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Original Research Article

Bacteria associated with different legume species grown in heavy-metal contaminated soils

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This study was conducted on seven experimental soil samples from mining and agricultural sites in Tunisia. Four local legumes, *Vicia faba*, *Lens culinaris*, *Cicer arietinum* and *Sulla coronaria*, were tested to select appropriate legume–tolerant bacteria symbionts for specific metal contamination. Soil analysis showed that Cd, Pb, Zn and Cu concentrations were significantly higher in contaminated sites than in agricultural soils. Investigation of legumes response to contamination showed that the greatest reduction in the shoot and root dry weights was observed in *Sulla coronaria* upon Cd contamination due to highly metal accumulation; while *Vicia faba* and *Lens culinaris* contained Cu and Pb respectively in their organs. Metal tolerance analyses showed that isolates from *Vicia faba* could grow with maximum Cu, Pb and Cd levels of 2, 4 and 4.5 mM, respectively; however, isolates from the others tested legumes were more sensitive to heavy metals. Genetic characterization by PCR-RFLP of the 16S rDNA for 20% of the isolates revealed different species including *Rhizobium leguminosarum, Rhizobium phaseolus, Rhizobium etli* and *Agrobacterium.* Selected *Rhizobium* species were chosen with their appropriate legumes to form prospective symbiotic systems for eventual phytostabilisation purposes, which should be tested in contaminated areas.

Keywords: Heavy metals, legumes, Rhizobacteria, Soil, Symbioses

INTRODUCTION

Soil contamination by heavy metals is a widespread occurrence due to human, agricultural and industrial activities (Beladi et al., 2011). These activities result in the accumulation of trace metals in agricultural soils, creating a threat to food safety and overall public health (Dary et al., 2010).

Conventional methods used for reclamation of contaminated soils, namely, chemical, physical and microbiological methods, are costly to apply (Danh et al., 2009). Phytoremediation seems to be a cheap and environmentally sound option for reclaiming toxic metals and metalloids. The most important challenge is to improve the efficiency of phytoremediation by increasing the accumulation of metals in plants or by improving key plant biological traits that should enhance metal uptake (Wu and Tang, 2009).

Recently, there has been increasing interest in the use of legume plants associated with microorganisms for bioremediation of heavy metals (Carrasco et al., 2005). This system presents the advantages of using the *Rhizobium*–legume symbiotic interaction as an efficient soil improvement system through root nodule formation (Vance and Lamb, 2001). For a long period, plant growth promoting rhizobacteria (PGPR) were mainly used in bioremediation of heavy metal polluted soils (Zhuang et al., 2007). Recent studies suggested that these bacteria have the ability to produce plant promoting substances in metal-stressed environments (Wani et al., 2007). Several plant associated bacteria have been reported to accelerate phytoremediation in metal contaminated soils by promoting plant growth and health, and they play a significant role in accelerating phytoremediation (Compant et al., 2010; Dary et al., 2010).

In Tunisia, soils around opencast mines exhibit very high contents of Pb, Zn, and Cd (Schramel et al., 2000) including cases at ex-mining sites (Sebei et al., 2005), in agricultural regions (Ayari et al., 2010) and at industrial sites. So the average content of Pb, Zn and Cd detected in the plants of the Ghezala mines was 0.7%; 0.9% and 0.003% of dry weight (DW), respectively (Sebei et al., 2005).

The purpose of the study was to isolate *Rhizobium* species that are resistant to heavy metals from contaminated soils in order to use them in association with their symbiotic heavy metal-tolerant legumes for bioremediation experiments. This work was conducted by: (i) assessing the level of heavy metal contamination in some Tunisian mining areas; (ii) evaluating the growth and heavy metal accumulation performance of four local legumes, *Vicia faba*, *Lens culinaris*, *Cicer arietinum* and *Sulla coronaria*; and (iii) studying heavy metal tolerance of strains isolated from the tested legumes.

