# CHARACTERIZATION OF HEAVY METALS RESISTANT *Rhizobium* sp. ISOLATES FROM DIFFERENT REVEGETATION PLOTS ON MANDENA MINING SITE (FORT DAUPHIN-MADAGASCAR)

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*Abstract*

*This study was focused on nine rhizobial strains associated with Mimosa latispinosa, a fast-growing pioneer species native to the Mandena mining site and able to thrive on post-mining soils. The objectives were to: (i) evaluate the heavy metals tolerance of these isolates by comparing their growth on medium supplemented with different concentrations of heavy metals (Ni, Cd, Cu, and Pb); and (ii) screen the presence of the heavy metal resistance genes on the isolated DNA. While all the strains tested showed varying degrees of tolerance to the metals tested, significantly greater tolerance was observed for isolates RMN 4, RMN 5, RR 11 and RR 14. Regarding the presence of genes responsible for heavy metal resistance, only strains RMN 4, RMN 6 and RR 13 presented the specific amplicons in the case of pcoR gene amplification. The amplification of heavy metals resistance genes for other samples did not yield any specific PCR amplicons, probably due to the use of inappropriate primers or the presence of different mechanisms that allow them to survive under stress conditions.*

*Key words*: *heavy metals, rhizobia, mining sites, restoration, Mimosa latispinosa.*

## INTRODUCTION

Heavy metals released by mining and the use of chemicals in agricultural practices can have adverse effects on various ecosystems, including plants (Wani et al., 2008). In the specific case of post-mining sites, soil contamination by these metals, combined with poor soil structure, low water retention capacity, lack of organic matter, and associated nutrients such as nitrogen (N) and phosphorus (P), can hinder the reintroduction of plant species during revegetation and/or site restoration programs. In this regard, exploiting the symbiotic relationship between legumes and rhizobia is presented as an attractive and cost-effective alternative to improve nitrogen supply in the plant-soil system (Gómez-Sagasti & Daniel, 2015).
Rhizobia are Gram-negative soil bacteria that can form symbiotic associations with the *Fabaceae* family, during which they reduce atmospheric nitrogen to ammonia. The symbiosis between rhizobia and legumes has a fundamental contribution to the nitrogen cycle in natural and agricultural ecosystems and is a major reason why legumes are able to colonize marginal lands and nitrogen-deficient soils (De Hoff & Hirsch, 2003). However, several studies have shown that increasing concentrations of heavy metals such as Cu, Zn, and Pb, lead to a reduction in the bacterial count of *Rhizobium* sp. and a change in nodal gene expression (Stan et al., 2011; Chaudri et al., 2008), other authors have suggested that Rhizobium can tolerate high concentrations of heavy metals and play an important role in the remediation of contaminated soils (Carrasco et al. 2005; Teng et al., 2015; Mohamad et al., 2017).
Studies have proposed applying this symbiotic interaction to metal-contaminated soils to improve soil fertility and extract or stabilize

metals simultaneously (Ike et al., 2007; Dary et al., 2010). Indeed, rhizobia heavy metal tolerance mechanisms may include: (i) adsorption and accumulation of heavy metals; and (ii) microbial secretion of enzymes and bioactive metabolites to increase their bioavailability and sequester their toxicity. These mechanisms can directly enhance phytoremediation through nitrogen fixation and growth factor production, or adsorb and accumulate metals, indirectly contributing to phyto-stabilization (Hao et al., 2014).

So, in order to take full advantage of rhizobia-assisted legumes for metal phytoremediation, the first essential step would be to isolate and characterize metal-tolerant rhizobial strains. Many symbiotic rhizobial strains showing resistance to heavy metals have been found in legumes growing in polluted regions, such as mine deposits and serpentine soils (Vidal et al., 2009; Grison et al., 2015).

This study focused on rhizobial strains associated with *Mimosa latispinosa*, a fast-growing pioneer species native to the Mandena mining site (Madagscar) and able to thrive on post-mining soils. The objectives were to: (i) evaluate the heavy metals tolerance of these isolates by comparing their growth on medium supplemented with different concentrations of heavy metals (Ni, Cd, Cu, and Pb); and (ii) screen the presence of the heavy metal resistance genes on the isolated DNA.

