Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i14.6100

METHODOLOGY FOR ISOLATION OF ENDOPHYTIC BACTERIA AND EVALUATION OF HEAVY METAL REMEDIATION EFFICIENCY

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Submitted: 22 February 2023; Accepted: 30 April 2023; Published: 06 June 2023

ABSTRACT
The aim of this study was to show the different protocols for the isolation, evaluation of the resistance capacity and identification of endophytic bacteria associated with different plant tissues adapted to environments contaminated with heavy metals. The protocols used have allowed the isolation and evaluation of a diversity of endophytic bacteria able to tolerate high concentrations of mercury and nickel.

Key words: Endophytic bacteria, heavy metal, tolerance,

INTRODUCTION
To mitigate the effects of metal contamination, techniques such as phytoremediation are being used, which is an effective, economical and environmentally friendly technology that is receiving much attention worldwide. 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).
Endophytic bacteria living in the internal tissues of plants enhance the efficiency of the phytoremediation process and increase plant biomass production through three mechanisms: increased root surface area and root hair production; increased metal availability; and increased transfer of soluble metals from the rhizosphere to the plant (Weyens et al. 2009; Ma et al. 2011).
Furthermore, the restoration of these ecosystems is rare but urgent because it would reduce the associated risks and replenish soils for agriculture (Wuana & Okieimen, 2011). On the other hand, these contaminated environments possess a biota of interest (Colpaert et al. 2011) because organisms resistant to these metals can be used to clean contaminated environments through biotechnological applications (Colin et al. 2012) thanks to the fact that they possess survival and detoxification mechanisms (Muñoz et al. 2012) that allow them to tolerate and accumulate heavy metals (Colin et al. 2012); thus, tolerant microorganisms isolated from environments contaminated with heavy metals

are a good alternative to clean and remediate these ecosystems (Vargas-García et al. 2012, Krishna et al. 2013). Likewise, as a first step for the design of bioremediation processes, it is suggested to determine the existing microorganisms in the affected areas (Guo et al., 2010), because native microorganisms, in addition to tolerating metals, are also adapted to the environmental conditions of temperature, humidity, pH, etc. of the area of interest.
In this sense, strains of endophytic bacteria associated with plant tissues growing in soils contaminated with heavy metals may be resisting heavy metals in situ. In order to understand this reality, it was necessary to carry out the present study, with the aim of isolating and identifying endophytic bacteria with the capacity to tolerate heavy metals in vitro and evaluate their possible use in remediation programmers.

MATERIALS AND METHODS

Pre-diagnosis. Bibliographic information on sites contaminated with the presence of heavy metals should be collected. Sites and geo-referencing should be available for a pre-visit.

Sampling site. The name of the Corregimiento, the jurisdiction of the municipality, location in the respective department and country must be recorded. In each site to be sampled, the geographic coordinates shall be noted: Xº X'X" north latitude and XºX'X" east longitude.

Collection and identification of plant material. The collection of plant material at the respective sampling sites shall be carried out as described in figure 1 below.

