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# Characterization of Root-Nodule Bacteria Isolated from *Hedysarum spinosissimum* L, Growing in Mining Sites of Northeastern Region of Morocco

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## Abstract

The aim of this study was to identify bacteria present in the nodules of the legume *Hedysarum spinosissimum* growing in metal-contaminated soils; and to test whether these root-nodule bacteria are able to promote host plant growth and enhance their phytostabilization potential. Seventy-four bacteria were isolated from nodules of *H. spinosissimum* growing in 3 different mining sites in Morocco and were identified by 16S rDNA gene sequencing. They belonged to 8 genera affiliated to *Pseudomonas* (49 strains), *Pantoea* (11), *Rhizobium* (6), *Herbaspirillum* (3), *Bacillus* (2), and one strain of *Serratia*, *Agrobacterium* and *Azospirillum*. Seven strains, presenting a high tolerance to Zn and Pb, exhibited capacity of inorganic phosphate solubilization and ammonia production from peptone degradation. The inoculation of *H. spinosissimum*, growing in 2000 µM of Zn by *Pseudomonas putida* and *Herbaspirillum huttiense* suppresses Zn toxicity symptoms in plants and also enhances plant growth by significantly increasing plant shoot and root fresh weights. The maximal Zn accumulation was observed in roots of plants inoculated by *Pseudomonas putida* with a translocation factor of 0.05 (± 0.006). Our results, evidence that the selection of metal-resistant bacteria is a key step in polluted soils for the use of plants like *Hedysarum spinosissimum* for *in situ* phytoremediation.

**Keywords:** legumes; Phytoremediation; Nodulation; Mine tailings; Heavy metal resistance; Phytostabilisation

## Introduction

Soil contaminations with heavy metals are principally caused by mining activities and require strengthening the efforts to minimize their impact on the environment and human health [1]. Since metals are non biodegradable, they accumulate and persist in the environment [2]. To cleanup these degraded and polluted areas, metallic pollutants should be extracted and stored in some appropriate and secure sites. Conventional techniques, including

thermal processes, washing, physical separation, stabilization/solidification, etc ..., are generally very expensive and harmful to soil integrity and microbial diversity [3,4]. Phytoremediation appears as a good alternative as plants can indeed bio-concentrate (phytoextraction) as well as bio-immobilize (phytostabilization) toxic metals through *in situ* rhizospheric processes [1,2]. However, mining sites in semi-arid areas are characterized by low vegetation cover due to the unfavorable effects of a combination of environmental factors, including metals toxicity, nutrient deficiency, poor soil structure and low water retention [5,6], thus, the development of a vegetative cover in these environments is a challenge. Recently, several studies have shown that the combination of plant-associated bacteria in phytoremediation may lead to promising results, in particular the group of plant growth promoting bacteria [7,8]. This approach is furthermore perceived as cost-effective, eco-friendly, and with good public acceptance [2]. The choice of bacteria is usually based on their potential to produce phytohormones such as IAA, gibberellins and cytokinins that directly promote roots and plant growth [9]. Other bacteria can synthesize organic chelators (siderophores) to acquire iron [10] that will play a positive role in plant nutrition; some have great potential for phosphate solubilization or possess ACC deaminase activity [11] while others are able to fix nitrogen [1] or provide protection against viral diseases [12]. In addition, microorganisms can be implicated directly in metal mobilization/immobilization, by changing the metal bioavailability, solubility and toxicity by different mechanisms including alteration of soil pH, releasing of chelators (organic acids, biosurfactants, siderophores, polymeric substances or glycoprotein, etc), metal biosorption or by oxidation/ reduction reactions [4,13]. The use of plants for the remediation of contaminated soils by heavy metals obviously also depends on the plant species, and on its anatomical, physiological and molecular characteristics

[14]. Furthermore, these plants need to be drought-, and metal-tolerant in arid and semi-arid area, and preferentially native [5]. Beside the interest of legumes in soil regeneration, thanks to their capacity to increase soil nitrogen through atmospheric nitrogen fixation, many legumes are able to accumulate metals in their roots, representing good candidates for metal phytostabilization. They have also been found to be generally the dominant plant species in metal contaminated environments [15,16]. In this regard, several legume species have been proposed for metal phytostabilization [17,18]. *Hedysarum spinosissimum* is a forage legume, which is part of the indigenous flora adapted to semi-arid climate of the mining sites located in eastern Morocco. The soils in these sites are contaminated by metallic pollutants and constitute a potential source of toxicity for the environment and peoples in surrounding villages.