MATERIALS AND METHODS

Site selection and soil sampling analysis

The study was performed in northern and central western Tunisia. The sites were selected because of high soil concentrations of heavy metals. Seven soil samples (three replicates) were collected at depth of 15 cm from the soil surface from various locations, including El Kef (S1), Makther (S2), Jerissa (S3), Ghezala (S4), Tamra (S5), Menzel Bourguiba (S6) and Jebal Ressas (S7), which cover agricultural, industrial and mining areas.

The collected soil samples were air dried at 40 °C for 16 hours and sieved into coarse and fine fractions. Well mixed samples, each weighs 0.5 g, were dissolved with 6 ml hydrochloric acid and 2 ml of 2% nitric acid at 95 °C for 75 min, filtered and then diluted to 50 ml with distilled water. Total heavy metal concentrations were determined for Cd, Co, Cu, Pb, Ni, Zn and Cr ($mg.kg^{-1}$), which were analyzed using Atomic Absorption Spectrophotometry (Ioan et al., 2008).

Plant cultivation and metal accumulation

Seeds of *Lens culinaris*, *Vicia faba* and *Sulla coronaria* were obtained from UTAP (Union Tunisienne de l'Agriculture et de la Pèche) station of Manouba located to the northwest of Tunis. Seeds of the cultivar Amdoun of *Cicer arietinum* were obtained from the "Cooperative Centrale de Semences et Plants Sélectionnées" (CCSPS), Tunis. Seeds were then surface sterilized with 70% ethanol for 15 min and rinsed five times with distilled water. Seeds of the four legumes were germinated on agar plates at 28 °C in darkness and were planted into plastic pots (15 cm diameter) containing 1 kg of soil per one. After germination, plants were thinned at rate of three seedlings per pot, placed in growth chamber at temperature of 20 °C and drip irrigated. Each treatment was replicated 10 times.

Experimental plants were harvested after the flowering stage. To determine the metal uptake in different parts of tested plants, the roots and shoots were separated and dried on filter paper at 60 °C for 72 hours to determine the dry weight before crushing and storage in small flasks. The metal content was determined using inductively coupled plasma–mass spectrometry (ICP–MS). 20 mg of each dried plant material was digested by a mixture of nitric acid and perchloric acid (4:1, v/v) until dry at 100 °C, followed by 2 h in 25 ml HNO_3. The mixture was filtered through Whatman filter paper, and the results were expressed in $\mu g.g^{-1}$ DW (Asma et al., 2009).

Collection and isolation of root nodules bacteria

Rhizobium was isolated from soil samples by nodulation using tested legumes grown on samples soils or from nodules of native plants collected in situ. Nodules formed on plant roots after 45 days for *Cicer arietinum* and *Lens culinaris* and after 60 days for *Vicia faba* and *Sulla coronaria* were cut off and *Rhizobium* was isolated from them as follows: collected nodules were washed with sterile water; their surfaces were sterilized by 70% ethanol and 0.2% $HgCl_2$ and washed exhaustively with sterile distilled water. Nodules were then crushed and the resulting suspension was streaked on to yeast extract mannitol agar (YEMA) plates. After repeated sub-culturing, pure culture was obtained from a single colony and preserved in 40% glycerol at 80°C; for experimental purposes. Plates were incubated at 28°C for 48 h, and the lowest heavy metal concentration inhibiting isolate growth was defined as the maximal resistant level (MRL). Strain nomenclature indicates soil sample location, host plant and strain isolate.