## MATERIALS AND METHODS

### Isolation of Rhizobia

Nine (09) isolates of Rhizobia were collected from rhizospheres' soils of *Mimosa latispinosa* from revegetation plots of different ages (6 months, 1 year, and 3 years, respectively) at Mandena mining site (Fort-Dauphin, Madagascar) (Figure 1) according to the methods described by Vincent, 1970. Bacterial colonies were grown on YEMA (yeast extract mannitol agar) (HiMedia Laboratories Pvt. Mumbai-400086, India) supplemented with Congo red and incubated at 28°C for 3 days. The Congo red was used to check the colony colour that represents *Rhizobium* sp. One (01) strain from the 6-month plot, three (03) strains from the 1-year plot, and five (05) for the 3-

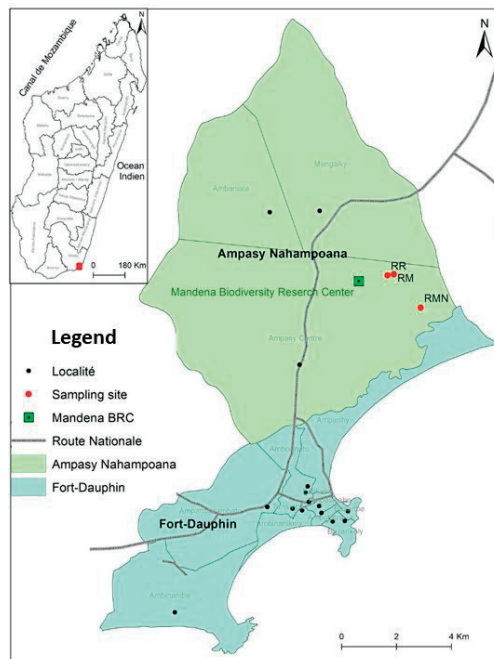year plot were used in further experiments (Table 1).



Figure 1. Origin of samples used in experiments.

Table 1. Origin of *Rhizobium* isolates

| Isolates cods | Age of plot | Localisation |
|---|---|---|
| RM4 | 6 months | S 24°57'819'' E 047°00'443'' |
| RR 11 RR 13 RR 14 | 1 year | S 24°57'960'' E 047°00'367'' |
| RMN 4 RMN 5 RMN 6 RMN 11 RMN 12 | 3 years | S 24°57'476'' E 047°01'159'' |

### Heavy metal resistance test by agar plate method

Heavy-metal-resistance of the isolates was tested by growing in YEMA media supplemented with one heavy metal at one concentration per plate. Three (03) concentrations of heavy metals (0.5 mM, 1 mM and 2 mM, respectively) were added to the medium using stock solutions of each heavy metal. The resistance against four different heavy metals (Ni, Cu, Cd, and Pb) was tested. The stock solutions of heavy metals were added to sterile agar as follows: Ni, Cd, Cu, and Pb were used respectively in the form of nickel chloride ($NiCl_2$), cadmium chloride ($CdCl_2$),

copper sulphate (CuSO4) and lead acetate [Pb (C2H3O2)2]. The agar plates were inoculated with bacterial cells and incubated at 28°C for 3 days. Isolates were considered resistant if growth was observed or sensitive if otherwise.

## Screening for enzymatic activity

Arginine- and ornithine-decarboxylase, activities were performed by inoculating the rhizobia strains on specific substrates. Thus, the enzymatic activity was performed on solid media containing 0.2% L-arginine hydrochloride, or 0.2% L-ornithine hydrochloride, respectively, using phenol red as an indicator dye (Sicuia et al, 2015). After one day of incubation, the result (enzymatic activity) was evaluated due to the colour change of the medium from yellow to pink.

In order to evaluate phytate solubilization, TS agar medium (5 g/L glucose, 10 g/L bacto-peptone, 5 g/L yeast extract, 1 g/L magnesium sulfate, 1 g/L calcium chloride, and 2 g/L phytic acid sodium salt, final pH 7.0) (Demirkan et al, 2014) was used. The bacterial strains that developed a clear halo around their colonies were phytase producers.

## DNA extraction

The pellet of the centrifuged liquid culture of bacteria was mixed with 400 µl distilled sterilized water. The samples were incubated on thermo-block (Dry Block Heating Thermostat Bio TDB-100, Biosan Ltd, UE) at 98°C for 10 min. and then incubated instantly on ice, for 5 min. The supernatant was used directly in PCR reactions.