The objectives of this study were to isolate and characterize metal tolerant bacteria from *H. spinosissimum*'s nodules and to propose a new model for phytoremediation of mining tailing by using plants and associated symbiotic bacteria.

## Material and Methods

### Soil collection and characterization

Soil samples were collected, in triplicate, from 12 points distributed among the 3 mining sites: Oued El heimer smelter (34°26'38"N/1°53'54"W), Touissit (34°28'06"N/1°46'18"W) and Sidi Boubker (34°28'23"N/1°42'55"W) mines. Soil debris was removed by sieving using a steel sieve (2 mm pores diameter). Each soil was then ground in a mortar until obtaining a fine powder. Samples were sent for ICP-AES analysis in Eurofins-France laboratory, under ISO/ IEC 17025:2005 norm and accreditation by the COFRAC 1-148.

### Bacterial trapping and isolation

Nodules were obtained from *Hedysarum spinosissimum* plants, aged of 2 months, growing in soils sampled from the 3 mining sites, under growth chamber under day/ night photoperiod of 16h/ 8h and temperature of 25°C. The nodules were collected, surface sterilized by soaking in alcohol (ethanol 95%), washed several times in sterile distilled water and crushed. The suspension obtained was spread on solid YEM medium (yeast extract mannitol), and incubated for 24-48 hours at 28°C. Bacterial colonies were purified on the same media by streaking 3 to 4 times on fresh media. Bacterial isolates showing different morphological appearance on agar media were selected, grown in liquid YM and stored at -80°C in 20% glycerol.

### Molecular identification

Genomic DNA was extracted from liquid cultures of bacterial isolates and 16S rDNA was amplified by Polymerase Chain Reaction (PCR) using the universal primers, 41F (5'-GCTCAGATTGAACGCTGGCG-3') and 1488R (5'-CGGTTACCTTGTTACGACTTCACC-3') [19]. The PCR mixture (25 µl) contained 1 µl of DNA (10 ng/ µl), 5 µl of 5xTaq DNA polymerase buffer (BIOLINE), 0.5 µl of Taq DNA polymerase (2.5 U), 1.25 µl of 10 pmol primers, 0.125 µl of Taq polymerase and

bidistilled water. The PCR was performed in a Veriti® 96-Well Thermal Cycler (Applied BioSystems®) with a hot starting at 94°C for 5 min, followed by 35 cycles of 94°C for 45s, 64°C for 90s, and 72°C for 90s, followed by a final extension at 72°C for 7 min. The sequencing was performed at Genoscreen Company (France). The 16S rDNA sequences were compared with those available in GenBank database using the BLASTN program through the National Center for Biotechnology Information server. The 16S rDNA sequences have been deposited in GenBank under the accession numbers from KP263459 to KP263517 and KP677886 to KP677900.

### Phylogenetic analysis

Multiple alignments of consensus sequences were done with the program ClustalW [20]. The resulting alignment was used for construction of phylogenetic trees using MEGA software version 6, using the Neighbor-Joining method [21]. The robustness of the phylogenetic tree was evaluated according to the bootstrap analysis based on 1000 re-samplings of the sequences.