180 isolates resistant to heavy elements were tested on agar plates containing YEM media (Vincent, 1970), with increasing concentrations of the specific heavy-metal salt. Survival of *rhizobium* strains is severely affected by heavy metal contaminated soils, as described by Gusmão-Lima et al. (2005). Thus, maximal concentrations used for resistance testing were similar to those of corresponding elements found in contaminated soils. Pb (in the form of lead chloride) was used at concentrations of 0.3, 0.4, 0.675, 1, 1.35, 2, 2.7, 2.97, 3.25, 3.375 and 4.05 mM. Cu (in the form of copper sulfate) was used at concentrations of 0.02, 0.03, 0.04, 0.05, 0.075, 0.1, 0.15, 0.25, 0.35, 0.4, 0.5 mM, 1 mM and 2 mM. Zn (in the form of zinc sulfate) was used at concentrations of 0.6, 0.8, 1, 1.2, 1.28, 1.32, 1.36, 1.4 and 1.6 mM. Cd (in the form of cadmium chloride) was tested at concentrations of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mM.

Isolate identification and PCR-RFLP analysis of the 16S rRNA genes

The specific primers used for 16S rRNA gene amplification were fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGCTTAAGGTGATCCAG-CC-3') (Weisburg et al., 1991). PCR amplification was carried out in a 25 µl reaction volume containing bacteria template DNA (10 to 50 ng), 2.5

Table 1. Total heavy metals concentrations of (Cd, Co, Cu, Pb, Ni, Zn and Cr) in the investigated soil samples

Soils	Cd(mg.kg-1)	Co	Cu	Pb	Ni	Zn	Cr	As
El Kef	<0.27	3.44	2.43	<4.4	9.11	26.7	31.9	<0.71
Makthar	<0.27	2.02	5.04	<4.4	9.78	27.9	19.6	<0.71
Jerissa	<0.27	2.60	10.6	27.2	7.37	31.2	8.76	<0.71
Ghezala	6.98	10.7	28.5	10100	35.3	235	59.2	8.54
Tamra	<0.27	12.2	18.9	797	9.39	293	53.2	<0.71
Menzel bourguiba	<0.27	2.53	20	426	66.8	84.5	36.7	<0.71
Jebal Ressas	<0.27	5.92	29.2	116	13.7	211	30.7	<0.71
Frensh Norm	0.7-2	25-30	35-100	60-100	50-70	150-300	150	1-25

[C] : Baize D (1996) Détection des contaminations modérées en " métaux lourds " dans les sols agricoles. Intérêt et limites de la norme AFNOR U 44-041.

mM 10X buffer, 1.5 U Taq polymerase, 1.5 mM $MgCl_2$, 200 µM dNTP and 1 µM of each of the primers.

PCR amplification was performed with a PerkinElmer model GeneAmp PCR System 2400. The reaction conditions were as follows: Initial denaturizing of 15 min at 94 °C was followed by 34 cycle of denaturizing of 1 min at 94 °C, annealing of 1 min at 55 °C and extension of 1 min at 72 °C. Final extension has also performed at 72 °C for 10 min. Reaction efficiency was estimated by horizontal agarose gel electrophoresis (1% $w.v^{-1}$) and colored in an aqueous solution of ethidium bromide (1 $mg.ml^{-1}$).

Aliquots of 9 µl PCR products were checked by restriction fragment length polymorphism (RFLP) analysis. Three restriction endonucleases, *Msp*I, *Nde* II and *Hae* III, were used for their highly level of discrimination. The reaction mixture was then incubated at 65 °C for 10 min to inactivate the restriction enzyme. Reaction products were separated by agarose gel (3% w/v) electrophoresis in TAE buffer run at 20 $V.cm^{-1}$ for 3 hours and stained with 1 $µg.ml^{-1}$ ethidium bromide (Trabelsi et al., 2009).

RESULTS

Samples soils analysis

The results showed that the highest concentrations of heavy metals were found in Ghezala (S4) located close to an old mine. The maximum value of Cd was 3 times higher than the permissible levels for agricultural soil according to French legislation (Baize, 1996) and 7 times more than the Cd concentration in the agricultural site of El Kef (S1) (Table 1).

Lead was the main contaminant metal in S4, S5, S6 and S7. The concentration of Pb exceeded in S4 and S5 the limits of the French legislation by 100 times and 8 times respectively. This metal level in S4 surpassed the value registered in agricultural soil S1 by 104 times. However, the Pb detected in the soil sample S3 does not exceed the limits of French laws (Table 1).

