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Heavy metal toxicity in *Rhizobium leguminosarum* biovar *viciae* isolated from soils subjected to different sources of heavy-metal contamination: Effects on protein expression

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Abstract

Heavy metals adversely influence microorganisms, affecting their growth, morphology and activities. Metals also can exert a selective pressure on the organisms, resulting in microbial populations with higher tolerance to metals. Given the importance of legumes in animal and human consumption and their use in maintaining soil fertility, some attention has been given to the effects that heavy metals exert on *Rhizobium* isolates. In this context, *Rhizobium leguminosarum* biovar *viciae* was isolated from areas with different heavy metal contents and their tolerances were compared. Alterations in the protein pool of *Rhizobium* populations were also evaluated. Physicochemical parameters were determined and heavy metal concentrations in soils were analysed by ICP-AES. Isolates were screened for their tolerance in YEM media supplemented with different heavy metals (Zn, Pb, Co, Cd, Ni, Cr). Proteins were extracted and separated by SDS-PAGE. EI_1 and EI_2 (engineering industries) soils presented the highest metal concentration, and were therefore the most polluted soils. Isolates showed different growth responses to heavy metals. C (control soil) and M (mines) isolates were less tolerant than EI_1, EI_2 and CI (chemical industries) isolates. Metals influenced their protein profiles, most of the alterations corresponding to decreases in polypeptide expression. However, in tolerant isolates these alterations corresponding basically to increases, as occurred in CI isolates.

This work suggests that there is a relationship between *Rhizobium*'s tolerance, heavy metal soil contamination and alterations in protein pool. As a result, the analysis of protein alterations seems to be a good indicator to estimate the level of stress imposed on *Rhizobium* populations submitted to heavy-metal contamination.

Keywords: Metal tolerance; Protein alterations; Rhizobia; Soil contamination

1. Introduction

Heavy-metal contamination is now widespread (Nriagu, 1990). Soil may become contaminated with metals from a variety of anthropogenic sources, such as smelters, mining, power station industry and the application of metal-containing pesticides, fertilizers and sewage sludge (Giller et al., 1989; McGrath et al., 1995; Robinson et al., 2001). In recent decades there has been increasing concern with heavy metal contamination, not only because of their toxicity to animals, plants and microorganisms, but also because they become irreversibly immobilised in soil components (McGrath and Lane, 1989). The amounts of metals accumulated in soils are dependent on the emission

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levels, the transport of the metal from the source to the accumulation site and the retention of the metal once it has reached the soil (Alloway, 1995). Some metals such as Zn, Cu, Ni and Cr are essential or beneficial micronutrients for plants, animals and microorganisms, whereas others, such as Cd, Hg and Pb have no known biological and/or physiological functions. However, all these metals could be toxic at relative low concentrations (Gadd, 1992). When exposed to moderate heavy metal concentrations, soil microorganisms were found to be very sensitive (Giller et al., 1998). Several studies have shown that metals adversely influence microorganisms (Shi et al., 2002), affecting their growth, morphology and activities (Bååth et al., 1998; Lakzian et al., 2002; Khan and Scullion, 2002), including symbiotic N_2 fixation (McGrath et al., 1988). These metals exert a selective pressure on the organisms, resulting in microbial populations with higher tolerance to metals, but with lower diversity, when compared to unpolluted neighbouring areas (Bååth, 1992; Bååth et al., 1998).

Rhizobium spp. are gram-negative soil bacteria that have a profound scientific and agronomic significance due to their ability to establish nitrogen-fixing symbiosis with leguminous plants, which is of major importance to the maintenance of soil fertility (Somasegaran and Hoben, 1994). For this reason and taking into consideration the importance of legumes in animal and human consumption, some attention has been given to the effects that heavy metals exert on *Rhizobium* isolates as free-living organisms or symbiotically associated with legumes (Ibekwe et al., 1995).

