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# The rhizosphere of *Sulla spinosissima* growing in abandoned mining soils is a reservoir of heavy metals tolerant plant growth-promoting rhizobacteria

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## ABSTRACT

Heavy metals tolerant soil bacteria are known to play essential roles in biogeochemical cycles, biotransformation of metals, bioremediation processes and, plant adaptation. The objective of this study was to isolate and characterize the bacterial strains associated with *Sulla spinosissima*, a native legume species growing in three abandoned mining sites situated in the Oujda region (East Morocco). Globally, more than 370 bulk and rhizospheric soil bacteria were isolated. Their identification by 16S rDNA sequencing showed that dominant phyla were Firmicutes, Actinobacteria, and Proteobacteria, while at the genus level *Bacillus* dominated, followed by *Stenotrophomonas*, *Arthrobacter*, and *Rhodococcus*. All the soils contained strains possessing plant growth-promoting traits. The best-performing strains were LMR283 for auxin production (145  $\mu\text{g mL}^{-1}$ ), LMR291 for phosphate solubilization (67.6  $\text{mg L}^{-1}$ ), LMR280 for siderophore production (92.4%) and LMR326 for ACC deaminase activity (105  $\text{nmol } \alpha\text{-ketobutyrate mg}^{-1} \text{ h}^{-1}$ ). Among tolerant PGP bacteria, 17 isolates showed antagonistic activity against the pathogen *Fusarium oxysporum* and 26 produced lytic enzymes. It was relevant that the rhizospheric soils prospected compared to bulk soils contained more interesting isolates for all the studied properties, in particular for soils sampled from Oued El Heimer and Sidi Boubker sites (respectively 79 and 63% of performing strains). Data presented here indicate that *Sulla spinosissima* growing in heavy metal soils is associated with multifarious active bacterial populations that probably sustain plant tolerance and growth under the prevailing stressful conditions. Superior strains identified are good candidates to be used with selected plants in rehabilitation programs in the contaminated ecosystems.

## Keywords:

Abandoned mining site  
Cultivable bacteria  
*Sulla spinosissima* rhizosphere  
PGP traits  
Antifungal activity  
Heavy metal tolerance

## 1. Introduction

Environmental pollution is continuously increasing because of the acceleration of human activities and economic development. In particular, industrial production increases the number of foreign and toxic substances released in the environment. Many of these

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pollutants exist in contaminated soils, landfills, and industrial facilities, because of intensive industrialization, urbanization, transportation, and agricultural practices (Dhaliwal et al., 2020). Many microorganisms can eliminate organic biodegradable pollutants, but heavy metals (like lead and zinc) persist in the environment for long periods and accumulate inevitably. Moreover, they are being continuously added into the soils through various activities and they can migrate to surface water or groundwater or enter the food chain through plants, to be found in animals and possibly in human bodies (Hembrom et al., 2020). Heavy metals can have adverse effects on bacterial populations, which can affect negatively the functioning of the ecosystems (Pringault et al., 2016; Stefanowicz et al., 2020).

The elimination of these pollutants can be achieved by some physicochemical methods, like soil replacement, solidification/stabilization and vitrification or soil washing. They are very effective for certain types of environmental contaminants but have many disadvantages. Interventions are cumbersome and expensive and generally leave a barren soil (Ullah et al., 2015). Bioremediation is one of the techniques that received a lot of interest in recent years (Rahman and Singh, 2020). It consists of using biological processes that can remove industrial pollutants that disturb the biogeochemical cycles, and reduce the level of pollution in the air, water, or soil (Katiyar et al., 2020). Bioremediation uses microorganisms and/or plants that are normally working as biological systems. This approach is particularly efficient for the elimination of organic pollutants: biological pollution of air, wastewater, and areas contaminated with polycyclic hydrocarbons, chlorinated compounds or explosives (Giannakis et al., 2016).

Many plant species were proposed recently for the extraction of toxic chemicals from the soil (especially heavy metals), in a process known as phytoremediation (Padmavathiamma and Li, 2007; Muthusaravanan et al., 2020). The low cost associated with phytoremediation technology and the possibility of recycling certain metals explain the growing interest in its development. The majority of the plants used in phytoremediation can tolerate high concentrations of heavy metals, accumulate them, and produce a large amount of biomass (Awa and Hadibarata, 2020). The efficiency of metal phytoextraction may be enhanced by using some plant growth-promoting rhizobacteria (PGPR) that have beneficial effects on plants growth (Glick, 2003; Liu et al., 2014; Son et al., 2014; Manoj et al., 2020). Some bacteria produce plant growth regulators such as indole acetic acid (IAA), cytokinins, and gibberellins (Etesami and Adl, 2020), others can suppress ethylene production through the synthesis of 1-aminocyclopropane-1-carboxylic acid deaminase (ACC deaminase) (Manoj et al., 2020). Other known mechanisms include enhancing nutrient uptake, N<sub>2</sub> fixers, phosphate solubilizers, siderophore-producers, (Manoj et al., 2020), inhibition of phytopathogens growth by lytic enzymes production such as chitinases (Seo et al., 2014), proteases, cellulases and lipases (Sreevidya et al., 2016), and induction of plant defense system (Pandey et al., 2016; Manoj et al., 2020). Besides, various microbial mechanisms are thought to be responsible of trace metal bioavailability: production of organic acids; siderophores or biosurfactants (Venkatesh and Vedaraman, 2012; Pirog et al., 2013; Manoj et al., 2020); and metal dissolution by bacterial production of strong acids (Glick et al., 1998; Weyens et al., 2013). Some bacteria can also achieve changes in metal speciation, toxicity, and mobility, as well as mineral formation or mineral dissolution or deterioration (Rajkumar et al., 2010). All these processes can contribute to an enhancement in soil fertility that will improve the installation of a vegetation cover in the contaminated sites. On the other side the interaction between microorganisms and plants plays a key role in the development of microbial communities in metal-contaminated sites. It is well known that microorganisms can tolerate the extremely toxic conditions of some mine sites and can survive in harsh environments where only few plants can survive (Liu et al., 2020).

Investigating microbial population in a contaminated environment can help to better understand the interaction between microorganisms and plants, and will further clarify the complex mechanisms behind plant-assisted soil remediation technology. Therefore heavy metals tolerant PGP strains could be suitable candidates for improving the stability and absorption of heavy metals, and plants adaptability, at least to a certain extent. The accumulation of elements can be influenced by selecting the appropriate microbial community in the rhizosphere.

In this general context, we were interested in studying the bacterial populations associated with the roots of a native forage legume, *Sulla spinosissima* L. (former *Hedysraum spinosissimum*), that grows in mining sites located in the eastern part of Morocco (Oujda region). The genus *Sulla* is one of the most important native legumes in the Mediterranean Basin and the largest genus of the tribe Hedysareae, Fabaceae, with 180 recognized species (Lock, 2005; Xu and Choi, 2010; Amirahmadi et al., 2014). This plant is known for its good agronomical traits both in terms of forage and for soil nitrogen supply (Allen and Allen, 1981; Sprent, 2001). However, there is little knowledge about the diversity and structure of the rhizobacterial populations associated with *Sulla* plants. It will be particularly interesting to study their potential beneficial biological activities and their role in *Sulla* establishment in stressful environments.

In a recent study, Sbabou et al. (2016) isolated diverse bacteria from root nodules of *Sulla spinosissima* growing in three Oujda mining site, where tons of waste, most of them abandoned in the open-air are accumulated after several decades of mine exploitation. Interestingly some of these bacteria were tolerant to Pb and Zn and possessed interesting *in vitro* PGP activities. They also had a positive effect on the tolerance and growth of *S. spinosissima* under toxic Zn concentrations. It was then interesting to study in more detail the population of rhizobacteria associated with this legume species that is naturally expanding in the Oujda mining soils, in order to access to the overall rhizobacterial diversity in terms of heavy metals tolerance and phytobeneficial properties. On the other side, it's interesting to study whether the presence of an adapted plant species such as *Sulla spinosissima* and or the soil properties, particularly the nature and extent of heavy metal soil pollution which related to the mining history of the site, may influence the bacterial populations recovered and their characteristics of these bacterial populations.

The aim of the present study is therefore to isolate, identify and characterize the bacterial strains associated or not to *Sulla spinosissima*, the naturally adapted legume mostly found in the heavy metals contaminated soils located in three different sites in the Oujda region. In the medium term, we hope that this research will provide a scientific basis for selecting some bacterial strains for individual or consortium inoculants to be used in soil phytoremediation and/or soil phytostabilization programs in these abandoned mining sites.