Copper was identified in just three out of seven locations, where the concentrations at S4, S6 and S7 ranged within the normal limits of 28.5, 20 and 29.2 $mg.kg^{-1}$, respectively. Moderate concentrations of Cr, Co and Ni were recorded in soils S4, S5 and S7, respectively, which did not exceed French legislation limits (Table 1). The study revealed that Ghezala was the most polluted area, while El kef was the least heavy metal contaminated site.

Legume growth in heavy metal contaminated soils

Response of legumes to soil contamination revealed different effects of heavy metals on shoot and root dry weights. Plants exposed to heavy metals showed noticeably a stunted growth compared to that cultivated in the control agricultural soil (Fig. 1). A decrease in root and shoot dry weights were proportional to the concentration of heavy metal. The greatest reductions of 81.8% (SDW) and 63.5% (RDW) were in *Sulla coronaria*, which could be due to Pb contamination in S4 and S5 (Fig. 1a). Similar decreases were also noted for shoot and root dry weights of *Vicia faba* for Cd and Pb contamination, with reductions of approximately 75% (SDW) and 60% (RDW) (Fig. 1b).

Contaminated S6 soil reduced the roots and shoots dry weight of *Vicia faba* by 63% compared to the control plant (Fig. 1b). However, *Lens culinaris* appeared to be the least affected legume by high Pb and Cd levels in S4 (Fig. 1c). Moreover, Pb and Zn had more adverse effect on *Cicer arietinum* in S5 than it was in S4, which is highly contaminated with only Pb and Cd (Fig. 1d). Higher levels of Pb and Zn in S5 reduced *Cicer arietinum* root dry weight up to 78.2%, whereas the dry weight reduction did not exceed 42% with both Pb and Cd contamination in S7 (Fig. 1d).

Heavy metal accumulation

The ability of legume species to absorb heavy metals varied widely, depending on the metal concentrations, on plant species and plant organ (Table 2). Results signified that legumes accumulated metals preferentially in their roots; except for *Lens culinaris* that demonstrated a translocation of 98% Pb from roots to shoots (Table 2).

Moreover, the highest Cd concentration was stored in the shoot and root of *Sulla coronaria,* while non significant Cd accumulation was recorded in both plant parts for *Vicia faba*. *Cicer arietinum* present significantly a greater Cd amount in the roots than in shoots. However, the content of Cd in the stems and leaves indicates an active metal