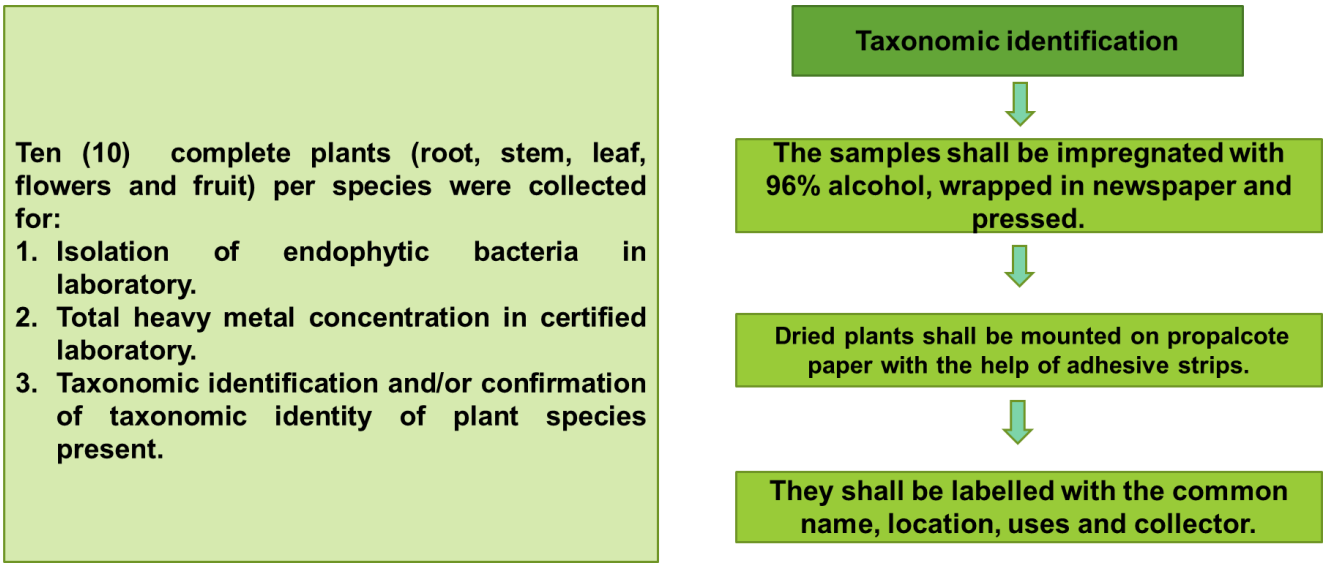


Figure 1. Outline of procedure for the collection of plant material from environmental sampling sites contaminated with heavy metals. Source: Pérez et al., 2016.

Sampling. At each site, 20 soil samples should be taken for physical-chemical, heavy metal concentration and microbiological analysis. The procedure shall follow the process as shown in figure 2.