## 2. Materials and methods

### 2.1. Characteristics of the studied area (Oujda)

The three studied sites are located in the region of Oujda (East Morocco), expanding over a mountainous strip of 100 km in length and 20–30 km in width. The region has an important mining potential due to the existence of large deposits of minerals (Smouni et al., 2010). Sidi Boubker and Touissit mining sites are located about 30 km south of Oujda city; they were closed respectively in 1975 and 2002 after 40 years and 75 years exploitation. The source of pollution in this area is mainly the result of waste washing sand dams extending over large areas (Argane et al., 2014). The foundry of Oued El Heimer is specialized in processing, smelting, and refining of lead and silver (Stowasser, 1973). The area of Oued El Heimer is exposed to different pollution forms such as SO<sub>2</sub> gas in the air, fine dust (especially areas under prevailing winds), and slags transported in particulate or dissolved form (Smouni et al., 2010).

The climate in the Oujda region is characterized by low precipitations with a fairly rainy winter and a hot and dry summer of variable duration. It is considered a region with a cold semi-arid climate (Koppen climate classification BSk). Rainfall is between 300 mm and 500 mm per year, while average temperatures are ranged between 26.1 °C in August (the warmest month of the year) and 8.4 °C in the coldest month (January) (Climat Oujda, Maroc). Precipitations and winds are effective means of disseminating heavy metals. There are two types of winds in the region: West to Northwest which is wet and rainy, and East wind that is hot and dry.

### 2.2. Soil sample collections and chemical analysis

Six soil samples were collected from the three different sites (Touissit, Sidi Boubker, and Oued El Heimer), with three samples analyzed from each site. The type of soil (rhizospheric or bulk) and GPS coordinates of the soil samples in each site are presented in Table 1. Soil samples pH was measured according to Kalra (1995), while their chemical composition was determined by atomic absorption spectrophotometry, performed by a Eurofins-France laboratory, under ISO/IEC 17025:2005 norm and accreditation by the COFRAC 1–148.

### 2.3. Isolation of bacteria from *Sulla spinosissima* rhizosphere and surrounding bulk soils

Soil samples of each sampling point were mixed and 4 g of the mixture were suspended in 16 ml sterile physiological water (0.9% of NaCl) and shaken for 30 min. Soil suspensions were diluted in 4-fold series and plated on Nutrient Broth (NB) agar medium supplemented with either, 1 mM or 5 mM of Pb (as Pb(NO<sub>3</sub>)<sub>2</sub>), or with 1 mM or 3 mM of Zn (as Zn(SO<sub>4</sub>)<sub>2</sub>·7H<sub>2</sub>O) (Becerra-Castro et al., 2012).

Soil suspensions were also plated on modified Pikovskaya (PVK) medium (Sundara Rao and Sinha, 1963), Modi medium (Modi et al., 1985) and YEM medium (Vincent, 1970) supplemented with 0.5 mg ml<sup>-1</sup> of tryptophan, to promote the growth of bacteria possessing benefit activities (respectively phosphate solubilization, siderophore production, and auxin biosynthesis). Modified PVK medium used contained 0.5 g natural phosphate instead of tricalcium phosphate. All the plates were incubated at 28 °C for at least 48 h. The bacterial colonies were sub-cultured at least three times to ensure purity and then preserved at –80 °C in culture media supplemented with 40% glycerol (v/v).

### 2.4. Molecular identification of the isolates

#### 2.4.1. Extraction of bacterial total DNA

Fresh bacterial suspensions of each strain were transferred to 1.5 ml-sterile tubes and centrifuged at 13000 g for 15 min. The resulting pellets were dissolved in 1 ml buffer (20% SDS, 2 M sodium acetate, 3 M Tris-acetate, 20 mM EDTA) and transferred to 1.5 ml-sterile tubes, then treated with NaCl and incubated for 10 min at 65 °C. After that, each suspension was extracted with 1.2 ml phenol-chloroform-isoamyl alcohol (25:24:1) followed by extraction of the aqueous phase with 1.2 ml chloroform-isoamyl alcohol (24:1). DNA was precipitated by addition of 0.6 ml isopropanol to the aqueous phase, incubation for 10 min at room temperature and centrifugation at 12 000 g for 10 min. The pellet was washed with 70% ethanol, air-dried, and dissolved in TE (Tris-EDTA, pH 8.0) buffer.

#### 2.4.2. PCR amplification of 16S rRNA genes and sequencing

For the specific amplification of bacterial 16S rRNA genes, we used the pair of primers 27f (5-AGAGTTTGATCCTGGCTCAG-3) and FGPS1509 (5-AAGGAGGGGATCCAGCCGA-3). Each 25 µl of the PCR mixture contained the DNA template (20–50 ng), 1 × Taq reaction buffer, 25 pmol of each primer and 1.5 U of Taq DNA polymerase (Bioline). PCR amplifications were performed using the following temperature cycling program: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C (1 min), 55 °C (30 s) and 72 °C (1 min), followed by a final extension at 72 °C (10 min). The PCR products were separated in 1% agarose gels by electrophoresis and sent

**Table 1**

Type and location of the soil samples studied.

Sites	Sampling points	Type of soil	GPS coordinates
TOUISSIT	Touissit N (TN)	Bulk soil	34°28'06"N/1°46'18"W
	Touissit 5 (T5)	Rhizosphere of <i>Sulla spinosissima</i>	
SIDI BOUBKER	Sidi Boubker 1 (B1)	Bulk soil	34°28'18"N/1°42'59"W 34°28'18"N/1°42'59"W
	Sidi Boubker 3 (B3)	Rhizosphere of <i>Sulla spinosissima</i>	
OUED EL HEIMER	Oued El Heimer 2 (OH2)	Rhizosphere of <i>Sulla spinosissima</i>	34°26'37"N/1°53'54"W
	Oued El Heimer 36 (OH36)	Bulk soil	

for sequencing (Genoscreen, France). Nucleotide sequences obtained in this study have been deposited in the NCBI database under accession numbers KU182753 to KU182861.

#### 2.4.3. Phylogenetic analysis

Sequences of the 16S rDNA were matched against nucleotide sequences present in NCBI GenBank using the BLAST tool to obtain the nearest phylogenetic neighbors (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences showing more than 97% similarity were considered to belong to the same operational taxonomic unit (OTU). The sequences were aligned using ClustalW Algorithm (Thompson et al., 1994) and Neighbor-Joining phylogenetic trees were constructed after calculation of a distance matrix with the software MEGA X (Tamura et al., 2007). The robustness of the phylogeny was evaluated by bootstrap analysis with 1000 replicates.

#### 2.5. Bacterial heavy metal tolerance

To estimate the levels of strains resistance to heavy metals, the minimal inhibitory concentration (MIC) of zinc and lead of the bacterial collection was determined. The isolates were plated in triplicate on NB plates supplemented with increasing amounts of the metals (5–35 mM of Zn and Pb). The MIC was defined as the lowest metal concentration at which bacterial growth was not observed (Becerra-Castro et al., 2011).

#### 2.6. Phytobeneficial activities of the isolates

##### 2.6.1. Plant growth-promoting traits

All the bacteria previously isolated on different nutritive media were screened for their PGP traits by using appropriate qualitative tests. Their capacity to produce auxin was determined according to the methods of Gordon and Weber (1951). Droplets of each bacterial suspension were dispersed on the YEM agar medium supplemented with 0.5 mg ml<sup>-1</sup> of tryptophan and incubated for 48 h at 28 °C. The colonies obtained were covered with a sterile filter paper disk impregnated with Salkowsky's reagent. Petri dishes were incubated in the dark for 30 min. The appearance of pink color indicates the production of auxin.

Siderophore production was studied by using a modification of the method of Schwyn and Neilands (1987). Strains were plated in triplicate on Modi plates and incubated for 2 days at 28 °C, after what chrome azurol-S (CAS) reagent was added in the plates. Orange zones around the colonies on blue agar were considered as positive reactions for siderophore production.

The dissolving phosphate ability of the isolated bacteria was tested according to the method described by Nautiyal (1999). The isolated microorganisms were plated on modified PVK solid media. After seven incubation days at 28 °C, colonies surrounded by a halo were considered positive for phosphate solubilization.