### Effects of heavy metals on bacterial growth

The tolerance of each bacterial isolate to Zn and Pb of selected isolates was evaluated on Tryptone-Yeast extract (TY) medium [22] supplemented with increasing concentrations of both metals. The choice of concentrations used for tolerance tests was based on the concentration of these metals in contaminated soils. ZnSO<sub>4</sub> and PbNO<sub>3</sub><sup>2-</sup> forms were used at concentrations ranged from 1 to 30 mM. The strains were grown in liquid medium for 24 h at 28°C. At the exponential growth phase of each strain, the optical density at the wavelength of 600 nm was determined and standardized at 0.06. Ten µl of the bacterial culture were deposited onto TY agar plates and incubated at 28°C for 24–72 h. Three replicates were made for each bacterial strain. Strains tolerance was also tested in liquid medium.

### Bioaccumulation of Zn in bacteria

A total of 100 ml of bacterial cultures (in TY medium) were prepared in 250 ml flasks, incubated at 37°C. After 48h incubation, the metal (5 mM of ZnSO<sub>4</sub>) was added to the bacterial cultures, which were then re-incubated under the same conditions. The cell mass was collected by centrifugation (8000 rpm for 10 min at 4°C) and washed twice with Tris-HCl buffer (0.1 M, pH 7.2) to remove elements adsorbed onto the cell surface [23]. The samples were then dried at 70°C until a constant dry mass was obtained. The bacterial pellets were mineralized using the following procedure: the pellets were introduced into tubes which contained 10 ml of nitric acid (HNO<sub>3</sub>) 60%, 0.5 ml of sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and 4 drops of perchloric acid (HClO<sub>4</sub>) were added. After incubation at room temperature for 24 h, the tubes were placed in a heating block at 140°C for 4 h. Finally, the volume was reduced by evaporation for 24 hours by slightly opening the tubes. The concentration of Zn in the samples was finally determined by ICP-AES method (Ultima 2- Jobin Yvon).

### Motility Assay

The procedure described by Murray, et al. [24] was followed with slight modifications. Bacterial motility was evaluated on a

semi-solid TY (0.3%) medium. Liquid cultures were standardized to a bacterial concentration of  $10^5$  to  $10^6$  cells/ ml. Five  $\mu$ l of each bacterial suspension were inoculated in TY semi-solid agar medium by gently pressing the tip into the medium. Three repetitions were performed for each strain for both treatments: metal stress treatment (5 mM of Zn) and a control treatment (no metal added). Petri dishes were incubated at 30°C for 24-48h depending on the strain. The bacterial motility was evaluated by determination of the diameter of the halo formed.

### Mineral phosphate solubilizing activity and ammonia production

Phosphate solubilization was first roughly estimated on modified solid PVK medium [25]. 10 $\mu$ l of each strain were deposited on the media in triplicates and incubated at 28°C for 5 days. The solubilization was evaluated by the halo diameter obtained. To precisely quantify the solubilized phosphate, flasks containing 100 ml of PVK medium were inoculated with 1 ml of pre-cultures and incubated at 28°C for 5 days with stirring at 150 rpm. The determination of soluble phosphate was done according to the phosphomolybdate method [26].

Bacterial strains were also tested for the production of NH<sub>3</sub> in peptone water [27] using Nessler's reagent (0.5 ml/ tube). The appearance of a faint yellow color indicates production of a small amount of NH<sub>3</sub> whereas a deep yellow to brownish color indicates high NH<sub>3</sub> production.

### Influence of selected strains on plant growth and Zn uptake

For inoculation, two strains (LMR23 and LMR51) were grown at 28°C for 24h in TY medium on a shaker at 150 rpm. The bacterial suspensions were washed twice and resuspended in sterile distilled water after determination of optical density (i.e. roughly  $10^6$  bacteria ml<sup>-1</sup>). After inoculum preparation, the experiment was conducted with 3 replicates for each of the 3 following treatments: (1) plants grown in nutrient solution without metal and inoculum, (2) plants grown in nutrient solution supplemented with the Zn MIC (Zn Minimal Inhibitory Concentration) of plants growth and (3) plants grown in nutrient solution with different combinations of strains with Zn at the MIC. Before the start of the experiment, plants were grown in normal conditions for 15 days. After treatment, plants were grown under the different conditions for 15 days and their roots and shoots were separated and rinsed several times in 0.2 mM CaSO<sub>4</sub> and then with distilled water. Samples were dried at 70°C for 48h and then treated according to the acid hydrolysis protocol described by Temminghoff, et al. [28]. Zn concentration was determined by ICP-AES method. The Translocation Factor (TF) for metals within a plant was expressed by the ratio of concentration of metal (shoot)/ metal (root), to estimate metal translocation properties from roots to shoots [29].

