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ORIGINAL ARTICLE

Physiological responses to cadmium, copper, lead, and zinc of *Sinorhizobium* sp. strains nodulating *Medicago sativa* grown in Tunisian mining soils

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Abstract The capacity of nodulating bacteria to survive in soil containing various heavy metal elements has been investigated with the aim of promoting the revegetation of mining sites with *Medicago* sp. Soil samples were collected from three different mining sites and one agricultural site at a location north of Tunisia. Heavy metal composition analysis showed that the soil samples were contaminated with different concentrations of cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn). The forage plant *Medicago sativa* was able to grow normally and to develop effective nodules in these contaminated soils. *Sinorhizobium* sp. strains nodulating *Medicago sativa* plants grown in these mining soil samples were isolated and characterized. The isolated strains were able to grow in soils containing up to 2.5 mM Zn, 0.3 mM Cd, 1 mM Cu and 2 mM Pb. The bioaccumulation was tested for two contrasting strains for each metal. For Cd, Pb, and Zn, strain S532 (tolerant strain) adsorbed lower amounts of metals than sensitive strain S112. For Cu, tolerant strain S412 absorbed more Cu than sensitive strain S112, even though adsorption was similar for these two strains. Our results support the use of *Medicago sativa–sinorhizobium* symbiosis for the regeneration and enrichment of moderately contaminated soils.

Keywords Heavy metals · Symbiosis · *Sinorhizobium* sp. strains · Soil regeneration · Alfalfa

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Introduction

Soils contaminated with heavy metals (HM) or organic pollutants represent a major risk because of their direct toxic effects, accumulation throughout the food chain, and the possibility of groundwater contamination. There are many sources of soil contamination with HM, such as smelters, sewage sludge, mine tailings, industrial activities and uncontrolled application of pesticides and fertilizers in the agricultural sector (McGrath et al. 1995; Robinson et al. 2001). When HM, such as cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn), are incorporated into the soil, they persist for a long time in the biosphere. In addition, several HM, such as Pb and Cd, are toxic to plants, animals, and microorganisms even at very low amounts (Gadd 1992).

Other HM, such as cobalt (Co), manganese (Mn) and Zn, are necessary microelements to plants and animals, but only at low levels as they are toxic at high concentrations.

In the anthropogenic context, polluted soils with HM contain low levels of organic matter which limits plant growth mainly by nitrogen deficiency. Thus, the regeneration of contaminated or degraded soils using revegetation strategies often uses leguminous species to improve the soil nitrogen content and quality (Gerhardt et al. 2009; Khan et al. 2009; Zhuang et al. 2007).

Rhizobia are Gram-negative bacteria which establish a nitrogen-fixing symbiotic relationship with leguminous plants, thereby allowing nitrogen fertilization and nitrogen fixation in the soil (Stacey et al. 2006). In order to use legumes for the rehabilitation of HM-contaminated soils, it is important to determine the effect of soil contaminants on the behaviour of both symbiotic partners. Legumes have been shown to have the potential to grow on soils contaminated with moderate levels of HM (Carrasco et al. 2005; Del Rio et al. 2002). However, it has also been reported that the contamination of soils with HM affects rhizobial survival, especially in the absence of the host legume (Broos et al. 2004, 2005). It is well known that host plants enhance microbial biomass and activity in the rhizosphere by producing root exudates (Brimecombe et al. 2001; Glick 2004). It has also been reported that under conditions of moderate soil contamination, legumes are still able to establish an effective symbiotic interaction with nodulating rhizobia, especially when appropriate HM-tolerant strains isolated from contaminated soils are used (Dary et al. 2010; Pajuelo et al. 2008).

The aim of the study reported here was to assess both the capacity of *Medicago sativa*-nodulating *Sinorhizobium* sp. strains in soil samples collected from mining sites to grow at various concentrations of HM, namely, Cd, Cu, Pb and Zn, and their ability to accumulate them.