ACC deaminase activity of the isolates was studied according to the method of Penrose and Glick (2003) and Onofre-Lemus et al. (2009) by using ACC- minimal media plates containing 3 mM of ACC as a sole nitrogen source. Bacterial colonies growing in these plates were considered as containing ACC deaminase activity.

In a second step, suitable quantitative methods were used to characterize more precisely the best strains recorded for each PGP trait.

**Auxin production:** It was measured according to the methods of Gordon and Weber (1951) and Sheng et al. (2008). Amounts of auxin produced were determined for the best strains only. Bacterial cells were grown in a YEM medium amended with 0.5 mg mL<sup>-1</sup> of L-tryptophan at 28 °C for 1 day in a shaking incubator at 180 rpm. YEM medium inoculated with sterile Milli-Q water served as the control treatment. Cultures were centrifuged at 12 000 g for 5 mn and supernatants filtrated through a Millipore filter. One mL aliquot of each supernatant was mixed with 1 mL of Salkowsky's reagent. The mixture was incubated for 30 min at 28 °C in the dark. Absorbance was measured at 540 nm and the concentration of auxin was determined using a standard curve prepared with known auxin concentrations.

**Siderophore production:** The estimation of siderophore production was performed according to the chrome azurol-S (CAS) analytical method (Schwyn and Neilands, 1987; Manjanatha et al., 1992). After growing bacterial strains on Modi liquid medium and centrifugation, one ml of supernatant was transferred into a tube and mixed with 1.0 mL of CAS reagent and allowed to stand for 1 h (Manjanatha et al., 1992). A reference was prepared by mixing 1.0 mL of the CAS reagent with 1.0 mL of the non-inoculated medium used for culturing bacterial strains. The absorbance of the samples was measured at 630 nm and compared to the control (Manjanatha et al., 1992).

Siderophore content in the sample was calculated using the following formula:

$$\text{Siderophore units (\%)} = (a-A)/A * 100$$

where A = absorbance of CAS reagent and a = absorbance of the sample at 630 nm (Tank et al., 2012).

**Natural phosphate solubilization:** The amount of phosphate solubilized by each strain was determined in Pikovskays's liquid medium (Zaidi et al., 2006) containing 0.5 g L<sup>-1</sup> of natural phosphate as the inorganic phosphate source. Triplicate 250 mL Erlenmeyer flasks containing 100 mL of medium were inoculated with the bacterial suspension to reach an initial OD<sub>600</sub> of 0.05. Flasks were incubated at 28 °C for 2 days in a rotary shaker at 180 rpm. Culture supernatants were collected by centrifugation at 12 000 rpm for 10 min and free soluble phosphate estimated by the vanadate-molybdate method (Watanabe and Olsen, 1965).

**ACC deaminase activity:** ACC-deaminase activity was measured by using a slight modification of the ACC-deaminase activity assay from Penrose and Glick (2003) and Li, et al. (2011). The ACC-deaminase activity was determined by the colorimetric measurement of the amount  $\alpha$ -ketobutyrate resulting from ACC degradation by the enzyme ACC deaminase. The unique modification consisted of using a minimal medium for growing bacterial cells instead of the DF salt medium. The minimal medium used contained: Mannitol (3.64 g), NaCl (0.05 g), K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O (0.35 g), MgSO<sub>4</sub> 6H<sub>2</sub>O (0.2 g), CaCl<sub>2</sub> 2H<sub>2</sub>O (0.05 g), FeCl<sub>2</sub> (2.7%) and 10 ml of a

microelements solution containing H<sub>3</sub>BO<sub>3</sub> (500 mg), MnCl<sub>2</sub> 4H<sub>2</sub>O (7.86 mg), NaMoO<sub>4</sub> 2H<sub>2</sub>O (27.62 mg), ZnSO<sub>4</sub> 7H<sub>2</sub>O (17.17 mg), CuSO<sub>4</sub>, (20.4 g). After adjusting the volume to 800 ml, the medium was autoclaved, then 1 ml biotin, 1 ml thiamine, and 1 ml pantothenic acid were added, and finally, the volume completed with sterilized distilled water to 1 L.

### 2.6.2. Antifungal activities

**Antagonistic activity:** was studied by using three *in vitro* confrontation methods between bacterial strains and a phytopathogenic fungus strain of *Fusarium oxysporum*.

Method 1 consisted of a direct confrontation, in which the fungus and the bacteria are cultivated side by side in Potatoes Dextrose Agar medium (PDA). The bacterial strains were first grown in glass tubes containing NB medium for 24 h at 28 °C followed by depositing 5 µl of the bacterial suspension in each PDA plate containing a 1 cm<sup>2</sup> fragment of thallus deposited at opposite on the edge of the box. In the control plate, a drop of sterilized distilled water was used instead of the bacteria.

Method 2 was designed to test if the bacterium inhibits the pathogen by secreting diffusible substances. Suspensions of bacterial strains prepared as before were placed on sterile nitrocellulose membranes (Millipore type, 0.45 µm) that were previously deposited on the surface of the PDA medium. After absorption of the drop, the dishes were incubated at 28 °C for 24 h. The membrane was removed from each plate and a 1 cm<sup>2</sup> fragment of the thallus placed on the agar surface. Plates were re-incubated for an additional period of one week (Lopes et al., 2015). The control dish was prepared in the same way but by depositing sterilized distilled water instead of the bacterial suspension.

In Method 3 we examined if the antagonistic effect of bacterial strains against the pathogen is due to volatile compounds emitted by bacteria. Bacterial suspensions prepared as before were striated in a Petri dish containing NB medium. A *Fusarium* thallus fragment of 1 cm<sup>2</sup> was placed in the center of a second plate containing PDA medium, then it was closed by the dish containing the bacteria. The 2 dishes were saddled with parafilm and incubated at 28 °C for one week (Lopes et al., 2015). The box serving as control was produced in the same way except that the bacteria were absent.

After incubation, the inhibition of the radial growth of *Fusarium oxysporum* thallus was evaluated by the following formula:

$$I = T-C / T * 100$$

T = Diameter of mycelium in the absence of bacteria

C = Diameter of the mycelium in the presence of the bacteria

**Protease production:** Bacterial isolates were spot inoculated on skim milk agar. Plates were incubated at 28 ± 1 °C for 96 h. Isolates showing clear zones around colonies were considered as protease producers (Ventosa et al., 1982; Smibert and Krieg, 1994). To

**Table 2**  
Chemical composition of soil samples from the sites studied.

Sites	Touissit	Sidi Boubker	Oued El Heimer
Pb mg kg <sup>-1</sup>	13 631	1412.5	2967
(SD)	(±10164.8)	(±311)	(±2193.4)
Zn mg kg <sup>-1</sup>	3178.5	8246	230
(SD)	(±33.2)	(±4459.4)	(±265.9)
Cu mg kg <sup>-1</sup>	479.7	72.9	40
(SD)	(±394.8)	(±19.9)	(±8.4)
Cr mg kg <sup>-1</sup>	17	4.65	32
(SD)	(±13.7)	(±7.3)	(±8.4)
As mg kg <sup>-1</sup>	74	9.86	42
(SD)	(±30.1)	(±1.3)	(±15.4)
N total	0.06	0.02	0.005
(SD)	(±0.02)	(±0.01)	(±0.007)
C total	6.7	12.1	1.9
(SD)	(±0.5)	(±0.1)	(±2.5)
P available	22.3	3.6	10
(SD)	(±15.4)	(±2.08)	(±2)
Ca meq%	19.3	2.9	30.9
(SD)	(±1.4)	(±0.2)	(±24.5)
Mg meq%	3.5	1.3	0.9
(SD)	(±0.3)	(±0.4)	(±0.2)
Na meq%	0.15	0.02	0.07
(SD)	(±0.2)	(±0.02)	(±0.03)
K meq%	0.3	0.02	0.4
(SD)	(±0.09)	(±0.01)	(±0.5)
CEC meq%	8.8	0.05	5.2
(SD)	(±4.4)	(±0.04)	(±7.6)
pH	8.5	8.5	8.8
(SD)	(±0.4)	(±0.5)	(±0.2)

**Normal values (NV)** (mg kg<sup>-1</sup>): Pb (35), Zn (90), Cu (30), Cr (70), As (0.35) (Bowen, 1979).

**Threshold values (TV)** (µg/g): Pb (300), Zn (300), Cu (140), Cr (70), As (20) (European Union, 2002; WHO/FAO, 2007).

\*SD: Standard Deviation.