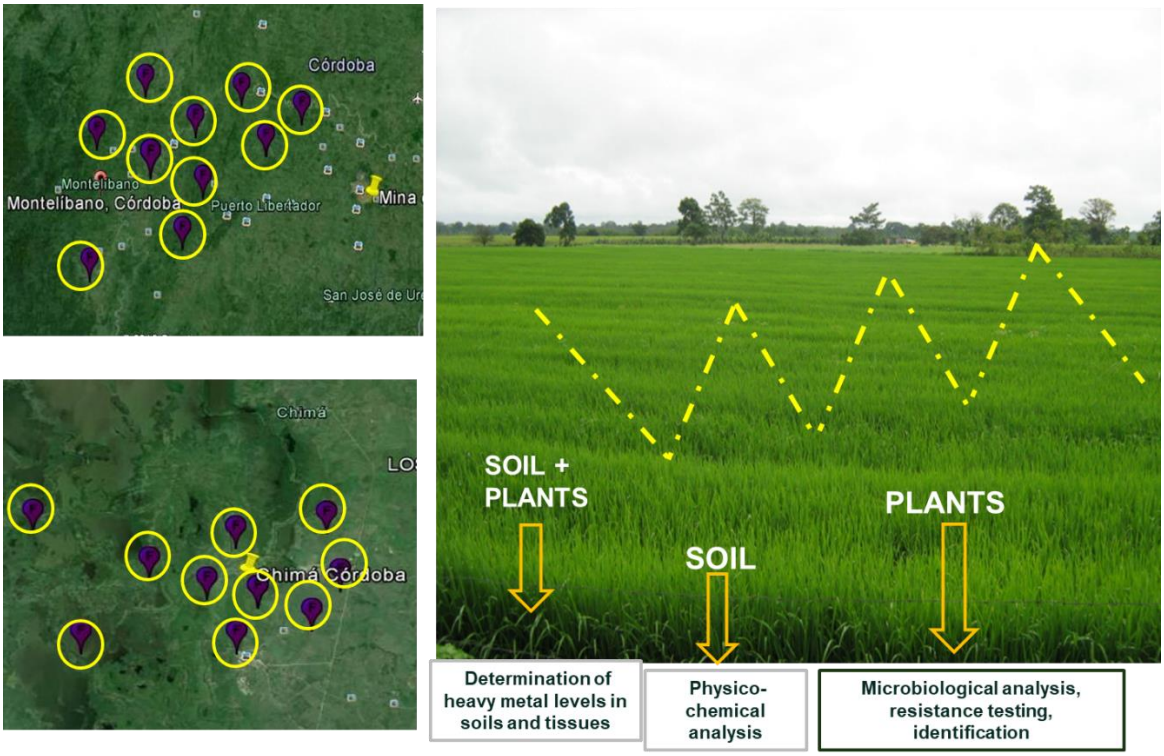


Figure 2. Scheme of procedure for collection of soil and plant, soil and plant tissues for soil physico-chemical analysis, heavy metal concentration and isolation of endophytic bacteria. Source: Perez et al. 2015.

In vitro heavy metal tolerance test. To assess the tolerance capacity of endophytic bacteria to heavy metals, the following protocol shall be followed as shown in figure 3.

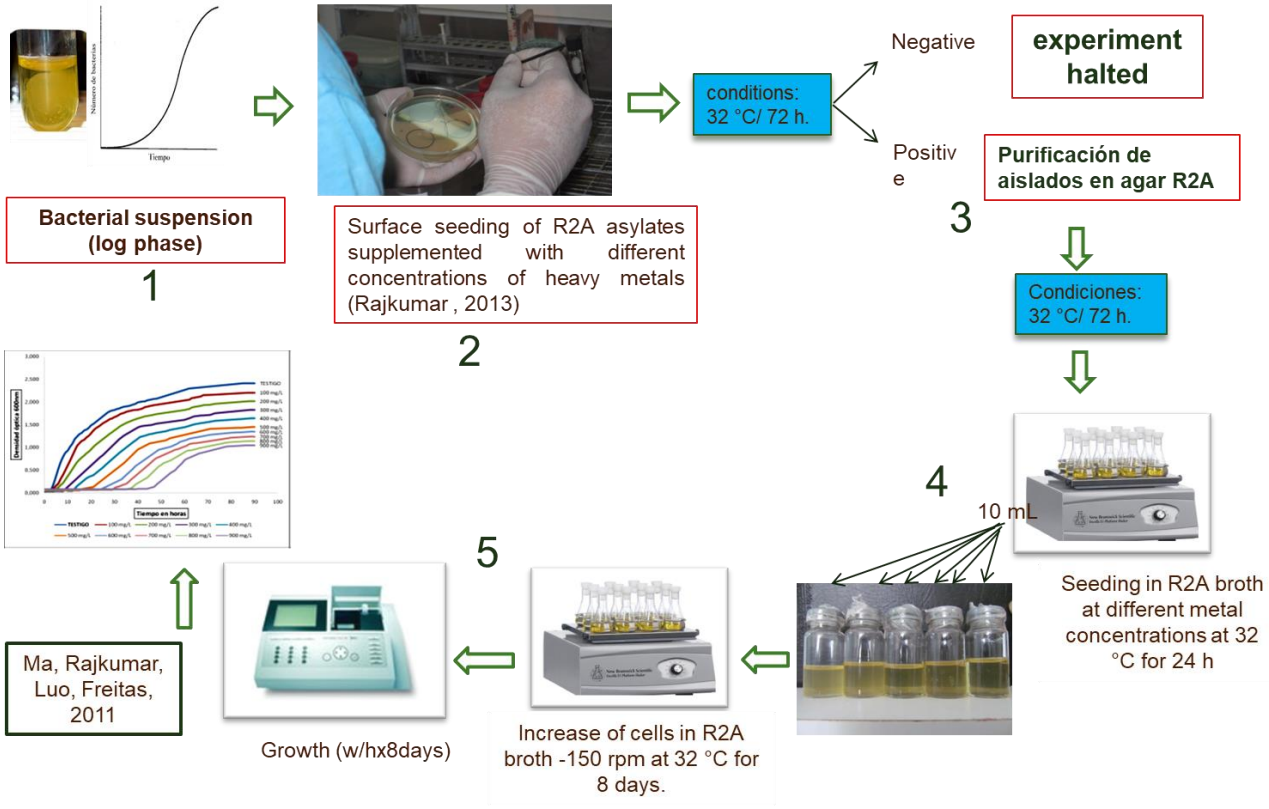


Figure 3. Schematic of the in vitro tolerance activity testing process of endophytic bacterial isolates at different metal concentrations. Source: Pérez et al., 2015.

