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Role of Some Chemical Compounds on the Detoxification of *Rhizobium leguminsarum biovar vicia* by Some Heavy Metals

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Abstract: The toxic effect of different concentrations of some heavy metals (Cadmium, Zinc and Lead) on the growth of cultured *Rhizobium leguminsarum biovar vicia* was studied and their LD_{50} toxicity were determined. The 50% inhibition of bacterial growth was achieved by contaminating the culture with 2.1 μM of Cadmium, 30 μM of Zinc or 290 μM of Lead. In attempts to counteract the toxic effect of these heavy metals, several compounds were tested to reactivate the *Rhizobium* growth and to abolish the toxic effect, either partially or totally, which is caused by the presence of heavy metals (as in case of presence of sewage sludge). Positive results were obtained from the addition of some of these compounds as: Mannitol and Glutamate, 5,7 dihydroxyflavone, Thiamine hydrochloride, Calcium chloride and Calcium ionophore A23187. Their most effective concentrations were determined for each compound in the presence of each heavy metal at its concentration of LD_{50}. An average recovery in bacterial growth, in presence of each of Cadmium, Zinc and Lead, individually, were as fellows: 97, 90, 88, 84 and 83%, due to the individual addition of 214 mM mannitol + 42 mM glutamate, 0.96 mM thiamine hydrochloride, 7.79 mM calcium chloride, 2.98×10^{-3} mM calcium ionophore A_{23187} and 0.69 mM of 5,7-dihydroxy flavone, resepectively.

Key words: Calcium ionophore, dihydroxyflavone, heavy metals, *Rhizobium leguminsarum biovar vicia*, thiamine

INTRODUCTION

The fixation of atmospheric nitrogen represents a major source of nitrogen input in soils. The nitrogen fixing systems are symbiotic systems, which can play a significant role in increasing soil fertility. The *Rhizobium* legume symbiosis is superior to other nitrogen fixing systems due to its high potential. Therefore, this system had been examined extensively. Moreover, the behavior of this system under harsh environmental conditions such as salt and drought stress, acidity, alkalinity and nutrient deficiency, showed a great suppress in the growth and in the symbiotic characteristics of *Rhizobium*[1]. The application of sewage sludge to agricultural soil was found to contain a variety of toxic heavy metals.

These heavy metals are known to persist in the soil over long periods and have ecotoxicological effects on plants and microorganisms[2]. *Rhizobia* are considered the most important soil bacteria because of their ability to supply legumes with nitrogen, through the mechanism of nodulation and the symbiotic atmospheric nitrogen fixation. Unfortunately, sewage sludge contains compounds, which is potentially toxic like heavy metals (Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn)[2].

The symbiotic interaction between the *Rhizobium* and the leguminous plants results in the production of root nodule, which in turn infect the bacteria via infection thread which results in nitrogen fixation. This signaling mechanism between the *Rhizobium* and the host plant occur through the secretion of the host plant root to flavonoids[3]. These compounds induce the transcription of *Rhizobium* node genes (early nodulation gene), which is responsible for the secretion of a lipo-oligopolysaccharide compound. This node gene produces several flavones compounds. One of these is dihydroxyflavone, which was added to *Rhizobium* culture, in absence of heavy metals and showed enhancement in *Rhizobium* growth[4,5]. Therefore, this lipo-sugar produces several signals, which is responsible for nodule formation[6]. Since soil *Rhizobium* has the ability to produce the acidic Exopolysaccharide (EPS), which is required for invasion of leguminous plants root nodule by *Rhizobium*[7]. Therefore, its synthesis was increased by the addition of 10 g L^{-1} mannitol and 2 g L^{-1} glutamate to cultured *Rhizobium*[8]. Moreover, this treatment resulted in the maximum production of 2 g L^{-1} EPS.

Although Zinc and Copper are required for *Rhizobium* growth, high concentrations of these two

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elements were considered toxic and that *Rhizobium* tolerance was only to a contaminant of 2,000 mg of Zn kg^{-1} of soil and 300 mg of Cu kg^{-1} of soil[9,10]. It appears that small amounts are required for *Rhizobium* growth, while larger amounts were found to be toxic[11,12].