In the present study, we investigated the effects of increasing levels of heavy metals on indigenous *Rhizobium* populations isolated from soils subjected to different anthropogenic activities, such as mining, chemical effluents and engineering contamination. In order to reach this goal we aimed to establish a relationship between the tolerance levels and the natural conditions experienced by the populations in their place of origin. Since protein synthesis is a cellular process very sensitive to changes in cell homeostasis (Yeo, 1998), we also examined alterations in protein expression in *Rhizobium* isolates from each location, with the purpose to evaluate the level of stress imposed on each isolate. These approaches can provide tools that could help to predict the impact of such activities on the microflora of neighbouring soils. Furthermore, *Rhizobium* can be used as an indicator organism to several toxic chemicals, including heavy metals as referred by Botsford (1999).

2. Material and methods

2.1. Site selection and soil sampling procedures

Five composite soil samples were collected from arable field in the centre of Portugal (Aveiro region). Soils were taken from the plough layer at 0–20 cm depth. When the soil samples were collected the host plant was absent from all soils.

A non-heavy metal contaminated agricultural soil was used as a control (C). The other soils have been exposed to different sources of heavy metals: EI_1 and EI_2 soils were collected from an area in close proximity (1 and 2 km, respectively) to engineering industries that have been in operation for nearly 30 years; M soil was placed in a river margin directly influenced by lead mining for 100 years, however, this activity ceased 30 years ago; CI soil has been influenced during the last 30 years by effluents resulting from chemical industry, however, due to effluent pre-treatment in the last 10 years, the heavy metal input became quite smaller.

2.2. Isolation of Rhizobium

Rhizobium was isolated from the root nodules of 30 days old *Pisum sativum* L. plants grown in a greenhouse with natural light, at 22/15 °C day/night temperature and 55–75% relative humidity, in containers filled with contaminated (EI_1, EI_2, M, CI) soils and non-contaminated soil (C). Nodules were sterilized in 2.5% sodium hypochlorite for 2 min following a rinse in 95% ethanol and washed thoroughly in six changes of sterile water. Surface sterilized nodules were streaked on to the surface of yeast extract-mannitol (YEM) agar containing Congo Red and *Rhizobium* isolates obtained as described by Somasegaran and Hoben (1994).

2.3. Physicochemical analysis of soil

The soil pH and redox potential were determined in the field using an Orion pH/Eh meter model 290A. The water content was determined by drying 50 g of soil at 60 °C until the weight was constant. Soil organics matter content was determined by loss on ignition at 550 °C using a muffle furnace following the method of Castro (1999).

2.4. Determination of soil heavy metal content

For heavy metal concentration analysis representative soil sub-samples were air dried and sieved (<2 mm). Concentrations of Zn, Pb, Co, Cd, Ni and Cr were

determined, after extraction with aqua-regia (McGrath and Cunliffe, 1985), by Inductively Coupled Plasma Atomic Emission spectrometry (ICP-AES).

2.5. Screening for heavy metal tolerance

Heavy metal tolerance of *Rhizobium* isolates was screened by plating in YEM media supplemented with metal (Zn, Pb, Co, Cd, Ni and Cr) of increasing concentrations: 0, 0.065, 0.125, 0.165, 0.210, 0.250, 0.500, 0.750, 1.000, 2.000 and 3.000 mM. For growth measurements colonies were harvested, suspended in double-distilled sterile water (ddH_2O) and optical density (620 nm) was determined (Figueira et al., 2005). The highest concentration of heavy metals supporting growth was defined as the maximum resistance level (MRL). In order to determine the population's tolerance levels the MRL percentage of *Rhizobium* isolates from each location was determined to all metals.

2.6. Protein extraction and SDS-PAGE electrophoresis

Four isolates were selected from each location and were grown in YEM media, supplemented with different metal concentrations. According to their threshold of tolerance (around 70% of growth inhibition), cells were harvested by centrifugation for 15 min at 4000 × *g* and 4 °C, and ressuspended in 200 μl of treatment buffer (Hames, 1981). Samples were then sonicated for 1 min with an ultrasonic probe and boiled for 5 min at 95 °C. Lysates were centrifuged to remove cell debris and the supernatant was collected. Proteins were separated by SDS-PAGE, carried out in 12.5 and 18% acrylamide slab gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad) and screened in a Densitometer apparatus (Bio-Rad – Model GS 710). The molecular weight was determined comparing with a protein standard (Broad Range Prestained Standard, Bio-Rad) and relative amount of proteins corresponding to each band were calculated using Quantity One Program Software (Bio-Rad) (Figueira et al., 2005).