Materials and methods

Prospecting and soil sampling

Prospecting activities were carried out in the northern region of Tunisia, which is characterized by the presence of several mines involved in metal extraction. Soil samples were collected from one non-mining (agricultural soil) site in the Mateur region (Fig. 1a) and one mining site each in the Gzala (Pb/Zn mining site; Fig. 1b), Ain Allègua (Pb/Zn mining site; Fig. 1c) and Djerissa (iron mining site; Fig. 1d) regions, respectively. In Djerissa, three points of sampling were considered: C0 (on the mining rejects), C2 (50 m from the mining rejects) and C2 (100 m from the mining rejects).

Plant culture and bacterial isolation

Seeds of *M. sativa* cv. Gabès were surface sterilized with H_2SO_4 for 3 min and incubated on 0.9% water-agar plates at 25°C for 48 h. Plants were cultivated in 500-ml aseptic plastic pots containing soil samples in a growth chamber at 25°C and 80% relative humidity and under a 16/8-h light/dark photoperiod. Five replicates of each soil sample tested in this assay. Plants were harvested 2 months post-planting, and root nodules were counted and used to isolate the microsymbiont according to Vincent (1970). Twenty bacterial strains were considered in this study: two from Mateur, one from Ain Allègua, two from Gzala, six from Djerissa C0, two from Djerissa C1 and six from Djerissa C2, as well as the reference *Sinorhizobium meliloti* strain RCR2011. The ability of isolates to re-nodulate *M. sativa* was assayed according to Brunel et al. (1996). Symbiotic performances of alfalfa in each soil sample were evaluated by measuring shoot dry matter (SDM) and number of nodules per plant (NN).

Rhizobial density in soils

The most probable number (MPN) method using *M. sativa* as a trap plant was used to estimate the density of rhizobia in soils according to Vincent (1970).

Soil analysis and determination of metal levels

Physical and chemical parameters of soil samples were analysed in the Soil Direction department at INGREF (Institut National des Recherches en Génie Rural, Eau, Forêts, Tunisia). The preparation of samples for the determination of HM concentrations in sampled soils was carried out according to the protocol of Klavins et al. (2000). HM levels were determined by inductively coupled plasma–optical emission spectrometry (ICP–OES).

Tolerance of rhizobia to heavy metals

Bacterial tolerance to heavy metals was determined by growth inhibition plate assays. Rhizobial strains were first cultivated on yeast extract mannitol (YEM) liquid medium for 48 h under continuous shaking (150 rpm). Aliquots (20 µl) of this pre-culture were cultivated on YEMA–CR medium (YEM agar–Congo Red) plates (10 spots per plate, 3 replicates per isolate) containing increasing concentrations of $CuSO_4$ (up to 1 mM), $CdCl_2$ (up to 0.3 mM), $Pb(NO_3)_2$ (up to 3 mM) or $ZnSO_4$ (up to 3 mM). Ethylenediaminetetraacetic acid (EDTA; 25 mM) was added to the culture media in the lead tolerance test to avoid Pb precipitation. The plates were incubated at 28°C for 72 h. The parameter used to evaluate the levels of tolerance was the maximum tolerable concentration.