Other researchers[13] determined the potential impact of the sewage sludge (source of combined heavy metals) application to soil on the presence and activity of nitrifying bacteria. This sort of studies might have its difficulties in not being able to determine the harmful effect of each of the individual heavy metal. Besides, there might be other factors in the soil, which can interfere with the obtained results of the adverse effect of heavy metals on the soil *Rhizobium* activity. These factors, which might vary from one soil to another, are mainly: pH, other salts in soil, fungi and others. Therefore, we propose, in this present study, *in vitro* cultured experiments as the most appropriate approach to study the effect of each of the heavy metals of interest on *Rhizobium* activity, since, all the experiments are subjected to the same standard conditions of pH, salinity, nutrient composition and temperature. Therefore, carrying these studies *in vitro* showed the advantages of eliminating all of the previously mentioned interfering factors in the soil.

In cultured experiments of the bacteria *Rhizobium leguminosarum*[14,15] the researchers revealed that the addition of 3 mM calcium ion resulted in the recovery of the *Rhizobium* growth after being stressed by low pH. Other scientists showed that calcium played a significant role in increasing the K^+ uptake, through calcium-dependent cell surface components that influence the attachment of *Rhizobium* to root hair cells[16]. The nodulation capacity of legumes at low pH and in presence of 0.1 mM aluminum was decreased, particularly at low Ca^{2+} levels (0.3 M), while nodulation was recovered by increasing Ca^{2+} level up to (3.0 mM)[17].

Moreover, the proposed activators, used in this current study, were examined at different concentrations in order to find the most effective concentration, which shows a positive result in reverting, either completely or partially the inhibition effect of the studied heavy metals. The great advantage of the present study lies at the intracellular level, where the mechanism of action of these activators at the cellular level has to be further investigated. Therefore, cultured bacterial cells were used in this current study for two purposes. Firstly, the determination of LD_{50} of the chosen toxic heavy metal. Secondly, to test the ability of some chemical compounds in preventing the harmful toxic effect of these heavy metals, in order to recover the bacterial normal growth. The physiological mode of action of the successful compound (named activator in this present study) on the *Rhizobium* system can be further studied and used as a model for explaining the relationship between the inhibitory mechanism of these metals and the intracellular mechanism for glycoprotein synthesis and secretion.

MATERIALS AND METHODS

The study was conducted at the Female Faculty of Sciences for a period of about one year.

Chemicals: All chemicals and salts were purchased from B.D.H except for thiamine hydrochloride, calcium ionophore A_{23187} (hemi calcium salt) and 5,7-dihydroxyflavone was purchased from Sigma Company.

Bacteria: *Rhizobium leguminosarum biovar vicia* was kindly donated by Dr. Essam A. Koreish, University of Agriculture, Alexandria University. Its Strain was routinely identified on the basis of Gram's reaction, morphological and biochemical tests. This strain is sensitive to heavy metal toxicity.

Media and growth conditions: *Rhizobium* strain were grown in Yeast mannitol Broth, YMB[18] (0.5 g K_2HPO_4, 0.1 g NaCl, 10 g mannitol, 0.4 g yeast extract and 0.2 g $MgSO_4$) solid or liquid media. The growth rates were determined by inoculating 150 mL of YMB with 0.25 mL of *Rhizobium* culture. Cultures were grown at 28°C with shaking (200 rpm) and at 2 h intervals (up till 48 h), cell density of 3 mL aliquots were measured at 600 nm in spectronic 401 spectrophotometer.

Measurement of the generation time: Cells were prepared as previously, except that Yeast Mannitol Agar[19] (YMA) media was used and incubated at 28°C in a shaker water bath (200 rpm). The growth was followed every 24 h and for 6 days. Data was plotted and treated as described by Smaeegeran and Hoben[19].

Study of the effect of addition of Cd^{+2}, Zn^{+2} and Pb^{+2} on the growth of *Rhizobium leguminosarum biovar vicia*: Bacterial culture was prepared in YMB media and cell density was measured at 600 nm, divided into ten batches (one control and nine experimental). To the experimental batches, different amounts of heavy metal salts (named inhibitors) were added at different concentrations from a stock solution of the heavy metals. Cadmium was added into three batches in the form of $3CdSO_4 \cdot 8H_2O$ to a final concentration of ($1.9x10^{-3}$, $2.9x10^{-3}$ and $3.9x10^{-3}$ mM). While the metal Zinc was added in from of $ZnSO_4:7H_2O$ to a final concentration of

($20x10^{-3}$, $300x10^{-3}$, $40x10^{-3}$ mM.). Finally lead was added in form of $PbCl_2$ to final concentration of ($260x10^{-3}$, $390x10^{-3}$ and $520x10^{-3}$ mM). All batches were placed in a shaker water bath (speed of 200 rpm) at 28°C. Every 3 h and for 48 h, 3 mL aliquot was sampled and cell density was measured at 600 nm.