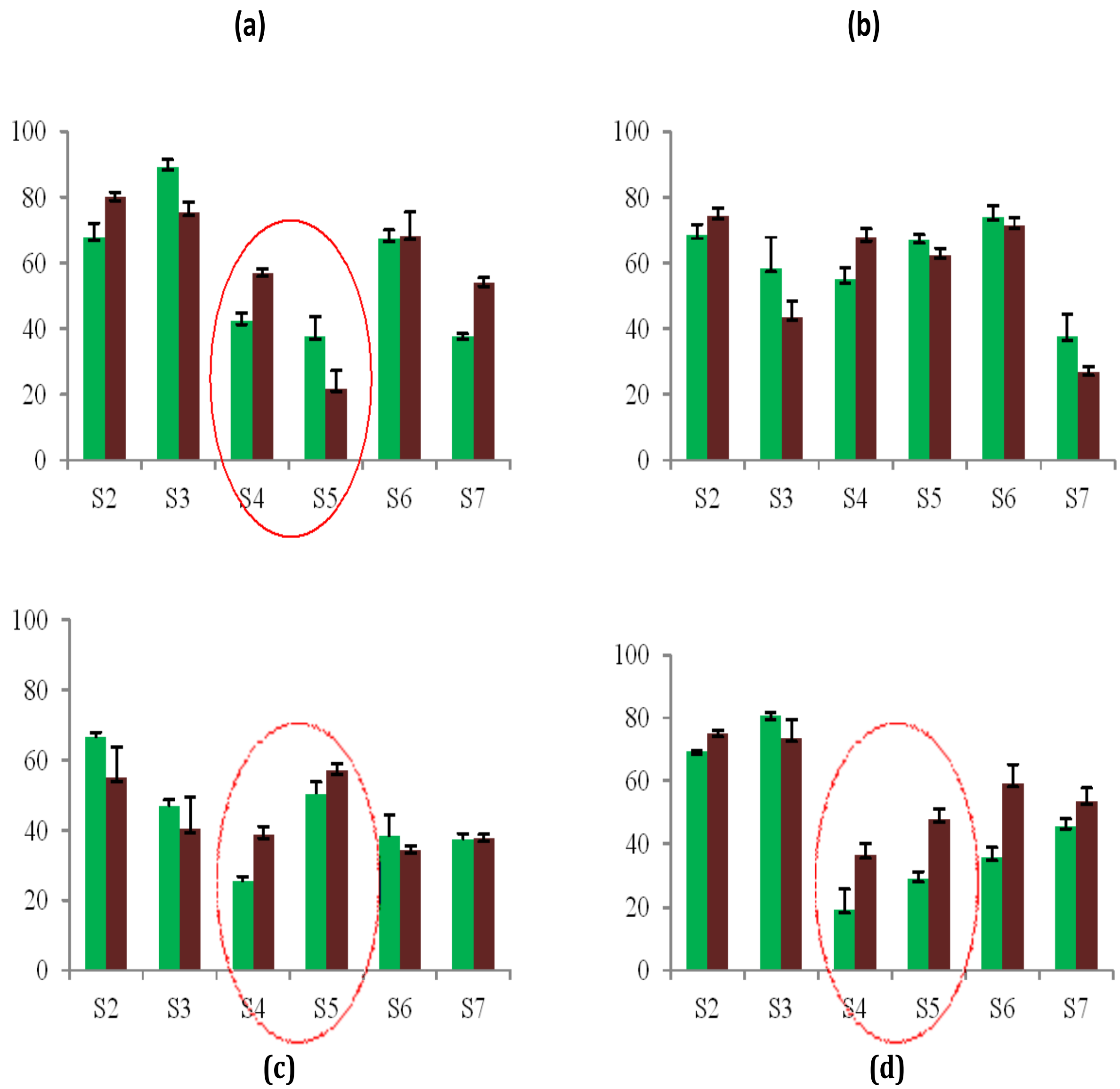

Figure 1:(SDW) and (RDW) percentages evolution of studied legumes, (**a**): *Cicer arietinum*; (**b**): *Lens culinaris*; (**c**): *Vicia faba*; (**d**): *Sulla coronaria*, after 8 weeks of planting on studied soils of **S2**: Makthar, **S3**: Jerissa, **S4**: Ghezala, **S5**: Tamra, **S6:** Menzel Bourguiba, **S7**: Jebal Ressas.

transfer from the roots to the aerial parts (Table 2).

Sulla coronaria tended to accumulate higher Cd level in the shoots and roots, with values ranging from 3 to 7 times more than in other tissues.

In general, experimental plants preferred to accumulate Cu in roots. The maximum quantity was observed in *Vicia faba* grown on copper contaminated S7 soil, whereas *Cicer arietinum* was at least, containing 90% of Cu less than *Vicia faba*. However, no significant differences in Cu content were recorded between *Sulla coronaria* and *Lens culinaris* (Table 2).

Legume plants did not differ significantly among each other in Zn levels and each plant species did not show a considerable variation in Zn contents between different plant parts (Table 2).

An increase in metal concentrations in legumes tissues was correlated with the elevation of total metal concentrations in the soil (r2 = 0.5) (Table 2). The results of growth and accumulation of metals suggest to test formed couples: *Vicia faba*-Cu, *Sulla coronaria*-Cd and *Lens culinaris*-Pb in a possible challenge of phytostabilisation / phytoextraction and fertilization of contaminated soils.