2.7. Statistical analysis

To assess the overall differences in the properties of the soils from various sites, statistically significant differences in the mean values of water content, pH, redox potential, organic matter and heavy metal concentrations were determined by one-way analysis of variance (ANOVA). Values are means of three replicates.

3. Results

3.1. Physicochemical analysis and heavy metal contamination of soils

The physicochemical properties of soils and heavy metal concentrations are shown in Tables 1 and 2.

Soils presented different physicochemical properties. pH values varied significantly ($P < 0.05$) between locations, with the M soil being quite acid. Furthermore, this soil also presented high levels of organic matter.

Heavy metal concentration of soils varied significantly ($P < 0.05$). Contaminated soils had higher concentrations of heavy metals than the control soil (C), with the exception of CI, where the concentrations determined were similar or slightly lower than C, evidencing a lack of contamination at the time of sampling. EI_1 and EI_2 showed the highest concentrations of metals especially of zinc where this metal was near or exceeding EC limits (CEC, 1986) and are therefore the most polluted soils.

3.2. Isolates heavy metal tolerance

Rhizobium leguminosarum biovar *viciae* isolates from different locations presented different responses to heavy metal stress. Isolates from C soil were in general

Table 1
Physicochemical analysis of soils from different locations

Soil	Physicochemical analysis			
	Water content (%)	pH	Redox potential (Eh)	Organic matter (%)
C	19.1 ± 0.67^{a}	$5.5 \pm 0.07^{a,b}$	$376.3 \pm 42.25^{a,b}$	5.87 ± 0.06^{a}
EI_1	$21.3 \pm 0.90^{a,b}$	$6.1 \pm 0.08^{b,c}$	$376.3 \pm 28.75^{a,b}$	6.24 ± 0.40^{a}
EI_2	$25.1 \pm 0.19^{b,c}$	$5.8 \pm 0.06^{a,b}$	$392.7 \pm 3.77^{a,b,c}$	8.39 ± 0.25^{b}
M	$24.8 \pm 0.09^{b,c}$	4.7 ± 0.04^{d}	457.8 ± 21.81^{d}	10.6 ± 0.58^{c}
CI	9.5 ± 0.49^{d}	5.3 ± 0.05^{a}	265.3 ± 45.54^{e}	5.0 ± 0.01^{d}

C, control isolates; EI_1 and EI_2, engineering industry isolates; M, mining isolates; CI, chemical industry isolates. Data are the mean ± S.E. from three replicate measurements. Different superscripts letters (a–d) represent significant differences ($P < 0.05$) between values at same column.

Table 2
Total metal concentration (mg kg^{-1}) and pH of soils of different locations

Soil	Metal concentration (mg kg^{-1}dry soil)					
	Zn	Pb	Co	Cd	Ni	Cr
C	37.85 ± 17.98^{a}	7.92 ± 3.25^{a}	1.48 ± 0.01^{a}	0.04 ± 0.01^{a}	4.93 ± 0.14^{a}	4.10 ± 3.75^{b}
EI_1	464.97 ± 7.10^{b}	49.33 ± 1.06^{b}	1.41 ± 0.03^{a}	0.17 ± 0.01^{b}	43.56 ± 0.26^{b}	62.54 ± 0.93^{c}
EI_2	299.49 ± 15.27^{c}	59.40 ± 2.29^{c}	4.27 ± 0.16^{b}	0.40 ± 0.02^{c}	37.17 ± 1.42^{c}	35.53 ± 2.06^{d}
M	88.09 ± 5.55^{d}	20.81 ± 6.43^{d}	9.00 ± 0.54^{c}	0.24 ± 0.01^{d}	17.71 ± 1.09^{d}	1.62 ± 1.14^{a}
CI	23.7 ± 5.20^{a}	8.0 ± 0.75^{a}	0.7 ± 0.09^{d}	0.04 ± 0.01^{a}	1.68 ± 0.13^{e}	2.66 ± 0.10^{a}
EC limits[a] (pH < 5.5)	150	50	–	1	30	50
EC limits[a] (pH 5.5–7)	300	300	–	3	75	200