### Statistical analysis

The mean and standard deviation of the 3 replicates for each treatment were calculated. Tukey's test was conducted to assess determine significant differences. Statistical analyses were performed with STATISTICA version 6.

## Results and Discussion

### Isolation and identification of bacteria

Overall, 74 endophytic bacteria were isolated from *Hedysarum spinosissimum* nodules. These isolates were selected based on their morphological differences observed between colonies on YEM solid medium and were identified using 16S rRNA gene sequencing. Most of the strains were isolated from Oued El heimer site (37 isolates) and Touissit (34 isolates), unlike Sidi Boubker where the number of isolates was limited to 3 (Table 1). The analysis of the 16S rRNA gene sequences revealed that the isolates belonged to 8 different taxonomic genera, including *Pseudomonas* genus (largest number of representatives, 49), *Pantoea* (11), *Rhizobium* (6), *Herbaspirillum* (3), *Bacillus* (2) and one representative of *Serratia*, *Agrobacterium* and *Azospirillum* (Table 1). The phylogenetic tree shows the relationships between the bacterial strains and related reference species (Figure 1). Phylogenetic analysis indicated that the bacterial strains isolated from the nodules of *H. spinosissimum* belong to 2 phyla: the *Firmicutes* represented by *Bacillus* sp. (2%) and *Proteobacteria* represented by the rest of the collection (98%), including the classes of  $\alpha$ -,  $\beta$ - and  $\gamma$ -*Proteobacteria* (Table 1).

Few studies have been made on wild legume *Hedysarum spinosissimum* nodule bacterial populations. Wei, et al. [30] suggested a high level of genetic diversity among isolates of *Hedysarum*. Benhizia, et al. [31] reported the presence of isolates belonging to bacterial genera retrieved in our study, *Pseudomonas* and *Pantoea agglomerans*, in addition to three other

**Table 1:** Identification, using 16S rDNA gene, of the bacterial isolates present in the nodules of the plant *Hedysarum spinosissimum*.

Bacterial species	Taxonomical division	Number of representatives	Sampling sites
<i>Pseudomonas</i> sp.	$\gamma$ - <i>Proteobacteria</i>	36	Touissit / Oued El heimer
<i>Pseudomonas putida</i>	$\gamma$ - <i>Proteobacteria</i>	8	Touissit
<i>Pseudomonas brassicacearum</i>	$\gamma$ - <i>Proteobacteria</i>	1	Oued El heimer
<i>Pseudomonas frederiksbergensis</i>	$\gamma$ - <i>Proteobacteria</i>	4	Touissit/ Sidi Boubker
<i>Pantoea agglomerans</i>	$\gamma$ - <i>Proteobacteria</i>	11	Touissit/ Sidi Boubker/Oued El heimer
<i>Serratia proteamaculans</i>	$\gamma$ - <i>Proteobacteria</i>	1	Oued El heimer
<i>Herbaspirillum huttiense</i>	$\beta$ - <i>Proteobacteria</i>	3	Touissit/ Oued El heimer
<i>Rhizobium leguminosarum</i>	$\alpha$ - <i>Proteobacteria</i>	4	Oued El heimer / Touissit
<i>Rhizobium galegae</i>	$\alpha$ - <i>Proteobacteria</i>	2	Oued El heimer
<i>Agrobacterium tumefaciens</i>	$\alpha$ - <i>Proteobacteria</i>	1	Oued El heimer
<i>Azospirillum lipoferum</i>	$\alpha$ - <i>Proteobacteria</i>	1	Oued El heimer
<i>Bacillus</i> sp.	<i>Firmicutes</i>	2	Oued El heimer

