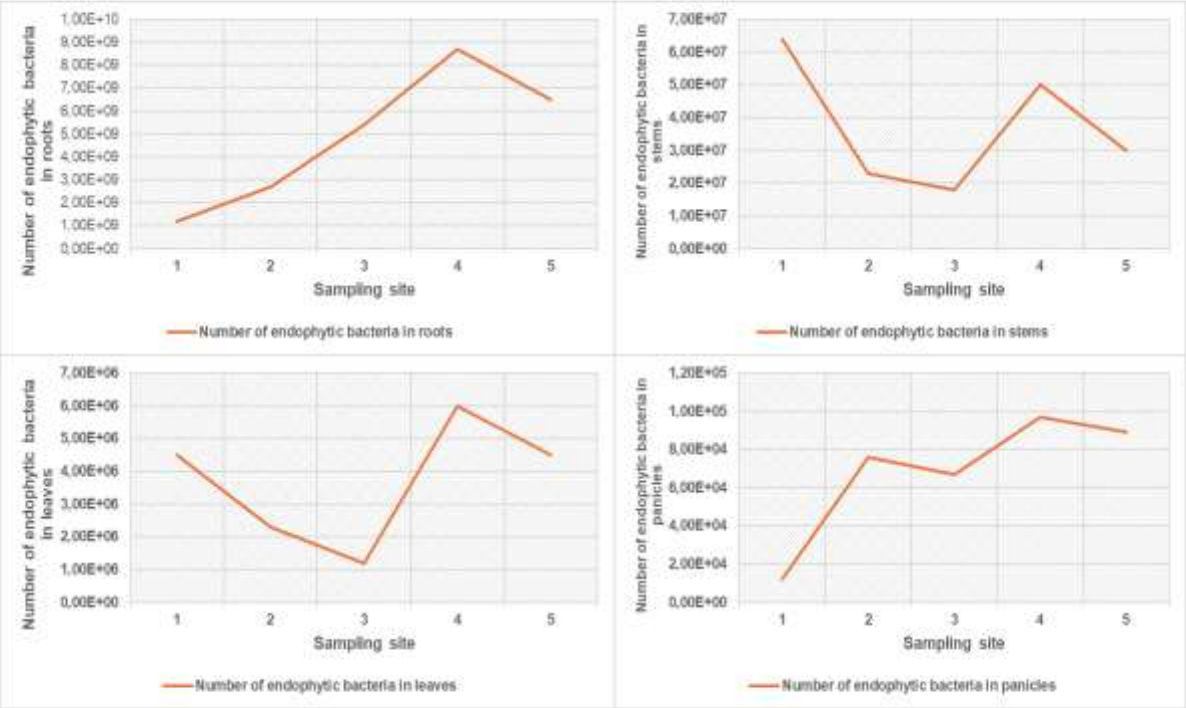

Figure 1. Amount of bacteria in CFU/g tissue associated with different rice variety tissues in the presence of nickel in the soil.

Comparative studies carried out by Perez et al, 2013, on the quantity of endophyte bacteria associated with the same rice varieties, carried out in the experimental farm La Victoria, located in the village of Mocarí, belonging to the municipality of Montería-Córdoba, in 2012, with the aim of evaluating the population density of endophyte bacteria revealed higher densities of endophyte bacteria in roots (3.2 x 10^{10} CFU/g tissue), with respect to stem, leaves, flag leaf and panicle; F733 (1.77 x 10^{10} CFU/g tissue) and Fmocarí (1.7 x 10^{10} CFU/g tissue) were identified as having the highest population density, compared to F473 and F2000, with densities of 2.0 x 10^7 and 1.56 x 10^7 CFU/g tissue, respectively.

However, the amounts found in the present study are below those found by Perez et al., 2013 under normal conditions of no heavy metal contamination, contrary to the present study where nickel levels are reported in rice soils. The amount of endophytic bacteria was variable and was affected by several factors such as: time of the year, type of tissue, species, variety of plants (Mocali et al., 2003) and the interaction of endophytic bacteria with other beneficial microorganisms (Araujo et al., 2002).

Figure 2 shows the averages of nickel concentration found per sampling site. The results indicate that the highest values found in rice soils were reported for site 5 with values of 3.4 and the lowest with 2.78 g/ha for site 1.