Isolation of endophytic bacteria - disinfection-counting process. The identification process shall be carried out according to the following protocol as described in Figure 4.

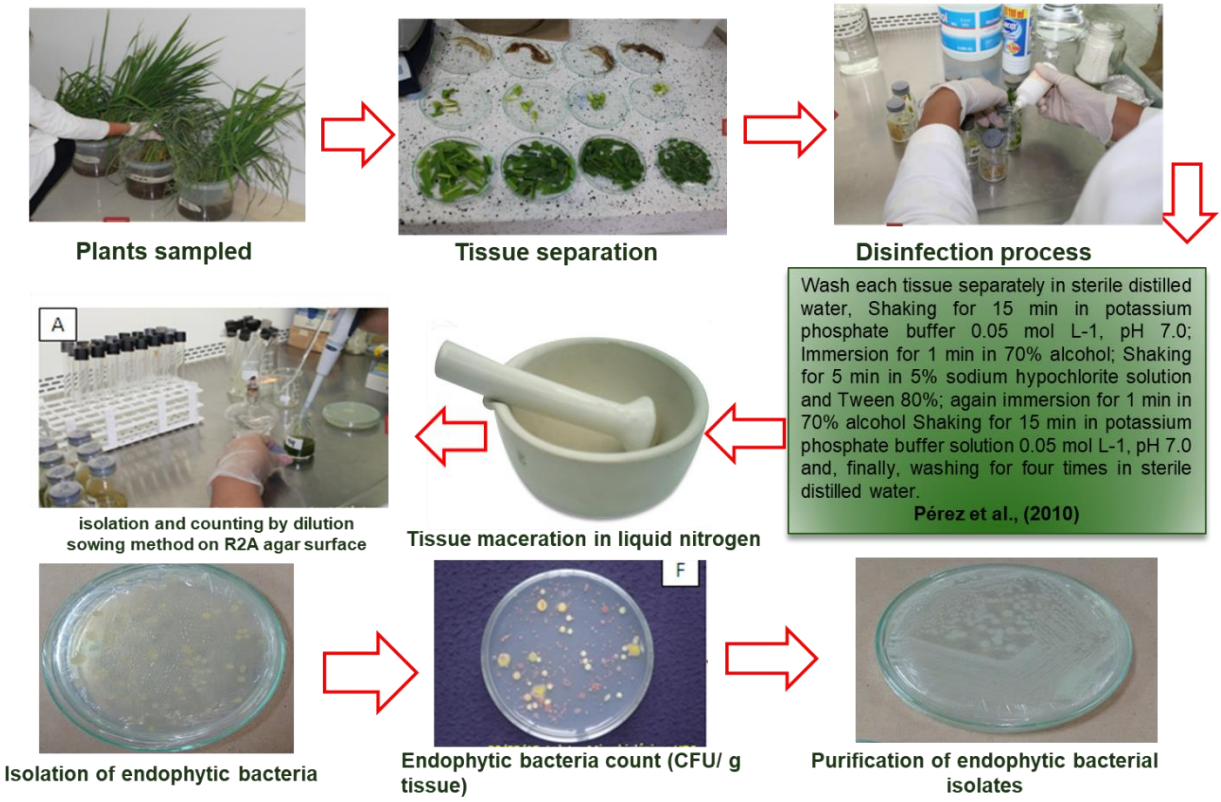


Figure 4. Schematic of the process of isolation of endophytic bacteria from plant tissues of plants growing in environments contaminated with heavy metals. Source: Pérez et al., 2015.