Effect of chemical compounds (named activators) on *Rhizobium* growth in presence of the heavy metal (named inhibitors) at their of LD_{50}: From the previous experiment, the LD_{50} concentrations (per million cells) for each metal (Cd^{+2}, Zn^{+2} and Pb^{+2}) were determined by plotting the percentage of bacterial growth from the different corresponding measured absorption values, relative to the control experiment, versus different concentrations of each added metal inhibitor). The used LD_{50} concentrations for these metals, are as follows: 2.1×10^{-3} mM Cd^{+2}, 30×10^{-3} mM Zn^{+2} and 290×10^{-3} mM Pb^{+2}.

Determination of the concentration of added activators: It was performed as described in the previous experiment (with activators) and in the presence of LD_{50} concentrations for these metals (Table 1).

RESULTS

Effect of heavy metals (Cd^{+2}, Zn^{+2} and Pb^{+2}) on growth of cultured *Rhizobium*: By using culture technique, the inhibition of bacterial growth by each individual metal was tested. This was achieved by incubating the cells with different concentrations of the tested metal. The magnitude of this inhibition was followed up till 48 h of incubation, where no more increase in growth was observed. The obtained absorption values from the experimental batches were divided by the corresponding absorption value for the control batch at the same sampling time. Therefore, the results shown in Table 1 are represented as percentage of bacterial growth relative to the control. All the presented data were treated in similar way. Table 1 shows the percentage of inhibition of bacterial growth at five different concentrations for each tested metal. The determination of the concentration of LD_{50} for each tested metal was carried out by plotting the concentration of each tested metal versus the percentage of inhibition in growth. A linear relationship was obtained. From that line, the corresponding concentration, which caused 50 % inhibition in growth, was recorded (Table 1).

Effect of some added chemical compounds (activators) on the detoxification of *Rhizobium* by heavy metals: The reactivation of bacterial growth was attempted by adding different chemical compounds to the cultured *Rhizobium*

Table 1: Effect of some metals (inhibitors) at different Concentrations on *Rhizobium* Growth after 48 h incubation with the inhibitor (metal salt) and their calculated LD_{50}

Type of inhibitor	Concentrations of inhibitors (mM)	Bacterial growth (%)	LD_{50} (mM)
$CdSO_4$	1.15×10^{-3}	91	2.1×10^{-3}
	1.50×10^{-3}	70	
	1.90×10^{-3}	55	
	2.90×10^{-3}	32	
	3.90×10^{-3}	24	
$ZnSO_4$	12×10^{-3}	95	30×10^{-3}
	15×10^{-3}	80	
	20×10^{-3}	60	
	30×10^{-3}	50	
	40×10^{-3}	33	
$PbCl_2$	150×10^{-3}	95	290×10^{-3}
	200×10^{-3}	72	
	260×10^{-3}	55	
	390×10^{-3}	34	
	520×10^{-3}	22	

Table 2: Values of different concentrations of added activators to *Rhizobium* culture in the presence of each heavy metal at it's concentration of LD_{50}

Type of activator		Concentrations (mM)		
Cd	Mannitol	71	143	214
Zn	+	+	+	+
Pb	Glutamic acid	14	28	42
Cd	Thiamine	0.38	0.96	1.9
Zn	hydrochloride			
Pb				
Cd	Calcium	7.79	16	20
Zn	Chloride			
Pb				
Cd	5,7-Dihydroxy	0.34	0.51	0.69
Zn	flavone			
Pb				
Cd	Calcium	2.99×10^{-3}	5.97×10^{-3}	8.2×10^{-3}
Zn	Ionophore A_{23187}			
Pb				

and in the presence of heavy metals at its concentration of LD_{50}. Among these chemicals, the following compounds were found to be highly effective: Mannitol and glutamate, thiamine hydrochloride, calcium chloride, 5,7-dihyroxyflavone and calcium ionophore A_{23187} (Table 2). Different concentrations of each of these effective compounds were tested to find the most effective concentration in abolishing the retarded growth. The obtained results are presented as comparison of the most effective concentrations (presented as % of bacterial growth) for each added activator, relative to the control and in the presence of heavy metals (Cd^{+2}, Zn^{+2} or Pb^{+2}), respectively (Table 3).