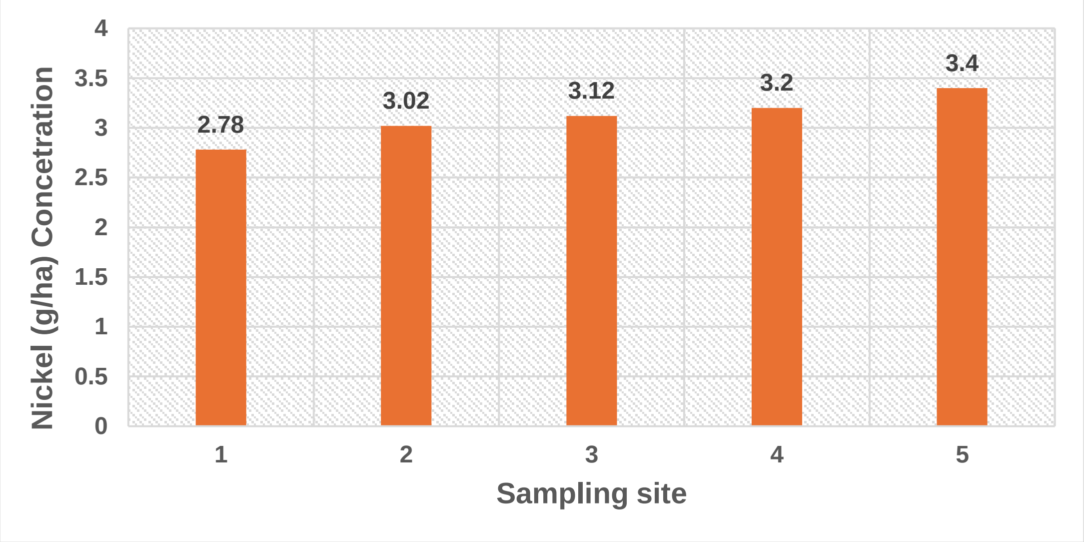

Figure 2. Nickel concentration in g/kg soil present in rice farms in the department of Córdoba, Colombia.

Figure 3 shows the concentration of nickel found per tissue in commercial rice varieties. The figure shows that the concentration of nickel found in the roots of the rice variety was 3.2, while in the panicle it was 2.6 g/kg of tissue.

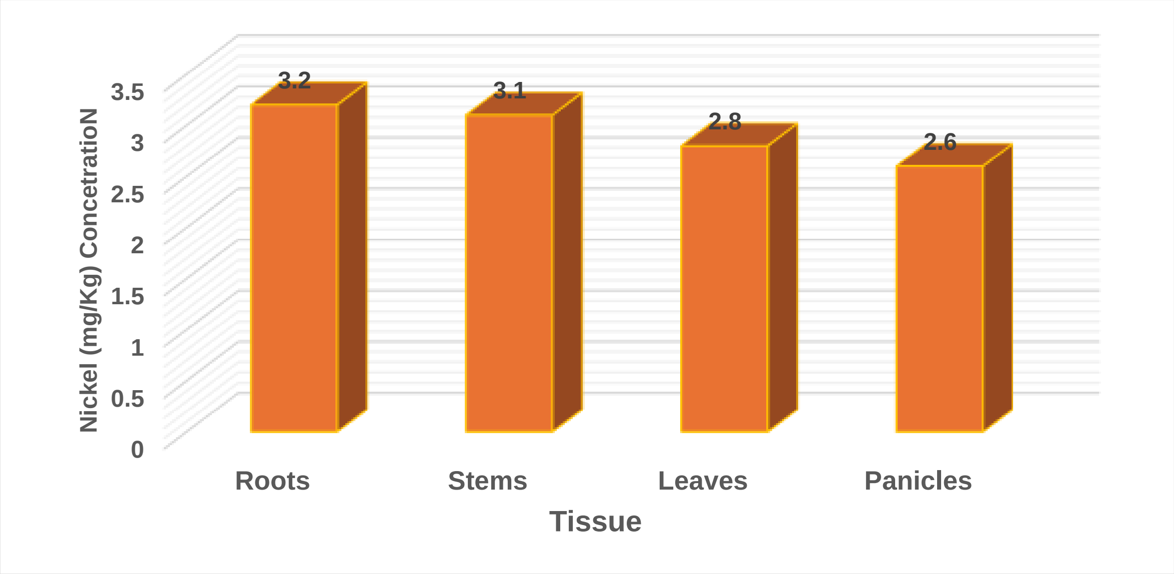

Figure 3. Nickel concentration (mg/kg) in different tissues of rice plants in the department of Córdoba, Colombia.