C, control isolates; EI_1 and EI_2, engineering industry isolates; M, mining isolates; CI, chemical industry isolates. Data are the mean ± S.E. from three replicate experiments. Different superscripts letters (a–e) represent significant differences ($P < 0.05$) between values at same column.
[a] EC limits for sewage sludge treated soils (86/278/CEC) (CEC, 1986).

less tolerant to all metals. CI isolates presented high tolerance to most heavy metals tested. The MRLs of the CI isolates were 3 mM for Pb, 1 mM for Zn and higher than 0.75 mM for Cd (Table 3). EI_1, EI_2 and M isolates showed similar responses to all heavy metals, however some EI_2 isolates were more sensitive to Ni and Pb than EI_1 and M isolates. On the other hand, some M isolates were more sensitive to Pb than EI_1 isolates (Table 3).

3.3. Alterations in protein expression

The effects of heavy metals on protein expression of isolates from different locations are shown in Fig. 1 as percentage of protein expression alterations (increases/decreases). Results were more influenced by isolates origin than by the heavy metal causing the stress. For every heavy metal, C isolates showed the higher percentage of decreases and the lower of increases, while CI isolates responded in the opposite way, with the higher percentage of increases and with the lower of decreases. In general, *Rhizobium* isolates showed similar quantitative alterations when exposed to different metals; however some differences were detected: in EI_2 isolates Zn and Pb enhanced protein expression, whereas Co mostly provoked decreases; when M isolates were subjected to Co, Ni, Cr and Cd most of the alterations corresponded to decreases of the protein expression, while Zn and Pb induced increases. All metals affected similarly the protein expression of C and CI isolates.

4. Discussion

Long-term metal deposition into soil results in high metal concentrations, which therefore affects negatively soil microflora (Smith and Giller, 1992; Matsuda et al., 2002). Soil is a complex environment where bacteria growth and development can be influenced by different edaphic factors, such as pH or organic matter content (Gadd and Griffiths, 1978; Saeki et al., 2002). Ibekwe et al. (1997) showed that under conditions of lower soil pH, the numbers of rhizobia were significantly reduced and the number of nodules observed on plants was also significantly lower. In this study, the pH values of most soils were below the optimal pH (6–7) for rhizobia growth (Table 1), however *Rhizobium* was able to withstand these pH values.

Hirsch et al. (1993) and Ibekwe et al. (1997) reported that the distribution of *Rhizobium* isolates in agricultural soils is affected by the presence of the host plant, which generally leads to an increase in population size that will persist in soil for some years. Rhizobia were isolated from soils where no host plants were grown on the last few years, which lead us to conclude that rhizobia was far more vulnerable to the direct influence of heavy metal contamination. In other reports (Chaudri et al., 1993), although the majority of metal concentrations determined in soils were below the EC limits (CEC, 1986) they could be enough to eliminate *Rhizobium* from soils. Nevertheless, in this work, we were able to isolate rhizobia from all soils, indicating that these bacteria were able to survive under the metal concentrations found. According to Ibekwe et al. (1997) and Giller et al. (1998) survival can be related with the physical protection of clay minerals and organic matter or with the existence of microsites where metal contamination may be minimal. These ‘‘niches’’ may harbour rhizobia that are not resistant to heavy metals. When analysing metal tolerance in different isolates, we chosen the highest metal concentration that the isolates could tolerate, rather than using the soil concentrations. This was made in order to test if their previous environmental exposure provided them a high tolerance in superior degrees of exposure, since we already new

Table 3
Percentage of MRLs of *Rhizobium leguminosarum* biovar *viciae* isolates from C, EI_1, EI_2, M and CI soils in YEM supplemented with different heavy metals concentrations (0–3 mM)