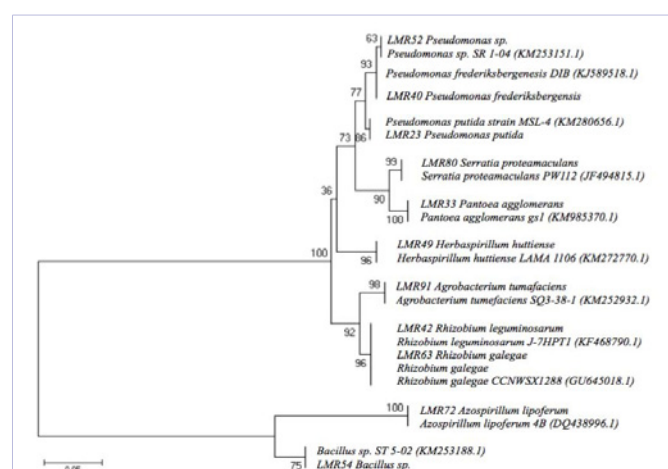


genera (*Enterobacter*, *Leclercia* and *Escherichia*), while Zakhia, et al. [32] only isolated one strain related to *Sinorhizobium*. Symbiotic *Rhizobium* species have been already isolated from nodules of another leguminous plant, *Anthyllis vulneraria*, growing in contaminated soils from a different mining district in Morocco [18]. The presence of *Agrobacterium* in nodules of plants in polluted soils was also previously described [33,34]. *Herbaspirillum* and *Azospirillum* are 2 root associated nitrogen fixing bacterium [9,35,36]. Species belonging to *Herbaspirillum* genus are known root endophytic Plant Growth Promoting Bacteria (PGPB) especially in cereals. In addition, *Herbaspirillum* strains have a considerable potential to produce auxin and siderophores, and to solubilize inorganic phosphates [37]. In a bioremediation frame, *Herbaspirillum* strains showed also an important resistance to As, Zn, Cu and Pb, and some good abilities for the leaching of Cu from contaminated soil [38]. Plant-growth-promoting *Bacillus* strains were also isolated from soybean root nodules [39]. This genus was also able to accelerate root and shoot growth by increasing chlorophyll content [40]. Different strains of *Bacillus* species such as *B. methylotrophicus*, *B. aryabhattai*, and *B. licheniformis* isolated from *Spartina maritime* rhizosphere exhibited multiple plant growth promoting properties and were selected as performing strains for restoration programs [41]. The presence of *Serratia* sp. in legume nodules of *Lupinus luteus* was previously reported [17]. This genus has also been shown to have some PGPR properties like ACC deaminase activity [42].

The combination of both harsh conditions encountered by the roots in metal polluted soils combined with the tolerance of *Hedysarum* to co-infestation of nodules by rhizospheric bacteria should explain the large taxonomic diversity we recovered in this study. Such diversity gives the opportunity for the selection of various strains, with different properties, that could be used either alone or in combination for inoculation.

### Effect of Pb and Zn on bacterial growth

To select strains tolerant to heavy metals, we conducted



**Figure 1:** Phylogenetic tree showing the relative position of isolates based on 16S rDNA partial sequences, using the neighbor-joining method. Bootstrap values for 1000 replicates are shown.

tests on solid and liquid TY media supplemented with ascending concentrations of Pb and Zn (from 1 to 30 mM) that were the most abundant metals present in the mining site of Oujda. The experiment on solid TY medium demonstrated contrasted responses of the isolates to the different amounts of heavy metals tested. Seven highly tolerant bacteria were selected for further studies. In liquid media, the effect of both metals on the growth of these strains was estimated by measuring the optical density at 600 nm. As seen in figure 2, the 7 strains showed the same level of resistance to Pb (25 mM) and Zn (5 mM). However, metals affected negatively the growth of these bacteria by reducing the number of bacterial cells (Figure 2). Compared with control treatment the reduction of optical density was more pronounced in Zn treatment (approximately 2/ 3 of reduction) than in Pb treatment with 1/ 3 of OD decrease.