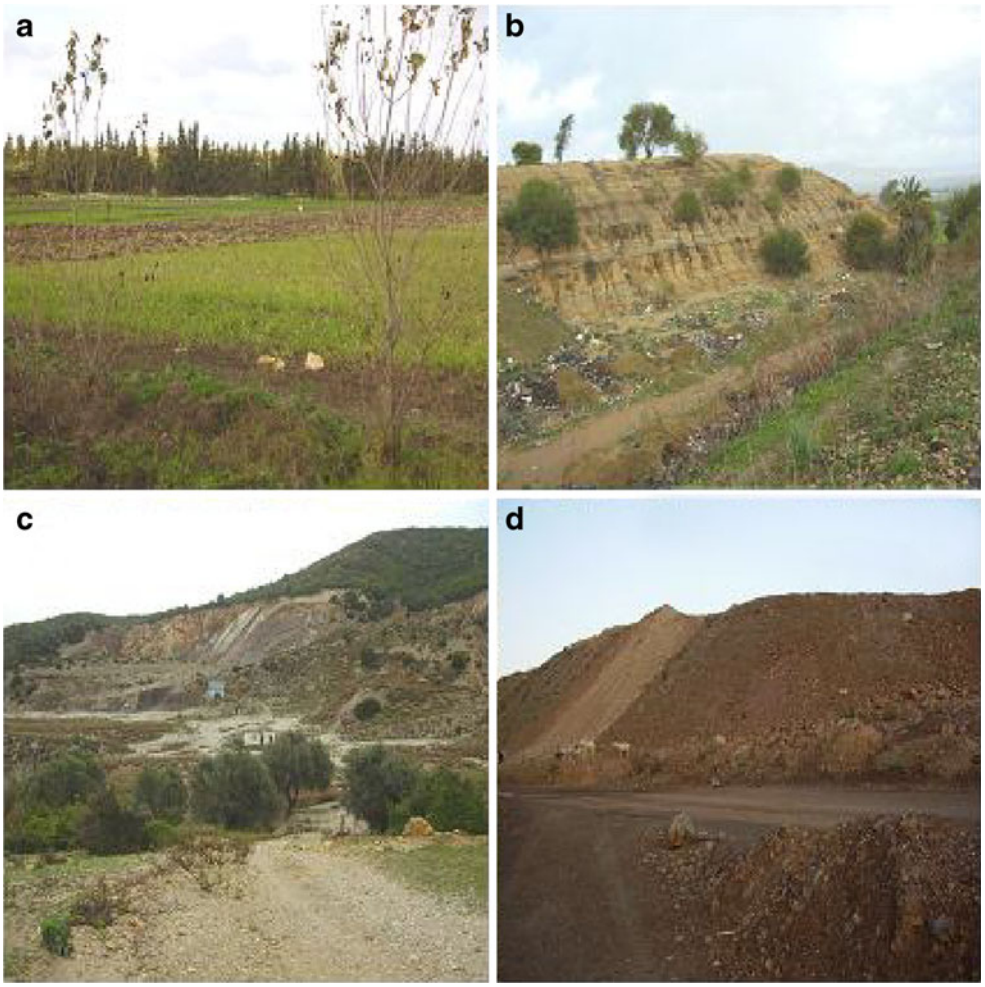

Fig. 1 Sampled sites in the surroundings of mines in northern Tunisia. **a** Mateur, non-mining site, **b** Gzala, Zn/Pb mine, **c**, Ain Allègua, Zn/Pb mines in the Nefza region, **d** Djerissa, iron mine

Determination of metal accumulation in bacterial cells

Accumulation of heavy metals in bacterial dried cells was determined basically as described by Rodríguez-Llorente et al. (2010). Strains S112, S532 and S412 were cultivated in YEM liquid medium for 48 h and the optical density was determined. Bacterial cultures were adjusted to a unique optical density of 0.8, and 1 ml of suspension was inoculated into 100 ml of YEM liquid medium supplemented or not with Cu (300 μM), Cd (50 μM) or Zn (500 μM). Strains S112 and S532 were tested for Cd and Zn accumulation, and strains S112 and S412 were tested for Cu accumulation. For each treatment, two independent cultures were inoculated and three replicates of each treatment were considered. After a 48-h incubation, the cultures were harvested and centrifuged at 10,000 *g* for 10 min at 4°C. One sample of cells of each metal treatment was washed first with distilled water, then with 0.2 M EDTA, pH 8.0, both for 5 min, prior to estimating the amount of metal absorbed in bacterial cells; the other sample was washed only with distilled water to estimate the total amount of metal accumulated by the cells. Finally, the cells were dried in the oven for 48 h at 60°C, and Cd, Cu and Zn accumulation were determined in the dried cells by ICP–OES.

Statistical analysis

Statistica software ver. 5.1 (Statsoft, Tulsa, OK; www.statsoft.com) was used to compare means using the Duncan multiple-range test. Significant differences among treatments were identified at $P=0.05$.