Molecular identification of endophytic bacteria

1. Genomic DNA extraction. This shall be carried out according to the scheme as shown in Figure 6.

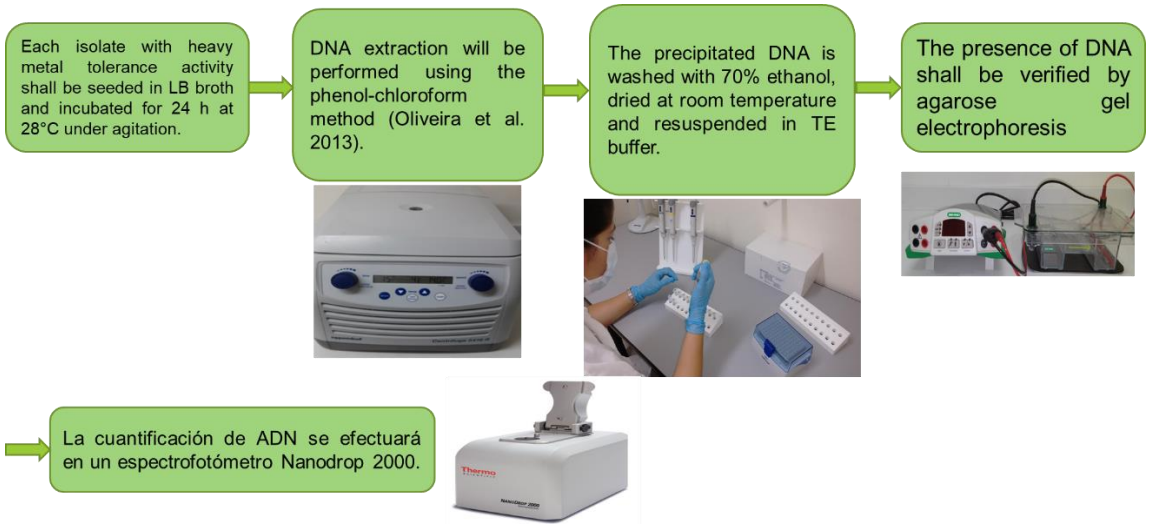
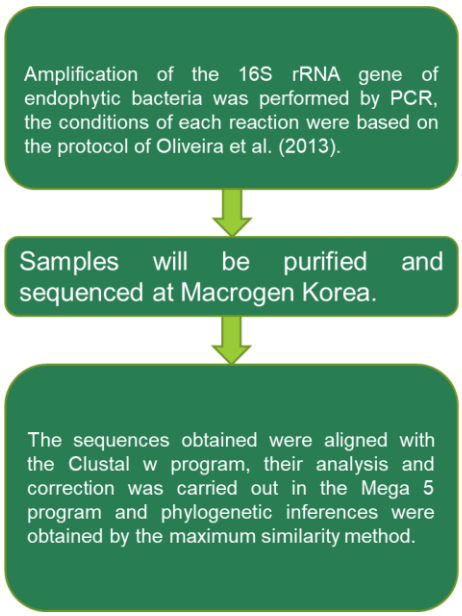


Figure 6. Step in the process of extracting genomic DNA from endophytic bacteria. Source: Pérez et al., 2016.

2. DNA amplification and identification of heavy metal tolerant endophytic bacteria. Identification shall be carried out as illustrated in figure 7.



Group	Primers	Sequences (5'-3')	Position in thegen 16S rDNA
Gamma proteobacteria	FD2	AGAGTTTGATCATGG CTCAG	γ-proteobacteria (18-47)
	RP1	ACGGTTACCTTGTTA CGCTT	Bacteria (1512-1406)
Beta proteobacteria	F 948β	CGCACAAGCGGTGG ATGA	β-proteobacteria (931-948)
	R 1492	TACGG(C/T)TACCTTG TTACGACTT	Bacteria (1492-1513)
Firmicutes	F BLS342	CAGCAGTAGGGAATC TTC	Firmicutes (342-402)
	R 1392	ACGGGCGGTGTGTA CA	Bacteria (1392-1406)

Figure 7. Stages in the process of DNA amplification and identification of endophytic bacteria with tolerance to heavy metals. Source: Pérez et al., 2016.