### Determination of Zn uptake of the most tolerant isolates

A deeper study has been done for these 7 resistant strains in order to evaluate their metal accumulation capacity. Zn was chosen for further investigations based on its important concentrations in the 3 sampling stations and also its standing as critical metal for the growth of living organisms, especially plants and bacteria.

As shown in Table 2, the *Rhizobium galegae* strain LMR64 or the *Herbaspirillum huttiense* strain LMR51 showed a very high level of zinc accumulation inside the cells (representing respectively 58 and 31 ppm), while other isolates showed a lower accumulation. The different levels of Zn accumulation in the strains were not correlated with the level of inhibition of their detected on TY medium containing high Zn concentration.

However, bioaccumulation of metals, inside the cell or on cell surface can lead to some applications in remediation by metal adsorption in soils [43-45] and could decrease the metal bioavailability around the plant rhizosphere and thereby reduce plant stress and metal accumulation [1,15,18,46].

### Motility assay

In their environment, bacteria are either mobile or bound to a substrate. In the present work, we were interested in swimming bacteria, because it is a physiological strategy developed by bacteria against stress [47]. The swimming was assessed by comparing the halo of motility between strains living under Zn stress and normal conditions (Figure 3). We found that motility was significantly reduced ( $p < 0.05$ ) under Zn treatment for some strains (23% for LMR27, 40% for LMR51 and 42% for LMR64), or completely inhibited for other strains (LMR80 and LMR54). Conversely, motility was unaffected for LMR23 and LMR79 with no significant differences ( $p < 0.05$ ). Under adverse environmental conditions, the maintaining of motility is useful to bacteria to find nutrients and to survive, and the movement via flagella appears to be important for the plant root colonization [48,49]. However, it has been reported that heavy metals could affect the motility [47]. In *Pseudomonas putida*, some proteins are involved in regulating motility reducing the migration of

bacteria and facilitating *Pseudomonas putida* biofilm formation, an important trait to protect plants against pathogens [48].

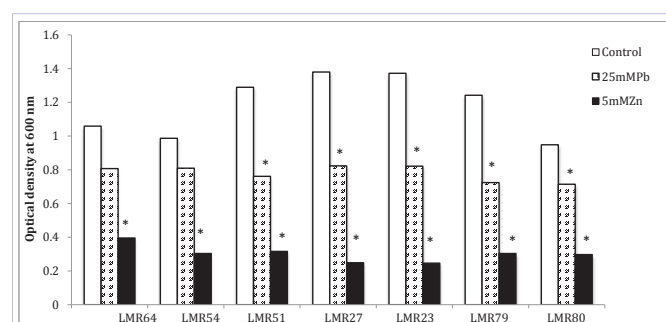
### Plant growth promoting properties

The determination of free phosphorus resulting from the activity of microbial solubilization of mineral phosphate was performed by the vanadate-molybdate method. The results are shown in Table 3. All strains tested showed a great potential of phosphate solubilization (with co-relation with pH decrease) from  $27.25 \pm 5.49$  ppm to  $90.91 \pm 7.95$  ppm depending on the strains and was maximum for *Pseudomonas putida*. The solubilization of insoluble phosphate into available form is a mechanism commonly observed in most metals resistant PGPB. This process is realized by means of organic acids secretion out of the cell, acidification, chelation and exchange reactions [1]. Pandey, et al [50] also showed the importance of the phosphate solubilizing activity of a strain of *Pseudomonas putida* isolated in Indian Central Himalaya associated with antifungal activity resulting in significant improvement of the plant biomass. Several other studies have also demonstrated the importance of phosphates solubilizing bacteria and their effects on plants by increasing the bioavailability of this macronutrient [1,51].