\*meq%: milli-equivalents per 100 g of soil.

confirm the results, positive strains were streaked on casein hydrolyzed medium and the plates were incubated at 28 °C for 24 h. The formation of a clear zone around the bacterial colonies indicated the proteolytic activity of the strain (Smibert and Krieg, 1994).

**Lipase production:** The minimal medium used contained: 0.3 g KH<sub>2</sub>PO<sub>4</sub>, 0.6 g Na<sub>2</sub>HPO<sub>4</sub>, 0.05 g NaCl, 0.1 g NH<sub>4</sub>Cl, 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 2 g agar, 2.5 g olive oil, and 100 mL distilled water. The medium was adjusted to pH 8 and autoclaved at 121 °C for 15 min. After inoculation by the isolates, plates were incubated at 28 °C for 48 h. A positive reaction was marked by the appearance of a halo around the colonies (Gupta et al., 2007; Salgado et al., 2014; Smibert and Krieg, 1994). The lipolytic activity of strains was confirmed by tributyrin and tween 20 agar plate methods (Kumar et al., 2012).

**Cellulase production:** Was determined according to the method described by Cattelan et al. (1999). M9 medium (Miller, 1972) supplemented with 10 g of carboxyl methylcellulose and 1.2 g of yeast extract was used to test the hydrolytic power of the strains. Isolates were plated and incubated for 8 days at 28 °C. The development of a clear halo around the colonies indicates a positive reaction (Kumar et al., 2004).

**Chitinase production:** Chitin agar plates containing 1% shrimp shell powder (SSP), 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, and 1.5% agar amended with 2% phenol red, were prepared. Plates were inoculated with aliquots of bacterial suspensions and incubated at 30 °C for 7 days. Clear zones around inoculants' spots indicate the presence of chitinase (Goswami et al., 2014; Wang et al., 2008).

## 2.7. Statistical analysis

Statistical analyses of the results were realized by R software, using the ade4 packages. Principal component analysis (PCA) was performed to explain the relationship between sites, environmental variables, and chemical composition and explore variations between them. One-way ANOVA was performed for each variable with XL STAT. Means with different letters are significantly different from each other ( $P < 0.05$ ) according to the Duncan test.

## 3. Results

### 3.1. Heavy metal contamination of the soils

Compared to normal heavy metal values in soils (Bowen, 1979) and known reported threshold values (European Union, 2002; WHO/FAO, 2007), Oujda mining soil samples analyzed by atomic absorption spectrophotometry are in general extremely polluted by heavy metals, depending on the metal measured and the location (Table 2). The three sites are highly polluted by the five heavy metals analyzed (As, Cu, Zn, Cd, and Pb).

All the soil samples analyzed are alkaline and showed low levels of carbon (C), nitrogen (N), and phosphorus (P), consequently their indexes of quality and fertility are low (Table 2). The cation exchange capacity (CEC) of the soils was also low, and therefore they can develop magnesium, potassium, and other deficiencies (Table 2).

A deep analysis of the results using the principal component analysis (PCA) showed that all analyzed elements are well correlated with each other except for total carbon (C) and (Zn). PCA revealed that soil samples from the Touissit site, which is an old polymetallic mine exploitation, and Oued El Heimer are grouped in the positive side of DIM1 closer to all variables except carbon and Zinc (Fig. 1).

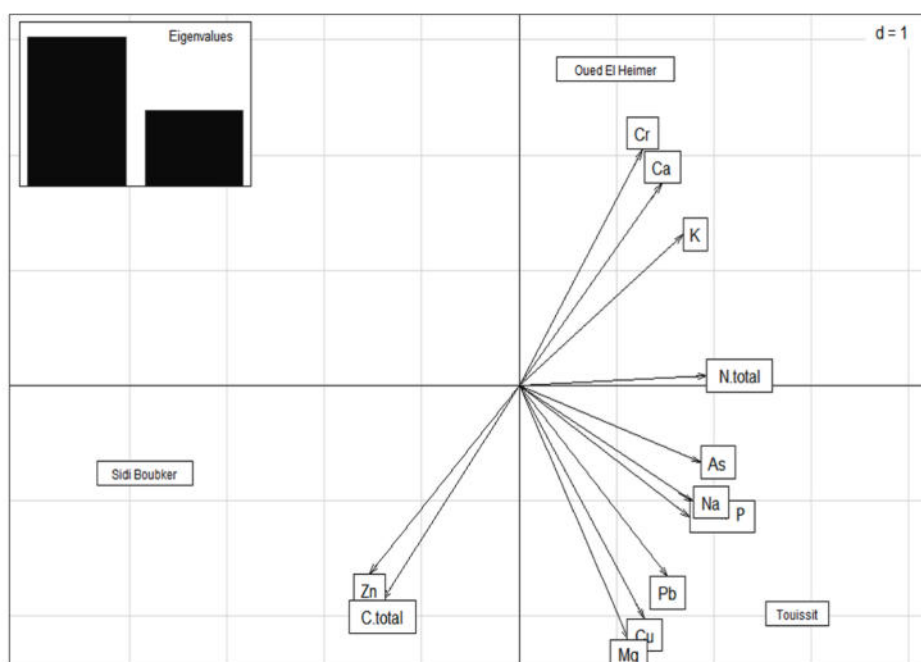


Fig. 1. Principal Component Analysis (PCA) of the chemical composition of soil samples (Touissit, Sidi Boubker, Oued El Heimer).

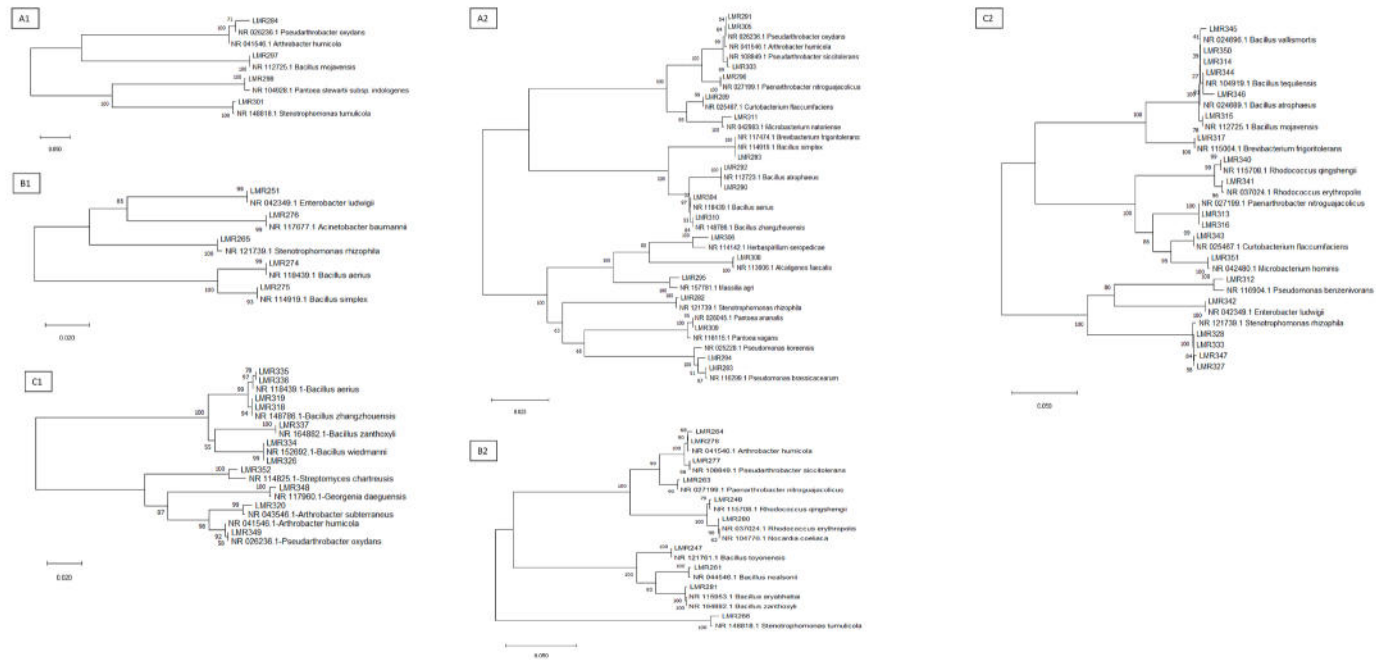


Fig. 2. Neighbor-joining phylogenetic trees of partial 16S rDNA sequences of representative isolates affiliated to the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes*. A: strains from Oued El Heimer, B: strains from Sidi Boubker, C: strains from Touissit, with A1, B1 C1: Bulk soils and A2, B2, C2: *Silla* rhizosphere soils.