## PCR for heavy metal genes

For PCR reactions DreamTaq green PCR master mix (Thermo Scientific™, Vilnius, Lithuania) was used. The total volume of the PCR reaction was 25 µl, containing: 12.5 µl buffer, specific forward and reverse primer 10 µM (Table 2), DNA 10 ng, and water. PCR program (Eppendorf Mastercycler gradient, Hamburg, Germany) was as follows: 95°C – 3 min, 35 cycles of - 94°C – 1 min, annealing temperature – 1 min (Table 2), 72°C – 2 min, and final extension 72°C – 7 min. Amplicons were visualized in 1.2% agarose gel, in UV light using the BioDoc-It Imaging System (Ultra-Violet Products Ltd., Upland, CA).

Specific primers were used as follows: pcoR, pbrA, nccA, czcD, chrB, chrA (Table 2).

Table 2. Heavy metal resistance genes used in PCR

| Heavy metal | Resistance gene | Annealing temp. | Reference |
|---|---|---|---|
| Cu | pcoR | 54°C | Fan et al. (2011), Abdel-Lateif (2017) |
| Pb | pbrA | 57°C | Wei et al. (2009), Abdel-Lateif (2017), Rahal et al (2023) |
| Cd/Ni | nccA | 57°C | Renitta et al (2015), Amer et al (2015), Rahal et al (2023) |
| Zn | czcD | 57°C | Wei et al (2009), Abdel-Lateif et al (2017) |
| Cr | chrB | 52°C | Ndeddy Aka &Babalola (2017) |

## RESULTS AND DISCUSSIONS

The nine (09) *Rhizobium* sp. isolates were tested for resistance to heavy metals using concentrations of 0.5, 1 and 2 mM, respectively, of Ni, Cd, Cu, and Pb (Table 3). At the lowest concentration (0.5 mM), all isolates were able to grow on media containing Ni, Cu and Pb, while for Cd, most isolates proved resistance except for RMN 12 and RM 4 isolates.

At 1 mM concentration, no growth was observed for isolates RMN 5 and RM 4 in the presence of Ni, RMN 12 and RM 4 in the presence of Cd and RMN 11 and RMN 12 in the presence of Cu. However, at the same concentration, all isolates tested showed resistance to Pb (Figure 2).
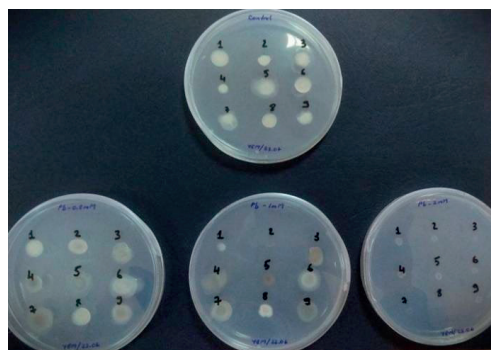


Figure 2. Growth of tested strains on YEMA medium with increasing concentrations of Pb: above: control; bottom, from left to right: 0.5 mM, 1 mM, 2 mM

At the highest concentration (2 mM), no isolate was able to grow in the presence of Pb. No growth was observed for isolates RMN 12 and RM 4 in the presence of Ni, Cd and Cu, RMN 4 in the presence of Ni and Cu, RMN 5 and

RMN 6 in the presence of Ni and RR 13 in the presence of Cd.

In general, the isolates' ability to resist the heavy metals decreased with increasing concentrations. However, strong tolerance was observed for almost all isolates from 3 years old plot: RMN 4, RMN5 and RMN 11 for Cd and Pb and RMN 6 for Cu. Isolates from one year plot also showed high tolerance for Ni and Cd (RR11 and RR 14) and Pb (RR 14) (Table 3).

Table 3. Effects of different concentrations of heavy metals on the growth of rhizobial isolates on YEMA plates

| Strain | C | Ni (mM) | | | Cd (mM) | | | Cu (mM) | | | Pb (mM) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | 1 | 2 |
| RM4 | +++ | ++ | - | - | - | - | - | + | + | - | +++ | ++ | - |
| RR 11 | +++ | +++ | +++ | + | +++ | +++ | ++ | +++ | ++ | - | +++ | + | - |
| RR 13 | +++ | ++ | + | + | ++ | - | - | +++ | ++ | + | +++ | + | - |
| RR 14 | +++ | +++ | + | + | +++ | +++ | ++ | +++ | ++ | + | +++ | ++ | + |
| RMN 4 | +++ | ++ | + | - | +++ | +++ | +++ | +++ | ++ | + | +++ | +++ | - |
| RMN 5 | +++ | ++ | - | - | +++ | +++ | +++ | ++ | ++ | + | +++ | +++ | - |
| RMN 6 | +++ | ++ | + | - | +++ | +++ | + | ++ | ++ | + | +++ | ++ | - |
| RMN 11 | +++ | ++ | + | + | +++ | +++ | +++ | ++ | - | - | +++ | +++ | - |
| RMN 12 | +++ | + | + | - | - | - | - | +++ | - | - | +++ | +++ | + |