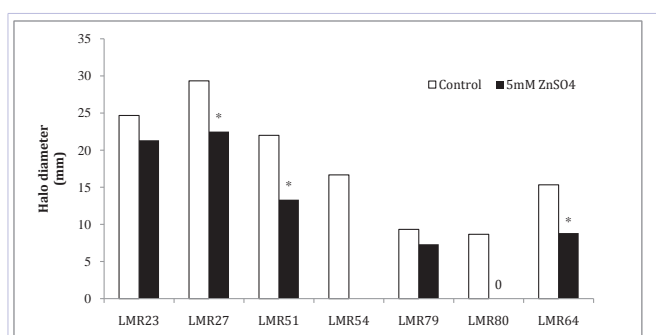
The detection of ammonia production was done using a qualitative method. As shown in table 3, all the 7 strains tested had a positive  $\text{NH}_3$  production but this production was more important for *Pseudomonas putida* and *Herbaspirillum huttiense*.

### Effects of the inoculation of selected strains on *H. spinosissimum* plants growth and Zn tolerance

The effects of the isolates LMR23 (*Pseudomonas putida*) and LMR51 (*Herbaspirillum huttiense*) on the growth of *H. spinosissimum* grown in control condition and in nutrient solution containing  $2000 \mu\text{M}$  of Zn (the minimal inhibitory concentration of Zn for *H. spinosissimum* plants) are shown in Table 4. The plants inoculated with the selected isolates showed a significant increase in plant fresh weight accompanied by the suppression of Zn toxicity symptoms and a high level of Zn accumulation in roots and aerial parts compared with the uninoculated control. The 2 endophytes used separately or together significantly ( $P < 0.05$ ) enhanced plants fresh weight as compared to control plants.



**Figure 2:** Growth of different bacterial strains in TY liquid medium under Pb and Zn treatments Asterisks indicate significant differences compared to the control ( $p < 0.05$ ).



**Figure 3:** Impact of Zn treatment on the motility of strains estimated by measuring the halo diameter (mm). Asterisks indicate significant differences compared to the control ( $p < 0.05$ ).

Strain ID	Closest relatives	Zn concentration in cells (ppm)	
		Control	Zn (5mM)
LMR23	<i>Pseudomonas putida</i>	$0.28 \pm 0.14$	$8.07 \pm 2.16^*$
LMR27	<i>Pseudomonas putida</i>	$0.11 \pm 0.04$	$14.54 \pm 3.24^*$
LMR51	<i>Herbaspirillum huttiense</i>	$0.24 \pm 0.04$	$31.19 \pm 17.54^*$
LMR54	<i>Bacillus</i> sp.	$0.51 \pm 0.14$	$4.24 \pm 0.57^*$
LMR64	<i>Rhizobium galegae</i>	$0.51 \pm 0.23$	$58.51 \pm 9.92^*$
LMR79	<i>Rhizobium leguminosarum</i>	$0.26 \pm 0.07$	$2.2 \pm 1.12^*$
LMR80	<i>Serratia proteamaculans</i>	$0.42 \pm 0.34$	$11.05 \pm 5.65^*$

Results were expressed as means  $\pm$  Standard Deviation (SD) ( $n = 3$ ). Asterisks indicate significant differences compared to the control ( $p < 0.05$ ).

Strain	Ammonia production	Phosphate solubilization	
		Soluble phosphate (ppm)	pH
LMR23	+++	$90.91 \pm 7.95$	$4.4 \pm 0.21$
LMR27	+++	$54.16 \pm 4.75$	$4.5 \pm 0.2$
LMR51	+++	$55.95 \pm 2.98$	$4.7 \pm 0.41$
LMR54	++	$51.79 \pm 11.18$	$4.4 \pm 0.61$
LMR64	++	$27.25 \pm 5.49$	$4.5 \pm 0.1$
LMR79	++	$54.54 \pm 2.35$	$4.7 \pm 0.17$
LMR80	++	$64.12 \pm 2.71$	$5.09 \pm 0.27$

Results were expressed as means  $\pm$  Standard Deviation (SD) ( $n = 3$ ). (++) intermediate, (+++) strong production