The phylogenetic analysis of the bacteria shows the presence of 7 species grouped in two subgroups (Gamma-proteobacteria and Firmicutes). Figure 4 shows the strains identified that showed the highest nickel tolerance (mg/L). Three species of endophytic bacteria were identified that showed homology with bacterial sequences present in the GenBack database. The results obtained relate Serratia marcescens isolated from roots of commercial rice varieties with the ability to tolerate 500 mg/L nickel. Aereomonas hydrophila tolerates 900 mg/L nickel while Bacillus cereus showed the highest tolerance up to 1200 mg/L nickel.

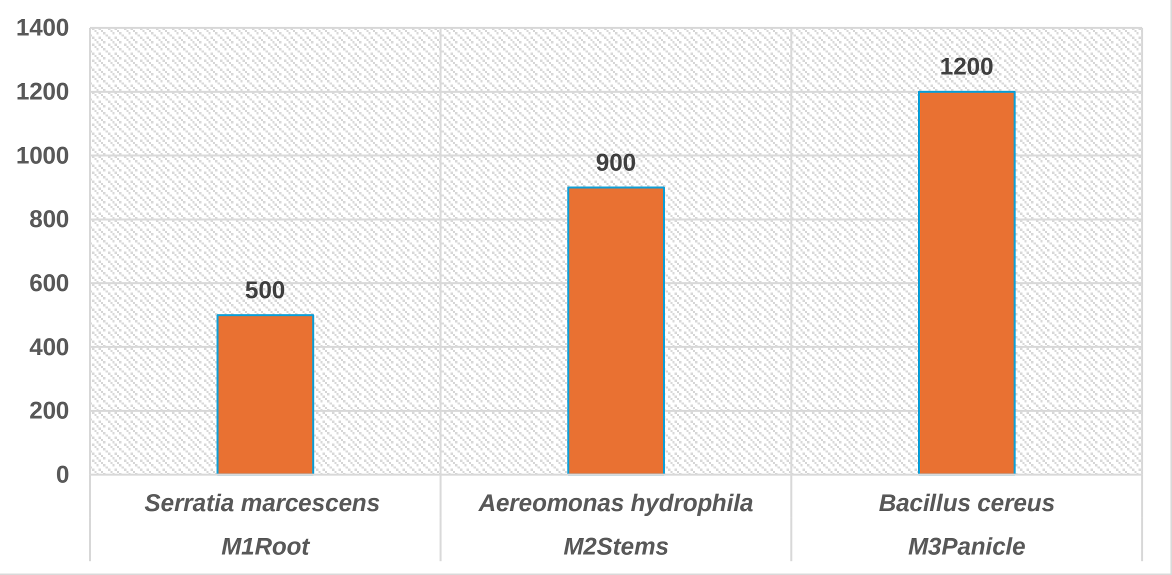

Figure 4. Identified strains of bacteria isolated from rice varieties with tolerance to different nickel concentrations.

Figure 5 shows the results of the in vitro siderophore production test using the azurol-S chromium medium (CAS) technique proposed by Schwyn and Neilands (1987).