RESULTS AND DISCUSSION

After the above procedures have been carried out and isolates of endophytic bacteria with heavy metal tolerance activity are obtained, the strains identified by sequencing are listed for referencing and construction of a genomic bank for further studies.

The results of the tests of endophytic bacteria associated with different plant tissues show the phylogeny of these bacteria as described in figure 8.

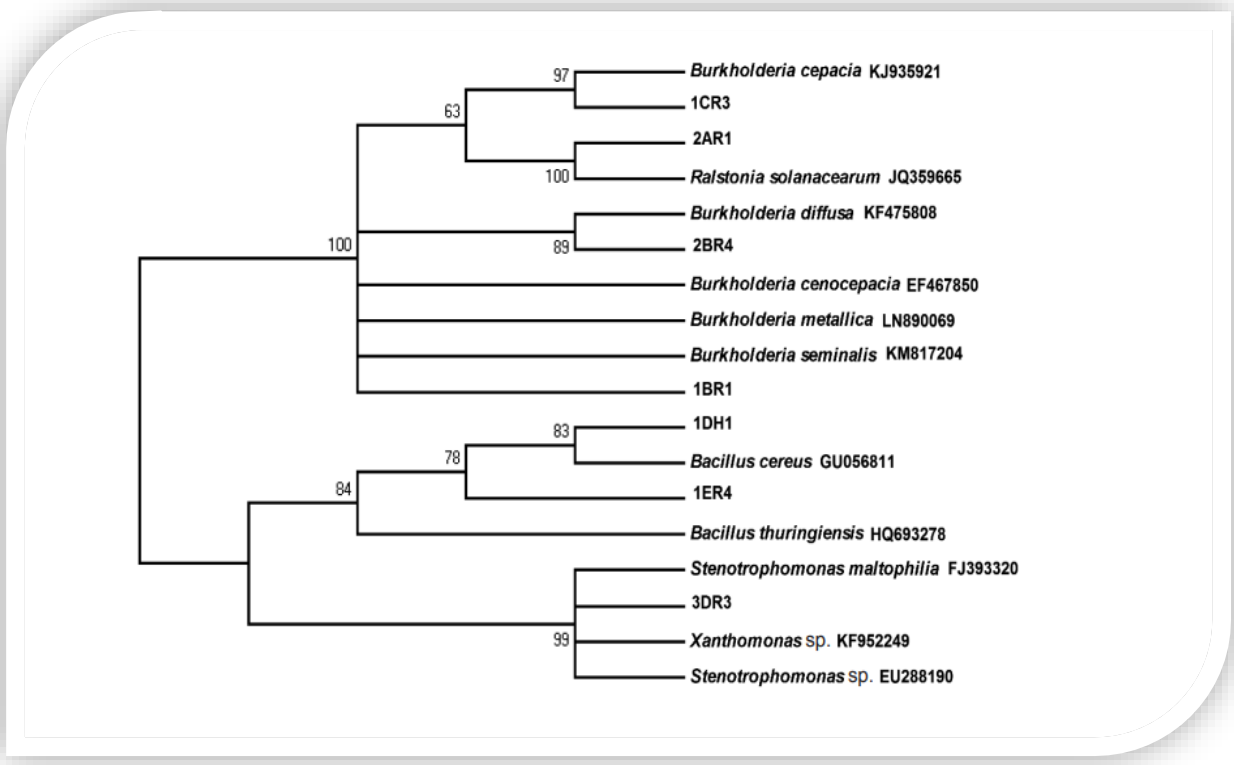


Figure 8. Phylogenetic maximum similarity tree of mercury-tolerant endophytic bacteria. Source: Perez et al., 2016.