Results and discussion

In this study, the quality of the soil, HM content and presence of rhizobia in soil samples taken from three mining sites located in northern Tunisia (Ain Allègua, Gzala and Djerissa) were determined and compared with those of an agricultural soil. Physical and chemical parameters as well as the density of the *Sinorhizobium* sp. strains in the soil samples are presented in Table 1. In terms of soil texture, the soil from Mateur had a silt–clayey

texture, while all of the others had a loamy texture. The agricultural soil from Mateur had higher levels of nutritive elements (phosphorus and potassium) and organic matter than the other soils, particularly the soil samples from Djerissa, which was characterized by the highest salinity level and lowest level of organic matter (Table 1). The soil sampled at Ain Allègua contained the highest level of nitrogen. Our comparison of the three sampling points in Djerissa showed that soil from sampling points C0 and C2 has the same texture. Soil collected at C0, which is in the mining rejects, contained the lowest level of K_2O (Table 1). The organic matter content in the mining soils was considered to be relatively sufficient for plant development when compared to other Tunisian soils analysed in previous works (Badri et al. 2007; Zribi et al. 2005).

The estimation of rhizobial density in these soils by the MPN method (Vincent 1970) showed that soil from Djerissa, which is not fertile (since it had the lowest level of nutritive elements and organic matter), had an important number of rhizobia, even more than the agricultural soil from Mateur region (Table 1). The difference in rhizobial density between soils could be related to the frequency of the host plants, the dynamic of natural microbial populations and the texture of the soil. The soil collected at Gzala contained the lowest number of rhizobia (Table 1). Moreover, there was no correlation between the MPN and the chemical parameters of the analysed soils, including nitrogen level (data not shown).

Our results showed that soil collected at Gzala contained the highest levels of Pb and Zn, while the agricultural soil from Mateur did not contain high levels of these two HM (Table 2). All of the soils sampled contained similar concentrations of Cd, except for the soil from Gzala, which contained the highest concentration of Cd (26 mg kg^{-1}). The soil sampled in the region of Djerissa at the mining rejects (C0) contained an important amount of Zn compared to the two other sampling points from the same site (Table 2). Nevertheless, the Cd, Cu and Pb levels at these three sampling points at the Djerissa site were similar (Table 2). According to directive 86/278/EEC(JOCE L 181/6 of 04/07/1986), soil at the mining site of Gzala is contaminated with Pb and Zn, and that at Djerissa sampling point C0 seemed to be moderately contaminated with Zn. The levels of HM in the soil samples varied strongly according to the source of contamination and the quality of the soils. Indeed, the high level of Zn (2050 mg kg^{-1}) and Pb (4050 mg kg^{-1}) in the soil samples from Gzala is likely due to the proximity of the sampling points to an ancient Zn/Pb mine.

The ability of *M. sativa* plants to grow and to be nodulated in soils containing different levels of metal pollution was tested (Table 3). *M. sativa* cultivated in the agricultural soil of Mateur showed a significantly higher number of nodules and aerial biomass production than plants cultivated in soils sampled from the mining sites (Table 3). Plants grown in the soil of Ain Allègua showed the lowest number of nodules and shoot dry matter (Table 3). Although the soils from Gzala and Djerissa contained different levels of HM (Table 2), *M. sativa* plants cultivated in these soils presented with a similar number of nodules and produced a similar amount shoot matter (Table 3). The highest growth and nodulation in mining soils were observed in plants cultivated in the Djerissa C2 samples. The behaviour of *M. sativa* plants in contaminated soils has been intensively analysed (Gardea-Torresdey et al. 1996; Lopez et al. 2005; Pajuelo et al. 2008). Our results indicate that *M. sativa* plants were able to grow and to be nodulated in the various mining soil samples tested in our study, but that both plant growth and nodulating parameters were generally lower in these plants than in those grown in the agricultural soil.