Fresh weights were increased by 44% (LMR23), 41% (LMR51) and 43% (LMR23/51) for these shoots and 59% (LMR23), 52% (LMR51) and 57% (LMR23/51) for the roots. Bacteria belonging to *Pseudomonas* genus are known for their beneficial effects that may ameliorate heavy metals phytoextraction [52]. It has been also shown that *Pseudomonas fluorescens* strain MH15 was able to increase the accumulation of Zn, Cd and Cu in the tissues of

**Table 4:** Effect of inoculation of *Hedysarum spinosissimum* with 2 bacterial isolates on the plants fresh weight and Zn uptake

Added Zn in plant medium (μM)	Treatment	Plant fresh weight (mg)		Zn concentration (ppm)		
		Shoots	Roots	Shoots	Roots	Translocation factor (Tf)
2000	Inoculated with LMR23	47.96 ± 22.1	219.9 ± 62.06*	1093.76 ± 143.84*	19695 ± 3677.73*	0.05 ± 0.006
2000	Inoculated with LMR51	51.83 ± 3.8*	251.76 ± 32.15*	1860.54 ± 405.06*	7100.03 ± 2735.83*	0.27 ± 0.08*
2000	Inoculated with LMR23/51	49.93 ± 9.15*	228.5 ± 43.41*	699.58 ± 150.38	5136.51 ± 1807.66	0.15 ± 0.08
0	Uninoculated	110.36 ± 39.57	606.93 ± 154.78	41.06 ± 4.65	98.26 ± 16.03	0.42 ± 0.04
2000	Uninoculated	21.23 ± 5.22	129.8 ± 20.13	397.47 ± 82.36	3590.12 ± 541.37	0.11 ± 0.01

Results were expressed as means ± Standard Deviation (SD) of three independent experiments. Asterisks indicate significant differences compared to the control (uninoculated treatments) at  $p < 0.05$

*Sinapis alba*, which that is consistent with our results. Many rhizobacteria and endophytic bacteria, including *Pseudomonas*, associated with yellow lupine, are also known to promote the growth of this plant and to increase its resistance and extraction capacity of Cd [53]. However, in our study, the accumulation of Zn was higher at the root level compared to the aerial part thus reducing translocation factor. Zn maximum accumulation was observed when inoculating the *Pseudomonas putida* strain alone, remarkably in the root level, which indicates a very low Zn translocation to aboveground biomass ( $Tf = 0.05 \pm 0.006$ ), suggesting an important phytostabilisation potential. In a recent study, the inoculation of poplar roots with a *Pseudomonas fluorescens* strain resulted in improved Cd absorption in the roots, showing the interest to use such bacteria in a Cd phytostabilisation program [54]. The use of several bacterial strains presenting different beneficial features is also often described as a very effective strategy to improve plant growth and activity of phytoremediation [55].

## Conclusions

The metal contaminated soils harbor a diverse group of microorganisms [56] among which some are capable of enhancing the effectiveness of phytoremediation [8,57]. This study analyzed nodule bacteria population of the legume *Hedysarum spinosissimum* and describes the potential of different bacterial strains to promote plant growth and enhance Zn phytostabilization potential. Growing in heavy metals-contaminated soils, this plant hosted a high diversity of endophytic bacteria inside their root nodules. These endophytes, that belong to 8 different genera, are resistant to high concentrations of Pb and Zn, produced multiple PGP traits such as, phosphate solubilization or ammonia production with a great potential of Zn bioaccumulation. Inoculation of *Pseudomonas putida* LMR23 and *Herbaspirillum huttiense* LMR51 of *H. spinosissimum* seedlings, grown in a nutrient solution supplemented with the minimal inhibitory concentration of Zn enabled plant survival and growth. By accumulating Zn mainly in the roots, these associations may further be tested for their phytostabilisation potential. The bacteria identified here may thus be used as bio-

inoculants *in situ* and may constitute a biological alternative to improve phytostabilization efficiency in the contaminated sites from where they originate. However, the molecular mechanisms whereby these bacteria help the plant to tolerate metals remain to be elucidated [58].

## Conflict of Interest

The authors declare that they have no conflict of interest.

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