These sites present the highest levels of heavy metals content. Soil samples from Sidi Boubker site, are grouped in the negative side of the two DIMs. PCA analysis characterized and classified the sites in terms of pollution with toxic heavy metals.

### 3.2. Genotypic characterization of the bacterial collection

After colonies purification, all morphologically different bacteria were identified by 16S rDNA sequencing and blasting (Weyens et al., 2009). The different bacterial species identified in the three soils are presented in three phylogenetic trees (Fig. 2). The analysis showed that bacterial sequences are related to at least 34 different known species and 18 genera that are associated with three bacterial phyla: Firmicutes, Actinobacteria, and Proteobacteria (Fig. 2). Considering all the soils in the three analyzed mining sites, firmicutes is

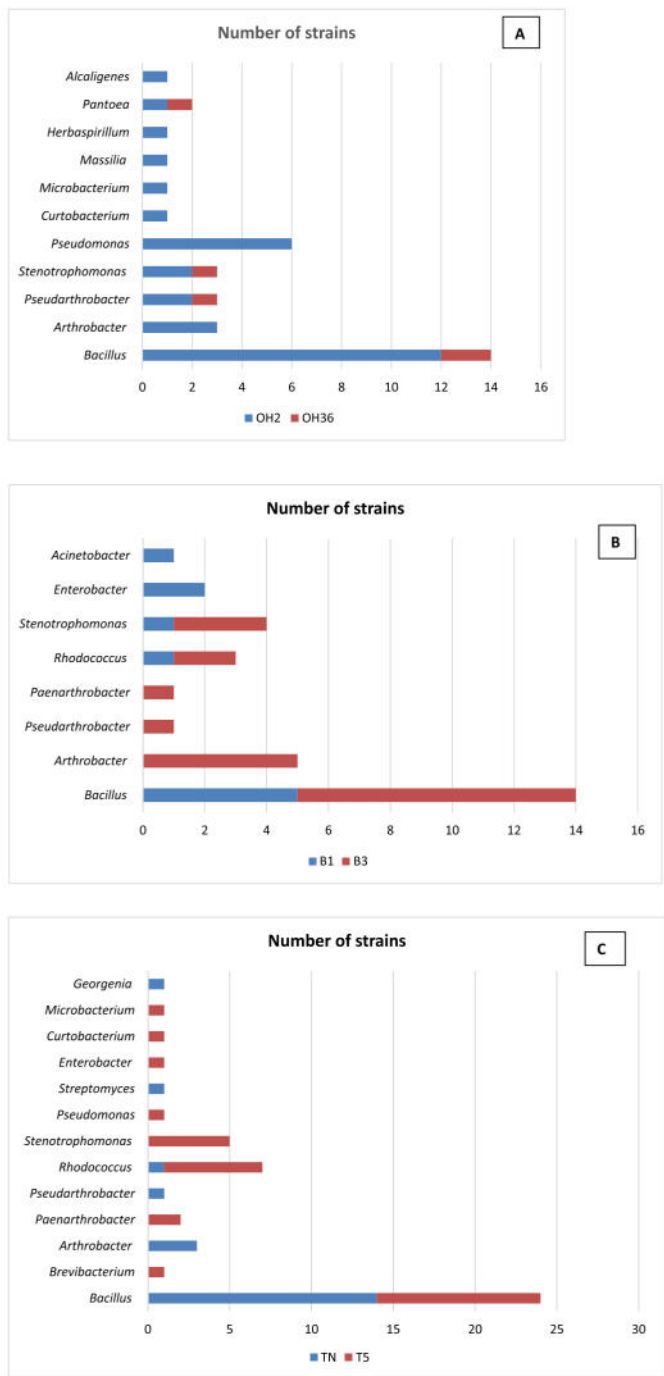


Fig. 3. Distribution of isolated strains at the genus level in the different studied sites (A: Oued El Heimer (OH), B: Sidi Boubker (B), C: Touissit (T)).

the most abundant bacterial phylum with 45.7% of total isolates (Supplementary Fig. 1), followed by Actinobacteria and Proteobacteria (respectively 30.2% and 24.1%). Strains identified in the proteobacteria phylum belong to 2 clades: Gammaproteobacteria and Betaproteobacteria. The latter was the less represented in all soils studied except for Oued El Heimer site.

The relative abundance of each genus in bulk and rhizospheric soils of each site is presented in Fig. 3. At this level, *Bacillus* was the most abundant with 49% of the total bacteria isolated from rhizospheric and bulk soils of Touissit. This genus was also clearly dominating in the bulk (B1) and rhizospheric (B3) soils in Sidi Boubker site. Genera of the group Arthrobacter (*Arthrobacter*, *Pseudarthrobacter*, and *Paenarthrobacter*) were also well represented in the *Sulla* rhizosphere (7 strains).

In the third prospected mining site (Oued El Heimer) *Sulla*-associated isolates belong mostly to the genus *Bacillus* sp. (12 strains), followed by 6 *Pseudomonas* strains. Contrasting with this, the bulk soil of the same site (OH36) led to the isolation of a reduced number of bacteria with only 2 *Bacillus* strains in addition to *Pseudarthrobacter*, *Stenotrophomonas* and *Pantoea* (one strain each).

### 3.3. Bacterial heavy metal tolerance

The number of Pb and Zn tolerant isolates among the 370 bacterial isolates of the collection was determined for each soil. Surprisingly, no Zn-resistant isolates were obtained from Oued El Heimer bulk soil (OH36), while only a small proportion of Pb-resistant isolates was recorded. This is contrasting with the high amounts of heavy metals measured in this soil (Table 2). However metal tolerant bacteria were isolated from the rhizospheric soil sample of *Sulla spinosissima* growing in this soil (OH2) with 18% for Pb tolerance, and 2.5% for Zn tolerance.

In Touissit site, the percentages of metal tolerant isolates obtained were generally lower than those recorded for Oued El Heimer soils. The majority of bacteria isolated from the rhizosphere of *Sulla* (T3) and bulk soil (TN) were weakly tolerant of Pb and Zn.

In the third soil-sampling site, Sidi Boubker, Zn-tolerant strains were a minority in both bulk (B1) and rhizospheric soils (B3). Interestingly 31% of the bacteria isolated from the rhizospheric soil were lead tolerant, while in the bulk soil Pb-tolerant bacteria percentage was lower (Fig. 4).

The level of heavy metals tolerance was assessed more precisely by determining the minimal inhibiting concentration (MIC) of each heavy metal studied for the 116 best strains. Results obtained showed that the most tolerant strains to lead and zinc belong to the genera *Rhodococcus*, *Stenotrophomonas*, *Brevibacterium*, and *Pseudomonas* with MIC values of 20–25 mM for Pb and 35 mM for Zn (Table 3).

### 3.4. Phytobeneficial activities of the isolates

#### 3.4.1. Bacterial PGP traits

Qualitative characterization of all isolates obtained showed that both bulk and rhizospheric soils contained isolates that possess plant growth-promoting traits. Their numbers differed depending on the soil origin (rhizospheric or bulk) and the site studied (Fig. 4).

Tests with Pikovskaya medium showed that 17.5% of the bacteria isolated from the rhizosphere of *Sulla* growing in Touissit soils (T5) were capable to solubilize rock phosphate. In the bulk soil (TN) of the same site, 12–14% of the isolates obtained were able to produce auxin or siderophores, or solubilize rock phosphate.