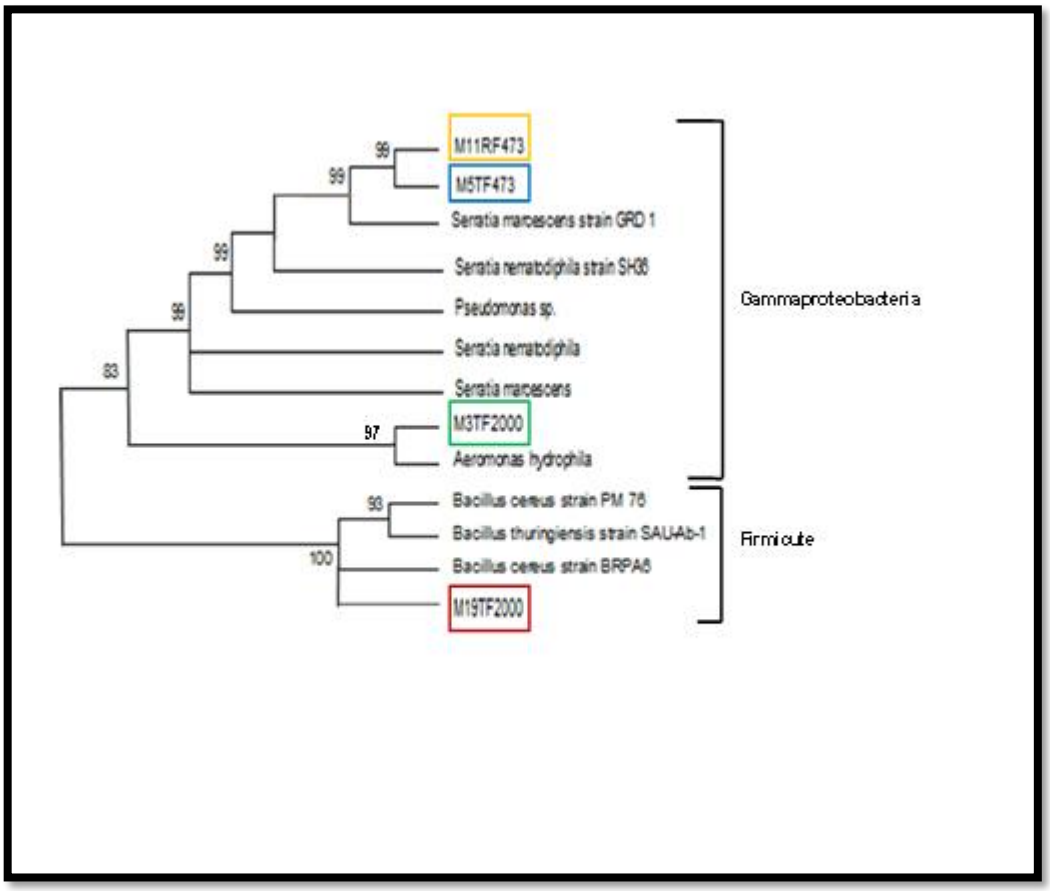


Figure 9. Phylogenetic tree of maximum similarity of nickel-tolerant endophytic bacteria. Source. Perez et al., 2015.

CONCLUSION

The results of 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. A total of 29 endophytic bacteria associated with the genera Cyperus and Paspalum grew at concentrations of 350 and 400 ppm of mercury. Fifteen morphotypes were associated with the species Paspalum arundinaceum, nine morphotypes with Cyperus luzulae and five with Cyperus laxus. Burkholderia cepacia, Burkholderia sp., Ralstonia solanacearum, Bacillus sp. and as a member of the family Xanthomonadaceae were identified.

As this is a preliminary study, we propose to continue with other studies in sites close to gold and ferronickel mines in order to monitor the diversity of endophytic bacteria in these environments. It is also recommended to carry out these studies at the ex situ level in greenhouses and in the field, testing different concentrations of mercury and nickel, determining the resistance of endophyte bacteria to higher concentrations.

ACKNOWLEDGEMENTS

The authors would like to thank the Microbiological Research Laboratory of the University of Sucre.

AUTHOR CONTRIBUTION. Alexander Perez Cordero: experiment execution, data analysis. Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. All authors have read and approved the manuscript.

CONFLICT OF INTEREST. All the authors of the manuscript declare that they have no conflict of interest.

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