Wild-types *M. sativa*-nodulating *Sinorhizobium* sp. strains were also isolated in this study. Among the 19 characterized rhizobial strains, only two were assigned to *S. medicae*—one

Table 1 Physical and chemical parameters and the rhizobial most probable number values of soil samples

Physical and chemical parameters and MPN	Mateur	Ain Allègua	Gzala	Djerissa[a]		
				C0	C1	C2
Clay (%)	31	27	28	17	23	17
Silt (%)	43	29	15	9	19	9
Sand (%)	11	24	34	43	28	42
pH (½.5)	7.95	7.93	7.63	7.45	7.65	8
EC (mmhos cm^{-1})	0.52	0.61	0.58	3.1	1.7	1.4
Organic matter (%)	2.5	3.2	2.2	1.1	1.6	1.2
Carbon (%)	1.5	1.9	1.2	0.6	0.9	0.7
P_2O_5 assimilation (mg kg^{-1})	45	18	23	18	8	13
K_2O (assimilation/1,000)	0.91	0.16	0.21	0.09	0.23	0.3
Nitrogen (%)	1.02	1.55	0.81	0.8	0.61	0.8
MPN (cells g^{-1} of soil)	44	160	32	890	1800	1300

EC, Electrical conductivity; MPN, most probable number

[a]C0, on the mining rejects; C2, 50 m from the mining rejects; C2, 100 m from the mining rejects

Table 2 Amounts of heavy metals in soil samples collected from the different sites

Heavy metals (mg kg^{-1})	Mateur	Ain Allègua	Gzala	Djerissa		
				C0	C1	C2
Cadmium (Cd)	10	10	26	11	10	10
Copper (Cu)	31	26	19	27	35	31
Lead (Pb)	75	202	4450	67	77	77
Zinc (Zn)	325	650	2050	1900	350	700

from the Djerissa C0 site and one from the Gzala site. The remaining strains were assigned to *S. meliloti* species, including strains S112, S412 and S532 (data not shown). The abundance of *S. meliloti* species found in *M. sativa* nodules (17/19 isolates) is in agreement with previous results showing that among 160 rhizobial strains isolated from four *M. sativa* cultivars growing in two different Tunisian soils, 158 strains belonged to *S. meliloti* (Saidi et al. 2009). The dominance of *S. meliloti* in nodules of *M. sativa* has been reported in several previous studies (Andronov et al. 1999; Eardly et al. 1990; Jebara et al. 2001).

The tolerance of the 19 bacterial isolates isolated from *M. sativa* nodules and the *S. meliloti* reference strain, RCR2011, was tested by growth inhibition on YEMA–RC plates. Approximately 90% of the rhizobial strains showed optimal growth in plates containing between 0.1 and 1 mM Zn (Fig. 2a). The percentage of isolates able to survive was reduced to 80% when the Zn level was between 1.2 and 2 mM. At 2.2 mM of Zn, the number of surviving strains was greatly decreased. The lethal concentration for Zn was estimated to be 3 mM. The level of tolerance to Zn shown by these *Sinorhizobium* sp. strains is lower than the amount of this metal in the soil samples. In terms of Cu, concentrations between 25 and 50 μM did not affect the growth of any isolate, but a 20% reduction in rhizobial growth was observed at Cu concentrations ranging from 100 to 600 μM. The drop of growth observed at 800 μM Cu was characterized by a 95% mortality (Fig. 2b).

All *Sinorhizobium* sp. strains were able to survive at 20 μM of Cd; however, 5% of strains were unable to grow at Cd concentrations of between 40 and 150 μM, and a 20 and 80% decrease in rhizobial growth was observed at 0.2 and 0.3 mM Cd, respectively (Fig. 2c). The levels of Cd and Cu measured in the soils therefore did not seem to be stressful for the rhizobia. For Pb, all isolates were able to grow at levels up to 2 mM, but no isolate was able to grow when the medium contained 3 mM Pb.