In Sidi Boubker site, the bulk soil (B1) contained lower percentages of strains showing PGP traits compared to those registered in the bulk soil of Touissit. However, in the rhizospheric soil (B3) percentages of siderophores producers and phosphate solubilizers were

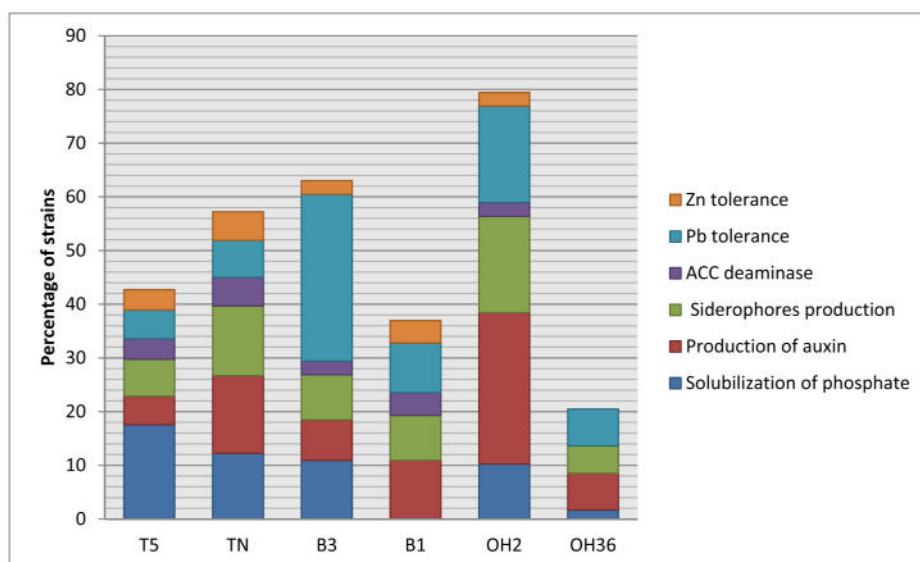


Fig. 4. Percentage of strains presenting phyto-beneficial activities (auxin production, phosphate solubilization, siderophore production, ACC deaminase activity), and tolerance to Pb and Zn. Oued El Heimer (OH), Sidi Boubker (B), Touissit (T), with T5, B3 and OH2 corresponding to rhizospheric soil samples of *Sulla spinosissima*, while TN, B1, and OH36 are bulk soil samples.

**Table 3**Minimal inhibiting concentrations of Pb and Zn for the best metal-resistant strains isolated from bulk soil and rhizosphere of *Sulla spinosissima*.

Code	Bacteria (Accession number)	Sites	Origin	Pb (mM)	Zn (mM)
LMR248	<i>Rhodococcus qingshengii</i> (KU182754)	B	Rhizosphere	25	35
LMR265	<i>Stenotrophomonas rhizophila</i> (KU182771)	B	Bulk soil	25	35
LMR266	<i>Stenotrophomonas tumulicola</i> (KU182772)	B	Rhizosphere	20	0
LMR280	<i>Rhodococcus erythropolis</i> (KU182786)	B	Rhizosphere	20	20
LMR282	<i>Stenotrophomonas rhizophila</i> (KU182788)	OH	Rhizosphere	20	10
LMR283	<i>Pseudomonas brassicacearum</i> (KU182789)	OH	Rhizosphere	10	5
LMR291	<i>Pseudarthrobacter oxydans</i> (KU182797)	OH	Rhizosphere	15	3
LMR293	<i>Bacillus simplex</i> (KU182799)	OH	Rhizosphere	5	3
LMR308	<i>Alcaligenes faecalis</i> (KU182814)	OH	Rhizosphere	15	3
LMR310	<i>Bacillus zhangzhouensis</i> (KU182816)	OH	Rhizosphere	15	10
LMR312	<i>Pseudomonas benzenivorans</i> (KU182818)	T	Rhizosphere	25	35
LMR317	<i>Brevibacterium frigoritolerans</i> (KU182823)	T	Rhizosphere	25	35
LMR326	<i>Bacillus wiedmannii</i> (KU182832)	T	Bulk soil	20	15
LMR327	<i>Stenotrophomonas rhizophila</i> (KU182833)	T	Rhizosphere	25	35
LMR333	<i>Stenotrophomonas rhizophila</i> (KU182839)	T	Rhizosphere	25	35
LMR340	<i>Rhodococcus qingshengii</i> (KU182846)	T	Rhizosphere	25	30
LMR341	<i>Rhodococcus erythropolis</i> (KU182847)	T	Rhizosphere	20	35

(B): Sidi Boubker, (T): Touissit, (OH): Oued El Heimer.

higher (8.4% and 11% respectively).

Concerning Oued El Heimer site, the *Sulla* rhizosphere (OH2) contained relatively higher percentages of PGP bacteria, with 28.2% of auxin producing strains, 18% of siderophores producers, 10.2% of rock phosphate solubilizers and a very low percentage of strains presenting ACC deaminase activity. At the opposite the bulk soil (OH36) contained a very small percentage of bacteria presenting the PGP activities studied, not exceeding 6%.

Generally, the obtained results showed that the number of bacteria producing auxin and siderophores or solubilizing rock phosphate was higher in the rhizosphere of *Sulla* plants than in bulk soil.

Quantification of the PGP activities for the best isolates showed that the most efficient strains able to solubilize rock phosphate were *Pseudarthrobacter oxydans* strain LMR291, *Pseudomonas benzenivorans* strain LMR312 and *Stenotrophomonas rhizophila* strain LMR282 with respectively 67.6 mg L<sup>-1</sup>, 50.7 mg L<sup>-1</sup> and 48.5 mg L<sup>-1</sup> of soluble P produced in modified PVK liquid medium (Table 4). For auxin production, twelve isolates were highly performant, mostly isolated from rhizospheric soil samples. They belong to different genera: *Bacillus*, *Stenotrophomonas*, *Enterobacter*, *Rhodococcus*, *Pantoea*, *Alcaligenes*, *Pseudomonas*, and *Curtobacterium*. The highest *in vitro* auxin producer was *Pseudomonas brassicacearum* strain LMR283 (144.9 µg mL<sup>-1</sup>), followed by *Alcaligenes faecalis* strain LMR308 (140.8 µg mL<sup>-1</sup>) and the lowest was *Bacillus wiedmannii* strain LMR326 with 2.8 µg mL<sup>-1</sup> (Table 4).

Among the bacteria tested, eight strains had high percentage values of siderophores production led by *Rhodococcus erythropolis* LMR280 and *Bacillus zhangzhouensis* LMR310 with respectively 92.41% and 92.1% (Table 4). Interesting results were also obtained for ACC deaminase activity measurements. Strains LMR326, LMR310, and LMR293 had the highest ACC deamination values (105, 104, and 93 nmol α-ketobutyrate mg<sup>-1</sup> h<sup>-1</sup> respectively) (Table 4).

### 3.4.2. Antifungal activities

The most performing PGP bacteria were also tested for their antagonistic activity against the phytopathogen fungus *Fusarium oxysporum* by using direct and indirect confrontation techniques. Only six strains exceeded 50% inhibition of fungal growth when using the direct confrontation method (Supplementary Table 1). The best results were obtained with strains LMR294 (*Pseudomonas brassicacearum*), LMR333 (*Stenotrophomonas rhizophila*) and LMR344 (*Bacillus tequilensis*) with respectively 81.4%; 90% and 88.3% of inhibition. Most bacteria tested were able to reduce the growth of *Fusarium oxysporum* at different levels by producing diffusible substances. Highly active strains were LMR292 (*Bacillus atrophaeus*), LMR294 (*Pseudomonas brassicacearum*), LMR303 (*Pseudarthrobacter oxydans*), LMR341 (*Rhodococcus erythropolis*), and LMR344 (*Bacillus tequilensis*).

The study of fungal inhibition by bacterial volatile substances showed 13 effective bacteria, with 2 strains displaying more than 80% inhibition of fungal growth. Best performing strains were LMR340 (*Rhodococcus qingshengii*), LMR293 (*Bacillus simplex*) LMR303 (*Pseudarthrobacter oxydans*) and LMR344 (*Bacillus tequilensis*).

The majority of strains studied were able to produce lytic enzymes, in particular chitinase and to a lesser extent cellulase, lipase, and protease. Results indicated also that only one strain was able to produce four enzymes (*Stenotrophomonas tumulicola* strain LMR266), 5 strains were able to produce three enzymes (14% of the strains tested), while 41% and 14% showed production of respectively two/one lytic enzymes, and 28% of the strains didn't produce any lytic enzyme. The best performing strains belong to the genera *Stenotrophomonas*, *Bacillus*, *Rhodococcus*, and *Microbacterium*, and most of them were isolated from *Sulla* rhizosphere (Supplementary Table 2).

## 4. Discussion

In the present study, we investigated the bacterial populations in three different abandoned mining sites in the region of Oujda (Touissit, Sidi Boubker, Oued El Heimer), especially their heavy metal tolerance and their PGP activities as influenced by the presence or not of *Sulla spinosissima* plants. Soil analysis revealed high levels of contamination by different heavy metals (As, Cu, Zn, Cd, and Pb)

**Table 4**PGP activities measurement for most interesting strains isolated from bulk soil and rhizosphere of *Sulla spinosissima*.