Metal tolerance of selected isolates

The effects of Pb, Zn and Cu metals on the development of 191 isolates obtained from nodules are illustrated in Table 3. The test of tolerance showed that the MRL of 8%, 16% and 8% of *Vicia faba* isolates reached ≤ 2 mM Cu, 4.05 mM Pb, and 1.75 mM Zn, respectively, while MRLs for the rest of

Table 2. Metal concentrations of Cd, Cu, Zn and Pb (µg.g-1 DW) in Soots (S) and Roots (R) of studied legumes (*Vicia faba*, *Cicer arietinum*, *Lens culinaris* and *Sulla coronaria*) grown in different heavy metals concentrations

SOILS	METAL	PLANTS SDW/RDW (µg.g-1)							
		Vicia faba		***Lens culinaris***		***Cicer arietinum***		***Sulla coronaria***	
S1	Cu	0.1773	0.0954	0.0247	0.0750	0.1162	0.0416	0.0247	0.0650
	Zn	1.4931	1.6686	1.4353	2.5084	0.8188	0.1825	1.0480	1.5877
	Pb	0.1444	0.2288	1.3238	0	0	0.1056	0.3424	0
	Cd	0	0.0043	0	0.0015	0	0.0065	0	0
S2	Cu	0.2527	0.1769	0.0276	0.1303	0.1128	0.0438	0.0276	0.1303
	Zn	1.5366	1.8710	3.1976	2.0776	0.6893	0.8942	0.7670	1.1039
	Pb	0.1365	0.1483	1.4222	0	0	0	0.7670	0
	Cd	0	0	0	0	0.0017	0.0008	0	0.0109
S3	Cu	0.0910	0.2001	0.0427	0.0569	0.0684	0.0613	0.0427	0.0569
	Zn	1.0115	1.1665	3.5567	0.5456	1.1158	1.4358	0.9225	1.4569
	Pb	0.5744	0.0760	1.6778	0	0	0	0	0
	Cd	0	0	0	0	0.0001	0.0026	0.0057	0.0036
S4	Cu	0.1320	0.1860	0.0559	0.1116	0.0336	0.0630	0.0659	0.0916
	Zn	7.4931	9.2658	10.8661	3.0009	1.6382	2.0512	0.6648	1.1058
	Pb	28.1163	3.5299	132.9804	1.4827	0.3501	58.7040	0	0.0298
	Cd	0.0048	0.0012	0.0005	0.0005	0.0043	0.0093	0.0031	0.0046
S5	Cu	0.0640	0.2245	0.0579	0.1851	0.0349	0.0548	0.0579	0.1851
	Zn	8.4109	8.4710	9.3344	1.3008	1.9387	2.1095	2.8564	2.0945
	Pb	6.1253	0.8985	86.5296	1.1348	0.0410	45.1609	0	0.1107
	Cd	0.0041	0	0	0.0041	0	0.0050	0.0072	0.0098
S6	Cu	0.1198	0.0536	0.0793	0.0600	0.0349	0.0343	0.0793	0.0600
	Zn	6.5616	7.4948	7.4612	2.3040	0.9382	1.0752	0.9765	0.6761
	Pb	3.4443	0.0170	22.3012	0	0	31.0320	0.4928	0
	Cd	0	0	0.0059	0.0049	0.0006	0	0	0
S7	Cu	0.6432	0.9555	0.1185	0.1248	0.0245	0.0305	0.1140	0.1267
	Zn	14.6301	17.4294	10.4112	1.4912	1.2559	1.4429	1.6191	1.1332
	Pb	1.3444	0	9.0299	0.0581	0	12.1437	0	0
	Cd	0	0	0.0171	0.0028	0	0	0	0