The toxic effects of HM on rhizobia is known to depend on the bioavailability of the HM, which in turn is primarily related to soil properties (pH, organic matter, among others), thereby explaining the differences between the quantities of HM in soils (only a part of the quantity available in solution comes into direct contact with plant roots) and the level of tolerance tested in the laboratory. Survival in soil can be a consequence of the physical protection provided by clay minerals and organic matter, which allow rhizobia to escape of the effect of HM (Giller et al. 1998; Ibekwe et al. 1997). The degree of rhizobial tolerance to HM previously reported is largely dependent on several parameters, such as rhizobial species, nature of the soils, sources and degree of contamination and growth medium (Carrasco et al. 2005; Delorme et al. 2003; Pereira et al. 2006).

Rhizobial strains S532 from Djerissa C1 and S112 from Mateur were chosen to evaluate Cd and Zn and Pb bioaccumulation due to their different levels of resistance to these metals (S532 was more tolerant than S112). For the same reason, Cu bioaccumulation was estimated in strains S112 and S412 from Djerissa C0 (S412 more tolerant than S112).

In the presence of Zn, the total amount of metal accumulated in S112 cells was fourfold higher than that in S532 cells (Table 4). In both rhizobial strains, most of the

Table 3 Number of nodules and amounts of shoot dry matter of plants cultivated on the soil samples

Parameters	Mateur	Ain Allègua	Gzala	Djerissa		
				C0	C1	C2
NN (*n* $plant^{-1}$)	27.8±7.7 a	5±2.5 c	11.8±2.5 b,c	10±0.7 c	8.8±1.7 c	18.8±4.0 b
SDM (mg $plant^{-1}$)	191.3±22.4	38.7±9.8 d	72.8±13.7 b,c	55.7±13.6 c,d	48.2±10.2 c,d	84±18.0 b

NN, Number of nodules; SDM, shoot dry matter

Values are given as the mean ± the standard deviation (SD). At the row level, means followed by different lowercase letters are significantly different at P=0.05 based on Duncan's multiple range test

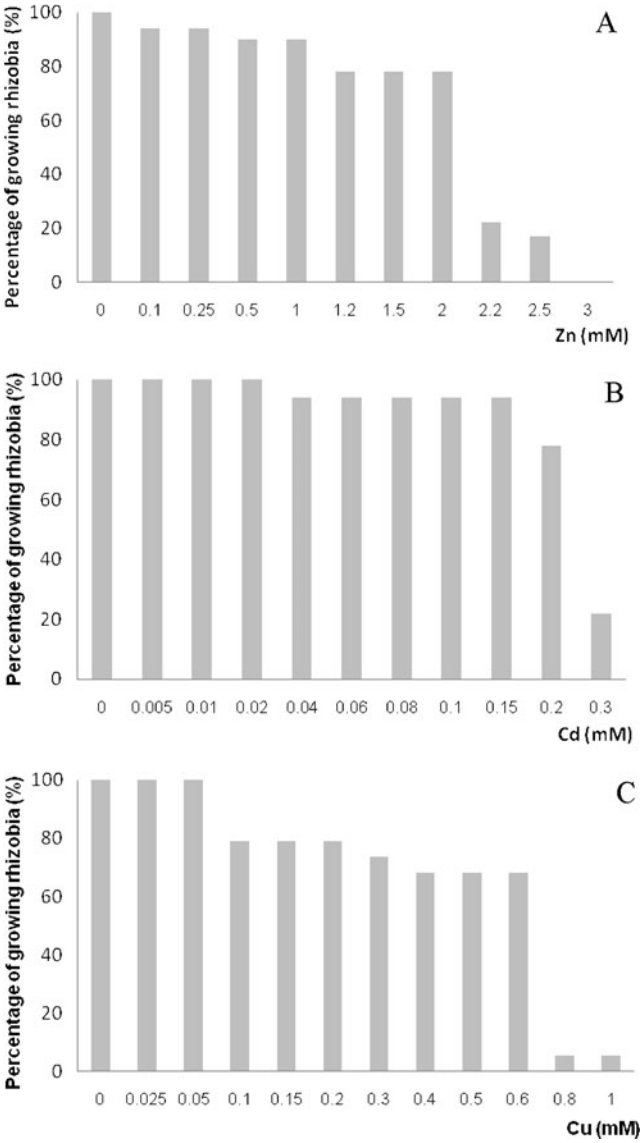

Fig. 2 Viability of rhizobia (20 strains) in the presence of increasing concentrations of Zn (**a**), Cd (**b**) and Cu (**c**)