Codes	Bacteria (Accession number)	Sites	Origin	Phosphorus (mg L <sup>-1</sup> )	Siderophore (%)	Auxin (µg mL <sup>-1</sup> )	α-ketobutyrate (nmol mg <sup>-1</sup> h <sup>-1</sup> )
LMR248	<i>Rhodococcus qingshengii</i> (KU182754)	B	Rhizosphere	ND	56,8 <sup>c</sup>	ND	ND
LMR266	<i>Stenotrophomonas tumulicola</i> (KU182772)	B	Rhizosphere	ND	86.8 <sup>b</sup>	19.5 <sup>h</sup>	ND
LMR274	<i>Bacillus aerius</i> (KU182780)	B	Bulk soil	ND	74,2 <sup>c</sup>	ND	ND
LMR280	<i>Rhodococcus erythropolis</i> (KU182786)	B	Rhizosphere	ND	92,4 <sup>a</sup>	ND	ND
LMR282	<i>Stenotrophomonas rhizophila</i> (KU182788)	OH	Rhizosphere	48,6 <sup>b</sup>	45,8 <sup>d</sup>	4.4 <sup>ij</sup>	ND
LMR283	<i>Pseudomonas brassicacearum</i> (KU182789)	OH	Rhizosphere	2,4 <sup>g</sup>	75.6 <sup>c</sup>	145 <sup>a</sup>	ND
LMR284	<i>Pseudarthrobacter oxydans</i> (KU182790)	OH	Bulk soil	ND	48,8 <sup>f</sup>	ND	ND
LMR289	<i>Curtobacterium flaccumfaciens</i> (KU182795)	OH	Rhizosphere	ND	ND	107,9 <sup>bc</sup>	ND
LMR291	<i>Pseudarthrobacter oxydans</i> (KU182797)	OH	Rhizosphere	67.6 <sup>a</sup>	ND	134.1 <sup>b</sup>	71.1 <sup>cd</sup>
LMR292	<i>Bacillus atrophaeus</i> (KU182798)	OH	Rhizosphere	ND	82,1 <sup>b</sup>	4.3 <sup>ij</sup>	ND
LMR293	<i>Bacillus simplex</i> (KU182799)	OH	Rhizosphere	ND	ND	5.3 <sup>ij</sup>	93 <sup>b</sup>
LMR294	<i>Pseudomonas brassicacearum</i> (KU182800)	OH	Rhizosphere	ND	ND	76,5 <sup>d</sup>	ND
LMR297	<i>Bacillus mojavensis</i> (KU182803)	OH	Bulk soil	ND	ND	25,6 <sup>g</sup>	ND
LMR298	<i>Pantoea stewartii</i> (KU182804)	OH	Bulk soil	ND	ND	55,6 <sup>f</sup>	ND
LMR303	<i>Pseudarthrobacter oxydans</i> (KU182809)	OH	Rhizosphere	23 <sup>cd</sup>	ND	ND	ND
LMR308	<i>Alcaligenes faecalis</i> (KU182814)	OH	Rhizosphere	ND	ND	140,8 <sup>ab</sup>	ND
LMR309	<i>Pantoea ananatis</i> (KU182815)	OH	Rhizosphere	15,5 <sup>de</sup>	ND	100,1 <sup>c</sup>	ND
LMR310	<i>Bacillus zhangzhouensis</i> (KU182816)	OH	Rhizosphere	ND	92,1 <sup>a</sup>	ND	104 <sup>a</sup>
LMR312	<i>Pseudomonas benzenivorans</i> (KU182818)	T	Rhizosphere	50,7 <sup>ab</sup>	ND	61,8 <sup>c</sup>	ND
LMR317	<i>Brevibacterium frigoritolerans</i> (KU182823)	T	Rhizosphere	ND	ND	21,1 <sup>h</sup>	ND
LMR326	<i>Bacillus wiedmannii</i> (KU182832)	T	Bulk soil	ND	ND	2.8 <sup>j</sup>	105 <sup>a</sup>
LMR333	<i>Stenotrophomonas rhizophila</i> (KU182839)	T	Rhizosphere	ND	ND	21,4 <sup>h</sup>	ND
LMR337	<i>Bacillus anthoxyli</i> (KU182843)	T	Bulk soil	ND	ND	36,1 <sup>fg</sup>	ND
LMR340	<i>Rhodococcus qingshengii</i> (KU182846)	T	Rhizosphere	ND	ND	26.7 <sup>g</sup>	58 <sup>d</sup>
LMR341	<i>Rhodococcus erythropolis</i> (KU182847)	T	Rhizosphere	16,9 <sup>de</sup>	ND	104,9 <sup>bc</sup>	ND
LMR342	<i>Enterobacter ludwigii</i> (KU182848)	T	Rhizosphere	ND	ND	104,7 <sup>bc</sup>	ND
LMR344	<i>Bacillus tequilensis</i> (KU182850)	T	Rhizosphere	18,7 <sup>d</sup>	ND	ND	ND
LMR345	<i>Bacillus vallismortis</i> (KU182851)	T	Rhizosphere	13,0 <sup>f</sup>	ND	ND	ND
LMR346	<i>Bacillus tequilensis</i> (KU182852)	T	Rhizosphere	22,3 <sup>cd</sup>	ND	ND	ND
LMR347	<i>Stenotrophomonas rhizophila</i> (KU182853)	T	Rhizosphere	23,7 <sup>cd</sup>	ND	ND	ND
LMR348	<i>Georgenia daeguensis</i> (KU182854)	T	Bulk soil	17,0 <sup>de</sup>	ND	ND	20 <sup>c</sup>
LMR349	<i>Pseudarthrobacter oxydans</i> (KU182855)	T	Bulk soil	14,0 <sup>f</sup>	ND	ND	ND
LMR351	<i>Microbacterium hominis</i> (KU182857)	T	Rhizosphere	ND	ND	ND	41 <sup>de</sup>
LMR352	<i>Streptomyces chartreusis</i> (KU182858)	T	Bulk soil	ND	56,7 <sup>e</sup>	ND	ND

(B): Sidi Boubker, (OH): Oued El Heimer, (T): Touissit, (ND): Non determined.

For each type of activity, were analyzed only the strains previously selected as positive for the qualitative tests.

and low level of soil fertility (carbon, nitrogen, phosphorus, and potassium). These results were normal regarding the origin of these soils that consisted mainly of waste from mining activity. However, heavy metals concentration in soils varies depending on the previous mining activities in each site and the nature of the releases. Therefore, it is interesting to see how these differences in metals composition will affect the diversity of bacteria present in these soils, their tolerance to heavy metals, or their beneficial activities. Effectively, many studies reported that heavy metals can change the composition and activity of the microbial community (Vera et al., 2019) and affect the morphology and metabolism of bacteria (Wang et al., 2007; Oliveira and Pampulha, 2006). They can also cause an increase in the level of resistance among the local bacterial community (Malik et al., 2002). Moreover, Nithya and Pandian (2010) reported that the higher the heavy metal concentration is, the higher will be the occurrence and selection of heavy metal-resistant bacteria. This was the case in the present study where a high number of tolerant strains to Zn and Pb were obtained from soils

affected by chronic heavy metal contamination.

The bacteria isolated from these soils were dominated by three phyla: Firmicutes, Actinobacteria, and Proteobacteria, which is consistent with the results reported in the literature for similar soil conditions (Janssen, 2006; Fajardo et al., 2019). Other studies indicated that Proteobacteria are dominant in contaminated sites (Ellis et al., 2003), while others reported that dominant groups varied according to the environment: Firmicutes in polluted marine sediment environment (Briceño et al., 2012) and Acidobacteria in heavy metal-contaminated soils and sediments (Nguyen et al., 2016).

Globally the molecular identification of the bacterial collection by 16S rDNA sequence analysis showed that the soils of the three mining sites contained populations of bacteria that are dominated by the genus *Bacillus* sp. This genus was found in each of the six soil samples prospected. Slight differences exist between the populations of the rhizospheric soils compared to those of the bulk soils. Some bacterial genera seem to be ubiquitous as they were found in the three sites, in bulk and rhizospheric soils, despite the different prevailing conditions such as soil texture, the existence or not of plants, the mining history of each site, the heavy metals type and concentrations. This is the case of the genera *Bacillus*, *Arthrobacter* and *Stenotrophomonas*. Other genera seem to be specific to some environments like *Pseudomonas* that were isolated exclusively from *Sulla* rhizospheric soils.