	Heavy metal concentrations (mM)									
	0	0.065	0.125	0.210	0.250	0.500	0.750	1.000	2.000	3.000
C isolates										
Zn	11.1	–	–	–	11.1	55.6	–	22.2	–	–
Pb	11.1	–	–	–	–	–	–	11.1	44.4	33.3
Co	11.1	–	11.1	–	44.4	–	–	33.3	–	–
Cd	–	–	–	66.7	22.2	11.1	–	–	–	–
Ni	–	77.8	22.2	–	–	–	–	–	–	–
Cr	–	–	–	–	22.2	–	–	77.8	–	–
EI_1 isolates										
Zn	–	–	–	–	–	10.0	15.0	–	75.0	–
Pb	–	–	–	–	–	–	–	–	30.0	70.0
Co	10.0	–	20.0	–	–	70.0	–	–	–	–
Cd	–	–	–	18.2	36.4	27.3	9.1	–	9.1	–
Ni	–	95.0	5.0	–	–	–	–	–	–	–
Cr	–	–	–	–	–	–	–	–	100.0	–
EI_2 isolates										
Zn	–	–	–	–	–	35.3	35.3	–	29.4	–
Pb	29.4	–	–	–	–	–	–	–	23.5	47.1
Co	5.9	–	5.9	–	–	82.4	–	–	5.9	–
Cd	–	–	–	28.6	21.4	28.6	14.3	7.1	–	–
Ni	5.9	88.2	–	–	–	5.9	–	–	–	–
Cr	–	–	–	–	–	–	–	–	100.0	–
M isolates										
Zn	–	–	–	–	–	29.4	23.5	–	47.1	–
Pb	–	–	–	–	–	–	–	17.6	64.7	17.6
Co	–	–	54.5	–	–	24.2	–	–	21.2	–
Cd	–	–	–	8.0	36.0	32.0	8.0	12.0	4.0	–
Ni	–	23.5	44.1	–	–	32.4	–	–	–	–
Cr	–	–	–	–	–	–	20.6	–	79.4	–
CI isolates										
Zn	–	–	–	–	–	–	–	100.0	–	–
Pb	–	–	–	–	–	–	–	–	–	100.0
Co	–	–	14.3	–	–	57.1	–	28.6	–	–
Cd	–	–	–	–	–	–	28.6	28.6	42.9	–
Ni	–	–	28.6	–	–	71.4	–	–	–	–
Cr	–	–	–	–	–	–	–	100.0	–	–

Isolates were obtained from root nodules of *Pisum sativum* L. plants as described. Data are the mean from three replicate measurements. The notation (–) indicated that MRLs did not fall in this concentration.

from previous experiments that they could tolerate the environmental concentrations without showing significant growth inhibition.

Rhizobium isolates tolerated higher heavy metal concentrations when compared with others studies. Angle et al. (1993) presented in their study Cd, Zn and Ni MRLs to *Rhizobium leguminosarum* biovar *viciae* 10, 3 and 14 times lower, respectively, than the MRLs observed in this work.

Engineering industries contributed to the increase of Zn, Ni, Cr and Pb concentrations in EI_1 and EI_2 soils. Mining also contributed to an increase of Zn, Ni and Pb in M soil. EI_1 and EI_2 were the most contaminated soils and presented the highest Zn concentration, in both cases EC limits were reached (Table 2). Positive relationships between the amounts of heavy metals in these soils and the levels of *Rhizobium* tolerance were evident, since EI_1 and EI_2 isolates were in general tolerant to all metals studied. This finding is consistent with the results of a study performed by Díaz-Raviña et al. (1994), who showed that the metal resistance patterns of bacterial populations were related to the total concentrations of metals in the soils. Furthermore, multiple heavy metal tolerance has been already