Table 3 Evaluation of the resistance heavy metals in legumes isolates

% Isolates	**Metals (MRL)(mM)**											
	Cu		**Zn**		**Pb**			**Cd**				
	0.25	**2**	**1.2**	**1.75**	**3.35**	**3.6**	**4**	**0.8**	**1. 8**	**2.2**	**3.2**	**4.05**
Vicia faba		6		8			16			3		
Cicer arietinum	8			34		20				38		
Lens culinaris	6		64		8					15		
Sulla coronaria	12		14			14				2.5		

The resistance to the different elements was determined on YEMA supplemented with increasing concentrations of the corresponding element.The resistance was expressed as % strains growing in MRL which is the maximal concentration of an element that does not affect bacterial growth

isolates did not exceed 0.25 mM Cu, 3.35 mM Zn and 1.2 mM Pb. 34% of isolates from *Cicer arietinum* tolerated up to 1.4 mM Zn, but only 8% of isolates from *Vicia faba* were resistant (Table 3). 9% of isolates from *Vicia faba* was able to grow with 4.5 mM Cd. 16% of isolates *Sulla coronaria* tolerated only up to 4 mM Cd, where the 48% resistance of *Cicer arietinum* strain not exceed 3 mM (Table 4). *Lens culinaris* strains showed the lowest resistance levels among all isolates at concentrations of 0.25 mM Cu, 3.35 mM Pb, 1.75 mM Zn and 0.8 mM Cd.

RFLP analysis of PCR-amplified 16S rDNA

The results showed that 36 isolates (20%) from tested

Table 4. Sampling sites, host legume, studied isolates selected, PCR/RFLP characterization

Strains	Host plant	Site/origin	Assignation PCR/RFLP
SV1	***Vicia faba***	El Kef	NI
SV3		El Kef	*R. etli*
SV6		Makthar	NI
SV7		Makthar	NI
SV13		Ghezala	NI
SV16		Tamra	*Agrobacterium*
SV20		Menzel Bourguiba	*R. Leguminosarum*
SV23		Menzel Bourguiba	NI
SV27		Jebal Ressas	*R. Leguminosarum*
SS44	***Sulla coronaria***	Ghezala	*R. Leguminosarum*
SS45		Ghezala	*R. Leguminosarum*
SS50		Ghezala	*R. Leguminosarum*
SS53		Ghezala	NI
SS54		Ghezala	NI
SS56		Ghezala	*R. phaseolus*
SS57		Ghezala	*R. phaseolus*
SS36		Jerissa	*R. phaseolus*
SS38		Jerissa	*R. phaseolus*
SS39		Jerissa	*R. phaseolus*
SS60		Ghezala	*Agrobacterium*
SS69		Menzel Bourguiba	*Agrobacterium*
SS79		Jebal Ressas	*R. phaseolus*
SL2	***Lens culinaris***	El Kef	*Rhizobium.sp*
SL11		Menzel Bourguiba	NI
SL12		Menzel Bourguiba	NI
SL19		Menzel Bourguiba	*Agrobacterium*
SL20		Menzel Bourguiba	*Rhizobium.sp*
SL27		Jebal Ressas	*Rhizobium.sp*
SL28		Jebal Ressas	*Rhizobium.sp*
SC5	***Cicer arietinum***	Makthar	*Agrobacterium*
SC15		Jerissa	*R. phaseolus*
SC16		Jerissa	*Agrobacterium*
SC29		Ghezala	*R. galegae*
SC30		Ghezala	NI
SC38		Menzel Bourguiba	*Agrobacterium*

samples had high tolerance to heavy metals.
Results revealed that 28% of isolates were equally related to *R. leguminosarum* and *R. phaseolus*. Approximately 20% of isolates were related to *A.* tumefaciens, whereas only two isolates from *Vicia faba* and *Cicer arietinum* were related to *R. etli* and *R. galegae*, respectively (Table 4). Finally, PCR/RFLP of the 16S rDNA gene could not discriminate 30% of the selected isolates, which were not identified.

DISCUSSION

In this study, an approach combining various complementary methods was used to identify beneficial associations of rhizobia and legumes for eventual cleaning and fertilization of heavy metal contaminated sites. The results of a preliminary investigation of heavy metal levels in seven sites showed high concentration of the metals such as Cd, Pb, Ni and Zn.