Dominant and abundant bacteria associated with heavy metals contamination may be indicators of multi-pollution and can be used in environmental and health monitoring (Jaiswal et al., 2019; Yogalakshmi and Singh, 2020; Caputo et al., 2019). The abundance of some types of bacteria in contaminated soils can be explained by different adaptations or resistance mechanisms to heavy metals toxicity, such as intracellular sequestration, mutations, siderophores production, cellular sensitivity reduction, membrane permeability, repairing mechanisms of the DNA stability, and living within a biofilm (Presentato et al., 2019). Each dominant genus identified in our study is known to possess several of these properties. Sporulation for example is a special mechanism found in *Bacillus* species, where dormant spores resistant to extreme conditions are generated in response to environmental stresses (Cortês et al., 2019; Gillet et al., 2019). Bacterial strains belonging to the Actinomycetales order, which includes the genus *Rhodococcus*, are known to overcome environmental stresses like very high concentrations of metal(loid)s especially arsenate and selenite. Many studies highlight how these microorganisms are worth exploring as candidates for bioremediation of heavy metals contaminated sites (Kong et al., 2019). Other Actinobacteria species belonging to the genus *Arthrobacter*, *Pseudarthrobacter*, *Paenarthrobacter* can survive under harsh and stressful conditions, including high metal levels (Ortiz-Ojeda et al., 2017; Balan et al., 2018; Igalavithana et al., 2019). *Stenotrophomonas* sp., also have a genome-enabled propensity to tolerate heavy metals with the implication of many resistance genes against different heavy metals such as mercury, cadmium, selenium, tellurium, cobalt, copper, zinc, nickel, and lead (Agarwal et al., 2019; Yu et al., 2019).

Despite their role in decontaminating mining soils, indigenous microorganisms are also supposed to play an important role in the functioning of these polluted ecosystems, particularly the bacteria possessing plant growth-promoting properties (PGP). These bacteria can help plants to settle and proliferate on these soils that are often characterized by their low fertility and an elevated content of toxic elements (Dubey et al., 2020). This may be the case of many bacterial isolates obtained from the mining soils of the Oujda region, which were found to have interesting PGP traits like inorganic phosphate solubilization, siderophore, or auxin production and ACC deaminase activity. The number of isolates harboring PGP traits is variable from a sampling site to another, between bulk and rhizospheric soils. Generally, the numbers of siderophores and auxin producing bacteria were higher in the rhizosphere than in bulk soils and they belong mostly to the genera *Bacillus*, *Stenotrophomonas*, *Arthrobacter*, *Pseudarthrobacter*, *Paenarthrobacter*, and *Rhodococcus*. Many species and strains belonging to these genera are known to possess at least one PGP trait and to tolerate many heavy metals at variable concentrations (Becerra-Castro et al., 2012; Singh et al., 2015; Singh and Jha, 2017; Ortiz-Ojeda et al., 2017; Balan et al., 2018). It was relevant that many strains in our study had high levels of PGP activities and high levels of tolerance Pb and Zn. This is the case of LMR280, LMR310, LMR283, and LMR326. Several of these strains could be potentially useful for improving plant growth and establishment in trace metal-contaminated soils and lead to a healthy environment.

In addition to their direct plant growth-promoting properties, some bacteria can influence indirectly plant growth by acting as biocontrol agents (Chenniappan et al., 2019). Different studies reported that many bacterial strains are potential biocontrol agents of *Fusarium*, such as *Paenibacillus polymyxa* WR-2 (Raza et al., 2015, 2016), *Bacillus amyloliquefaciens* JDF35 (Zhao et al., 2018; Lahlali et al., 2020) and *Bacillus subtilis* IBFCBF-4 (Zhu et al., 2020).

In the present study, strains LMR294, LMR303, LMR341, and LMR344 had different *in vitro* inhibition rates against *Fusarium oxysporum*, which may be related to the species and the modes of action involved. The main mechanisms of action that have been reported for bacterial antagonism include antibiosis through the secretion of antifungal metabolites and volatile organic compounds (VOCs) or parasitism by the secretion of lytic enzymes (Jadhav et al., 2017; Lopes et al., 2015). Lytic enzymes are known to play a key role in cell wall-degradation of the hyphal growth of fungal pathogens and other soil microorganisms. Bacterial proteases, lipases, cellulases, and chitinases activities were tested for all strains of our collection. They were found to be largely present among the strains and it was relevant that the majority produced more than one enzyme and about half of them produced two lytic enzymes. This diversified enzymatic activity can confer to this strains a competitive advantage for their survival among the other components of the microbial populations inhabiting the soil and at the same time allows them to have a protective effect against pathogens threatening plants with which they are associated.

Taken together the results obtained so far for heavy metals tolerance, PGP attributes, antagonistic activities, and lytic enzymes production indicate that the Oujda abandoned mining soils prospected and most particularly the rhizospheric soil of *Sulla* plants is a large reservoir of promising diversified bacterial strains. All of these properties allow bacterial strains to grow and survive under higher heavy metals concentrations and compete with the other soil microorganisms. At the same time, these strains can improve plants' growth directly by enhanced nutrient availability and phytohormones stimulation, and indirectly by controlling the level of plant's ethylene by the bacterial ACC deaminase activity and protecting plants against bacterial and fungal diseases. Many reports indicate

that strains with higher PGP activities and resilience to harsh conditions may be proposed as biofertilizer candidates (Arora et al., 2020) and for industrial applications (Raddadi et al., 2013). Seen that they were isolated from extreme environments, they may possess other interesting properties like the sequestration of heavy metals and can, therefore, be used for bioremediation of heavy metal contaminated soils (Hazen et al., 2019), especially under phytoremediation approaches for those strains capable to thrive in the root ecosystem (Santos et al., 2019). In this context, it will be interesting to test under controlled conditions the best PGP heavy metals tolerant strains identified in this study with selected metal tolerant plants, like *Sulla spinosissima*, to identify superior plant-bacteria pairs that can be introduced in the abandoned mining soils studied. Inoculants to be tested may consist of single elite strains possessing multifarious traits (like LMR310 and LMR341) or consortia of bacteria with complementary activities (LMR291 for phosphate solubilization, LMR280 for siderophore production, LMR283 for auxin production, and LMR310 or LMR326 for ACC deaminase activity), provided that the strains of the mixture do not exhibit antagonism or competition with each other. The last step will consist to test the best performing inoculants under field conditions. The objective will be to determine if these strains can affect positively the settlement and growth of selected plant species under the severe toxic conditions prevailing in the mining sites studied. Such approach has been proven to be efficient in many recent studies, in particular, it was reported that inoculation of plants with selected PGPRs can facilitate plants growth, enrich the soils and create a vegetation cover in contaminated sites (Glick et al., 2010; Aafi et al., 2012; Dubey et al., 2020).

In conclusion, our study made an advance toward providing insight into the distribution and activity of the bacteria in three multi-polluted abandoned mining sites in the region of Oujda. We have identified the major groups of cultivable bacteria inhabiting these soils, that were dominated by *Bacillus* species. The bacterial distribution seems to be related to Pb and Zn soil contaminants and especially to the presence or not of spontaneous plants (*Sulla spinosissima*) cover. Interesting strains, LMR248, LMR266, LMR280, LMR283, LMR291, LMR312, LMR326, LMR303, LMR310, LMR333, LMR340, LMR341, LMR344, that are heavy metal tolerant and possess high levels of PGP and antifungal traits were identified. The use of the selected bacteria as bio-inoculants for plants to be introduced in this region could be a sustainable promising nature-based approach aimed at controlling or stopping the dissemination of heavy metals from the contaminated sites (bioremediation or phytostabilisation). In addition, the approach adopted in this work could have applications in other regions of Morocco and/or other countries suffering from the problem of abandoned mining sites. For this purpose, the selected native heavy metal tolerant PGP strains should be tested with chosen plants under the field conditions in different sites to evaluate their potential as an essential component of a sustainable strategy for the recovery of heavy metal contaminated areas.

#### Author contributions

Malika Oubouhssaine: investigation, data curation, writing-original draft preparation. Ikram Dahmani: investigation, software, data curation. Laila Sbabou: methodology, conceptualization, investigation. Odile Bruneel: methodology, investigation, resources, project administration, funding acquisition. Jamal Aurag: methodology, conceptualization, validation, resources, project administration, funding acquisition.

#### Declaration of competing interest

The authors declare no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2021.102236>.

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