Zn was accumulated by biosorption to the cell surface (adsorbed), as can be seen by the amount of metal accumulated after washing with EDTA. The amount of Zn accumulated within the cells (absorbed) was higher in the S532 strain than in the S112 strain. The total amount of Cd accumulated and absorbed by bacterial cells was 2.4- and 2.1-fold higher, respectively, in strain S112 than in strain S532 (Table 4). In the presence of $Pb(NO_3)_2$, the total accumulation of Pb was twofold higher in S112 than in S532. In addition, the amount of biosorption of Pb was threefold higher in S112 than in S532 (Table 4).

Regarding Cu bioaccumulation, the amount of metal absorbed into both S112 and S412 cells was higher than that adsorbed to the cell surface. The adsorption in S112 was greater than in S412, but the latter strain was more efficient in terms of absorbing Cu (Table 4).

The variable degree of tolerance to HM described in our study is mostly related to the efficiency of mechanisms developed by rhizobia to detoxify these elements. Rough et al. (1995) reported that bacteria have developed five different mechanisms to detoxify HM: export, reduced permeability, extracellular detoxification, intracellular sequestration and extracellular sequestration. In our study, we tested two strains with different levels of resistance to the effects of HM, namely, strains S532 and S112 for Cd, Pb and Zn and strains S412 and S112 for Cu, in order to better understand the bacterial metal tolerance mechanism in these strains. The total bioaccumulation of both Cd and Zn was higher in the sensitive strain, S112, with most of the metal adsorbed to the cell surface (Table 4). The behaviour of this strain is probably related to its capability of Cd and Zn biosorption. On the other hand, it would appear that the tolerant strain (S532) avoids the external and internal bioaccumulation of Cd and Zn, suggesting the possibility that mechanisms of reduced permeability and/or export of the metal are being used by this strain.

In terms of the bacterial behaviour in the presence of Cu, the response of both *Sinorhizobium* sp. strains was the activation of the intracellular bioaccumulation of this

Table 4 Levels of Cd, Cu, Pb and Zn bioaccumulation in rhizobia strains nodulating *Medicago sativa*

Heavy metal treatment	Wash solution	*Sinorhizobium* strains[a]		
		S112	S412	S532
Cd	H_2O	11,192±446.7	ND	4,817±191.8 b
	EDTA	1,497±58.9 a	ND	796±30.8 b
Cu	H_2O	414±15.6 a	376±14.0 a	ND
	EDTA	297±10.9 b	333±12.3 a	ND
Pb	H_2O	13,420±535.9 a	ND	6,410±255.4 b
	EDTA	64.3±1.6 a	ND	22.1±0.9 b
Zn	H_2O	2,871±113.8 a	ND	719±27.7 b
	EDTA	109±3.3 b	ND	263±9.5 a

ND, Not determined

[a]Values are expressed in units of mg kg^{-1} of cell dry matter and given as the mean ± SD. At the row level, means followed by different lowercase letters are significantly different at $P=0.05$ based on Duncan's multiple range test

element; this was particularly evident for the resistant strain S412. In this case, most of the Cu remained within the cell (Table 4), since most of the metal accumulated was washed out with EDTA. Our results therefore suggest that the isolated strains used different mechanisms of metal resistance, although more experiments are needed to elucidate the specific resistance mechanism activated in each case.

In conclusion, *M. sativa* plants were able to grow and to be nodulated in the HM concentrations found in these soils. However, there was a reduction of growth and nodulation in these soils compared to growth and nodulation in the agricultural soil. In addition, we have isolated a collection of *M. sativa*-nodulating rhizobia with different levels of tolerance to HM and different HM bioaccumulation potential. The symbiotic efficiency, tolerance and biosorption of HM are essential criteria for the selection of rhizobial strains which could be of interest as inoculants for the regeneration and enrichment of moderately contaminated soils.

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