Lead concentrations values were greater than the threshold value justifying a contamination assessment for all samples except for the agricultural soils. This finding agrees with results obtained by Zribi et al. (2012).

The dry weight of different legume species retarded response to Pb, Cd and Zn stress. Azmat et al. (2006) reported clear symptoms of Cd and Cu toxicity such as growth retardation and a decrease in relative water content as a result of elevation of heavy metal concentrations in the soil. High level of Zn in the soil had a deleterious effect on the plant growth, especially on *Cicer arietinum* when Zn levels in soils exceeded 200 mg/kg, which is in agreement with the outcome of experiment conducted by Parvaze et al. (2008).

Outcomes of this study revealed that Cu, Pb and Cd mainly accumulated in the roots of *Vicia faba*, *Lens culinaris* and *Sulla coronaria*, respectively, with some translocation to the shoots. This variable accumulation of heavy metals in legume tissues at different sites may be attributed to an increased solubility of metals in contaminated soils and their mobility in plant tissues (Kumar et al., 2009). A significant positive correlations were found between Cd and Pb concentration in the soil and root tissue for *Sulla* *coronaria* and *Lens culinaris*, respectively, presumably due to the higher soil metal content, not to systematic changes in soil and foliar concentrations across species (r1=0.865; r2= 0.823).

Legumes play a crucial role in the biological fertilization of contaminated soils, especially when they are associated with rhizobacteria that are tolerant to heavy metals. The degree of metal resistance depended on the trace element. Pereira et al. (2006) reported that *Azorhizobium caulinodans* tolerated up to 5 mM Cd; while other *Rhizobium* sp. was very sensitive to Cd with a MIC of 0.1 mM (Wojas et al. 2007). The MIC of *Rhizobium leguminosarum* bv. *viciae* was found to be varied between 15 and 120 μM Cd (Belimov and Wenzel 2009). As a result, these isolates can be used in a bioremediation test to improve plant growth under trace metal stress conditions. Early investigation of Sousou et al. (2013) indicated that inoculation of *Anthyllis vulneraria* with symbiotic and nitrogen-fixing bacteria induced a significant decrease of Zn content in the plant tissue. Moreover, activation of the nodulation and growth of the biomass nodules have been described for several inoculated plants such as *Cicer arietinum* (Wani et al. 2008c), *Lens culinaris* (Wani et al. 2008b), and *Pisum sativum* (Wani et al. 2008a). These results suggested that selected metal resistant bacteria can serve as an effective metal sequestering and growth-promoting bioinoculant for plants in metal stressed soil.

The PCR-RFLP of 16S rRNA analysis led to the identification of many symbiotically effective rhizobia in polluted soils, while other authors reported the isolation of only symbiotically ineffective strains from clover plants grown on heavy metal polluted soils (Giller et al., 1989). Gene sequencing is needed to clarify the phylogenetic positions of these isolates and to establish whether the unclassified isolates represent new species or not, especially those that were able to induce nodule formation with *Vicia faba* and *Lens culinaris* (Yadegari et al. 2008). These findings suggest that co-inoculation of rhizobia and heavy metals tolerant isolates belonging to other generea, can respond better than single rhizobia inoculation which is in agreement with previous researchers demonstrating that microbial inoculation effects were greater on growth when seedlings were inoculated with combination of microbes rather than individually (Jetiyanon and Kloepper, 2002).

Conclusions

Phytoremediation is still a target for research and developmental phase, with many technical issues needed to be addressed. Outcomes of this research indicated that legumes plants, ~~of~~ *Vicia faba, Lentils culinaris* and *Sulla coronaria,* are efficient in remediation towards copper, lead and cadmium.

Several rhizobia strains and other nodules bacteria that displayed a resistance to heavy metals tested might be commercially formulated in the future in order to be applied to the soil in effort to promote plant growth.

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