(+++): very good growth; (++): moderate growth; (+): poor growth; (−): no growth.
C: Control; Ni: Nickel, Cd: Cadmium, Cu: Copper, Pb: Lead

The ability of *Mimosa latispinosa* to grow and develop in Mandena's post-mining soils is largely due to their association with rhizobia. These rhizobia are important ecological players, responsible for the entry of biologically fixed nitrogen into metal-contaminated areas and facilitating the activation of the restoration of the Mandena mining site (Mohamad et al., 2017).

The present study has shown that Rhizobium sp. isolates associated with Mimosa latispinosa grown on the post-mining soils of Mandena show varying degrees of tolerance to the tested heavy metals. This tolerance could be linked to the acquisition of metal resistance mechanisms, such as metal efflux transporters (Lakzian et al., 2002). However, it has also been shown that this tolerance tends to decrease with increasing metal concentration, in line with the results of previous studies which have shown that high concentrations of heavy metals can affect the growth, morphology, and nitrogen-fixing activities of microorganisms (Shi et al., 2002; Pereira et al., 2006).

The capacity of all nine (09) strains to decarboxylate arginine and ornithine was evaluated. The results showed that all strains have the capacity to decarboxylate the two amino acids tested, emphasizing the change of colour of the culture media, from orange to pink (Figure 3).
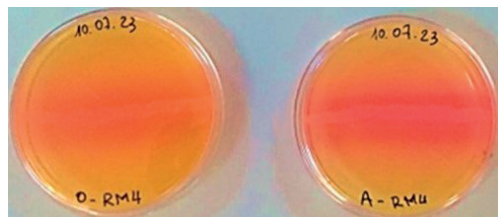


Figure 3. Enzymatic activity for arginine- and ornithine-decarboxylase for RM4 strain

The capacity of the strains to metabolise the phytate through organic phosphorus solubilization was qualitatively evaluated by the formation of halos around the colonies cultured on specific medium and incubated at 28°C for 10 days. Positive results (phytase producers) were obtained only for RMN 6, RMN 12 and RR 14 strains.

In these experiments, amplification based on heavy metal specific primers was used to screen and identify some heavy metal resistance genes (*nccA, pbrA, pcoR, chrB*, and *czcD*) in the tested samples. The *pbrA* gene encodes a P-type Pb(II) efflux ATPase in the lead resistance operon that is involved in the uptake, efflux, and accumulation of Pb(II) (Rahal et al, 2023). The czcD gene is a cation diffusion facilitator protein family transporter located in the cytoplasmic membrane and reduce ions accumulation ($Cd^{2+}$, $Zn^{2+}$ and $Co^{2+}$) in the cytoplasm to the periplasm. The nccA gene provides resistance to nickel, cadmium, and cobalt using a similar mechanism (Rahal et al, 2023).

In our experiments, only three strains (RMN 4, RMN 6, and RR 13) yielded specific amplicons (650 pb) after *pcoR* gene amplification. These results are in accordance with the microbiological analysis. Also, in the case of *nccA* gene amplification, the primers used in PCR reaction yielded a band with approximately 300 bp. This result differs from those mentioned in the literature (Ndeddy Aka & Babalola, 2017; Rahal et al, 2023).

The amplification of heavy metals resistance genes for other samples did not yield any specific PCR amplicons, probably due to the use of inappropriate primers or the presence of different mechanisms that allow them to survive under stress conditions. That includes transporting heavy metals out of the cell, metal precipitation outside or inside the cell, or heavy metal binding through exopolysaccharides (Wei et al. 2009; Rahal et al, 2023). Due to the poor amplification and reproducibility of results of most tested isolates with the nccA, pcoR, pbrA, czcD and chrB primers, sequencing and nucleotide translation for these genes were not followed up further.

## CONCLUSIONS

The isolation and selection of rhizobia strains resistant to stresses such as heavy metals are an important part of efficient nitrogen fixation and improved plant productivity, particularly in contaminated and severely depleted areas such as post-mining soils.
The absence of amplicons in bacterial isolates suggests that the tolerance/resistance to heavy metals could be due to other heavy metal resistance mechanisms.
The use of these strains in revegetation trials could not only increase the level of nitrogen fixation by *Mimosa latispinosa*, but also improve the efficiency of phytoremediation.

## ACKNOWLEDGEMENTS

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