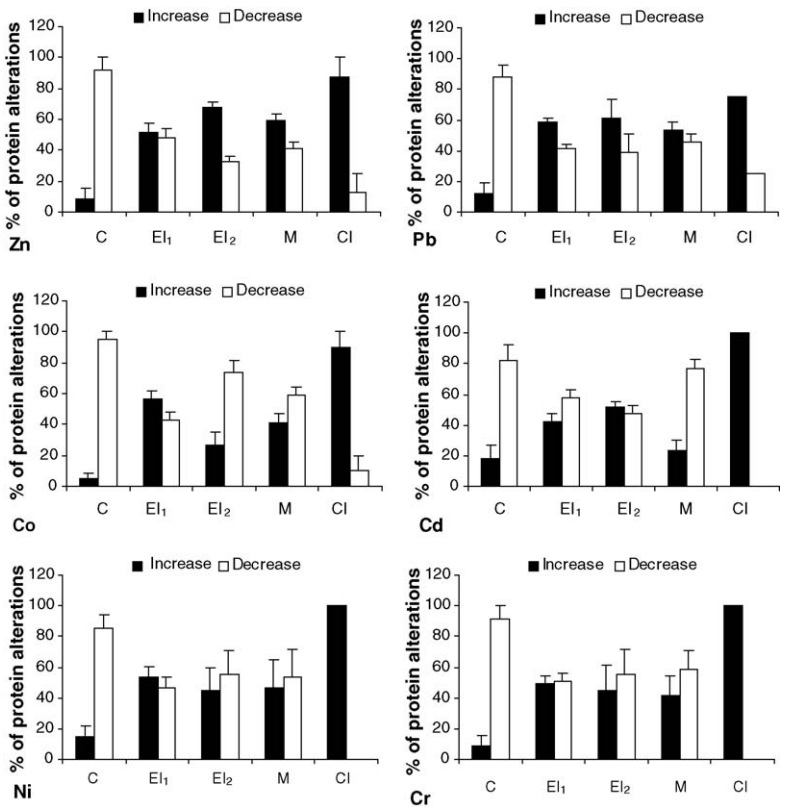

Fig. 1. Percentage of protein alterations induced by heavy metal stress in isolates from different locations: C, control; EI_1 and EI_2, engineering industry isolates; M, mining isolates; CI, chemical industry isolates. Black, increase protein expression; white, decrease protein expression. Data are the mean from three replicates. Vertical bars represent S.E.

demonstrated (Chander and Brookes, 1993; Díaz-Raviña et al., 1994; Doelman et al., 1994).

CI and C soils presented similar heavy metal concentrations, but metal tolerance was different between isolates from each location. CI isolates were extremely tolerant to all metals while C isolates were in general moderately tolerant. Bååth (1992) and Bååth et al. (1998) considered the possibility that those heavy metals exert a selective pressure in bacteria of contaminated soils, resulting in the presence of more tolerant organisms in these areas. CI isolates were well adapted to stress conditions, because heavy metal contamination occurred during the last 30 years. However, in the last 10 years the heavy metal inputs in CI soil, resulting from chemical industry were reduced due to pre-treatment of wastewater and for this reason the soil contamination has decreased drastically. The *Rhizobium* population of CI soil do not reflect these new conditions but instead, reflect a background of contamination. Therefore, the investigation of populations subjected to strong heavy metal contamination in the past is of major importance, not only to investigate the degree of stress imposed but also because we can estimate how many years populations need to recover and to adapt to new conditions.

Imposition of any stress to bacteria results in adaptive responses that lead to changes in the regular metabolic process in the cells, which are then reflected in the alteration of the protein profiles (Saxena et al., 1996). It is generally assumed that the increases of protein expression reflect a positive attempt of cells to adjust to the new environmental conditions, whereas the decreases of expression, in special if they occurred in a high number of proteins, is indicative of disruption of cellular metabolism. In the case of *Rhizobium*, under metal exposure it was observed increases/decreases of polypeptides expression (Fig. 1). C isolates were the less tolerant isolates and most of protein alterations corresponded to decreases, suggesting a deleterious effect of metals on basic cell metabolism. In EI_2 isolates Zn and Pb induced increases in the protein expression. Because the soil concentrations of both metals were

high, our findings lead us to suggest that EI_2 population is well adapted to the condition prevailing in its site of origin. CI isolates were the most tolerant and showed concomitant high increases of protein expression, which leads us to conclude that, these isolates expressed mechanisms, that allowed them to enhance resistance to metals. A higher protein expression was usually related to tolerance mechanisms in *Rhizobium* according to Saxena et al. (1996).

These findings lead us to conclude that there is a relationship between *Rhizobium*'s tolerance and the alterations in protein pool. Thus, the analysis of protein alterations seems to be a good indicator to estimate the level of stress imposed to *Rhizobium* populations. Furthermore, this work showed that *Rhizobium* is a sensitive species that can help to predict the impact of heavy metals on agricultural soils submitted to contamination until the present or that have cessed or drastically decreased some time ago.

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