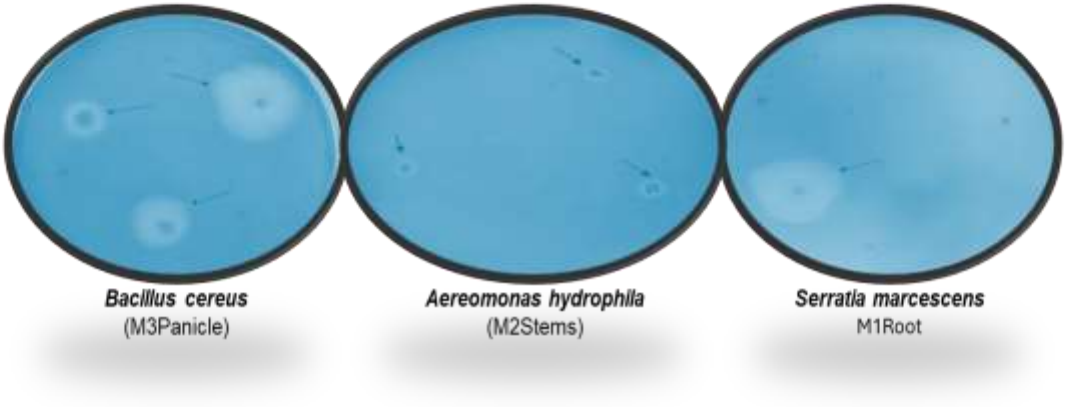

Figure 5. In vitro production of siderophores by rice endophytic bacteria at different nickel concentrations.

The present study isolated Bacillus cereus from rice crop panicles with the ability to tolerate 1200 mg/L nickel. Several results indicate that Bacillus cereus isolated from contaminated environments have shown tolerance to heavy metals as well as bioremediation capabilities (Zahoor & Rehman 2009, He et al., 2010, Guo et al., 2010, Valverde et al. 2011). Ahemad (2014) reports that several plant growth-promoting species of the genus Bacillus are being applied to detoxify heavy metal-contaminated environments; Gupta and Diwan (2017). Other studies also point to Bacillus cereus as bioremediators of heavy metals such as lead (II), copper (II), nickel (II) or zinc (II), silver (I), chromium (VI) or cadmium (II). Muñoz-Silva et al., (2019).

Aereomonas hydrophila was isolated from tillers of rice plants with the ability to tolerate 800 mg/l nickel. At laboratory level, Aereomonas hydrophila was reported as a phosphate solubilize. Lara et al., (2011) and It has a high efficiency in the removal of lead from synthetic wastewater. Ramírez et al., (2016)

Species of Serratia spp. have been isolated from cotton (Gossypium hirsutum) and sweet corn (Zea mays) plants (McInroy & Kloepper, 1995), as well as from stem rhizosphere (Rosales et al., 1993) and rice seeds (Mukhopadhyay, et al., 1996). In fact, non-pathogenic isolates of S. marcescens have been used as biocontrol agents in agricultural crops due to their chitinase activity (Kalbe et al., 1996; McInroy, et al., 1995; Rosales, et al, 1993) and their ability to induce systemic M11R F473 N2-binding M11 R F473 siderophore M5 T F473 PO4 solubilization M11 R F473 PO4 solubilization 53 resistance in plants (Press et al., 1997).

The studies carried out with endophytic bacteria associated with rice cultivation worldwide have focused on the presence of diazotrophic bacteria (Verma et al., 2001), rhizobial (Sturz & Nowa, 2000); anoxic phototrophic bacteria (Paolino & Scavino, 2004); endophytic bacteria and populations of fungi and actinomycetes, with role in growth promotion, potential nitrogen fixation and disease resistance (Azevedo, et al., 2000) and there are no studies that relate the presence of endophytic bacteria associated in rice soils with the presence of nickel and the tolerance capacity of these bacteria to different concentrations of Ni and the capacity to produce siderophores qualitatively in vitro, which undoubtedly makes this work the first literature report on this subject.

Among the microorganisms involved in heavy metal phytoremediation, rhizosphere bacteria deserve special attention because they can facilitate the phytoremediation process by changing the bioavailability of metals through alteration of soil pH, release of chelating agents such as e.g. organic acids, siderophores, among others, oxidation/reduction reactions (Ma et al., 2011). In addition, the rhizosphere provides a complex and dynamic microenvironment in which microorganisms, in association with roots, form unique communities that have considerable potential for the promotion of plant growth (Belimov, 2005) and the detoxification of hazardous waste compounds (Black, et al., 1993; De-Souza et al., 1999).

CONCLUSION

The results of the 16s rDNA gene sequencing identified with similarity sequences of the species: Aeromonas hydrophila, Bacillus cereus and Serratia macescens as endophytic bacteria isolated from commercial rice plants in the department of Córdoba with the ability to resist different concentrations of nickel and the capacity to produce siderophores.

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DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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