DISCUSSION

This present study was performed to examine the effect of the heavy metals (Cd^{+2}, Zn^{+2} and Pb^{+2}) on the survival and activity of *Rhizobium leguminosarum biovar vicia* in culture system. Also to elucidate further, the interaction between heavy metals in sewage sludge-

Tabel 3: Effect of different concentrations of activators on the percentage of bacterial growth in the presence of different heavy metals (inhibitors) at the concentration of LD_{50}

	Bacterial growth (%)		
Type end concentration of the activator	$CdSO_4$ (2.1 x 10^{-3} mM)	$ZnSO_4$ (30 x 10^{-3} mM)	$PbCl_2$ (290 x 10^{-3} mM)
Mannitol (71 mM) + Glutamate (14 mM)	90	90.4	90.8
Mannitol (143 mM) + Glutamate (28 mM)	92.3	93	93.3
Mannitol (214 mM) + Glutamate (42 mM)	96.4	96.8	97.2
Thiamine hydrochloride (0.38 mM)	81.1	82.2	82.8
Thiamine hydrochloride (0.96 mM)	88.8	90	90.4
Thiamine hydrochloride (1.90 mM)	69.9	70.3	72.9
Calcium Ionophore A_{23187} (2.98 x 10^{-3} mM)	83.5	83.9	84.7
Calcium Ionophore A_{23187} (5.97 x 10^{-3} mM)	71.8	72.6	73.2
5,7-dihydroxy flavone (0.34 mM)	58.8	59.4	59.6
5,7-dihydroxy flavone (0.51 mM)	67.1	67.6	68
5,7-dihydroxy flavone (0.69 mM)	83.8	84.3	85.3
Calcium chloride (7.79 mM)	87.3	87.8	88.1
Calcium chloride (16 mM)	73.3	74.8	75.1

treated soils and the above mentioned highly economically bacteria. These studied heavy metals (Cd^{+2}, Zn^{+2} and Pb^{+2}) are known of being abundant in soil and highly toxic to *Rhizobium*[20]. Specially, lead, which is known to be the most abundant toxic metal in the biosphere. In order to achieve maximum effect of these heavy metal ions, their soluble form were used, so cadmium and zinc were used in the form of sulphate, while lead was used in the form of chloride. Many researchers documented the inhibitory effect caused by heavy metals. Some researchers, instead, were looking for heavy metal resistant strains of bacteria[21]. But, in this present study, the researchers were focusing on finding some chemical compounds, which can be added to the soil in order to abolish the inhibitory effect of heavy metals. This present study consists of two major parts. The first part deals with the study of the effect of some heavy metals on *Rhizobium* viability. Therefore, three heavy metals were chosen for this study (Cd^{+2}, Zn^{+2} and Pb^{+2}), since they are commonly found as pollutants. The second part deals with testing several chemical compounds (mannitol and glutamic acid, thiamine hydrochloride, 5,7-dihydroxy flavone, calcium chloride and calcium ionophore A_{23187}) for their ability to recover the *Rhizobium* normal growth and viability. Other researches[22-24] had noted that *Rhizobium* toxicity by heavy metals not only depend on the concentration of heavy metals, but also on the presence of other components and factors like soil pH, drought, temperature, other organic and inorganic components and microorganisms. Therefore, this present study used the *in vitro* cultured system, for several reasons. First, in order to avoid the previously mentioned variability factors present in an *in vivo* system, which encountered other researchers. Secondly, the availability of studying the toxic effect of each individual heavy metal at several concentrations and in presence of different concentrations of the tested chemical compounds (activators). Thirdly, because many of these activators were used for the first time in combination with a specific heavy metal, therefore their proper effective concentration has to be determined experimentally.

In the present study, we observed the same phenomena, that cadmium was the most potent metal in retarding the generation time, followed by lead. Moreover, we observed that the generation time remained almost unaltered if the activators, especially mannitol + glutamate and thiamine, were added simultaneously with all of the studied heavy metals, at their concentration of LD_{50}. We revealed also that the level of toxicity depends heavily on the concentration of the added heavy metal. This level of toxicity ranges from being ineffective (at very low concentration) to being lethal (at high concentration) to the bacteria under investigation. The relative potency, from this present study, shows that, cadmium was the most potent inhibitor to the *Rhizobium* growth (LD_{50} of 2.1 μM). While lead was the least potent (LD_{50} of 290 μM). The explanation for these phenomena comes from the work of transmission electron microscope on Pseudomonas Bacillus[25] where lead was seen as the most excluded metal from the cell and may be *Rhizobium* behave in the same fashion. This could be explained, from our data that the requirement of 150 folds increase in the concentration of lead is needed to show a 50% inhibition in *Rhizobium* growth in comparison to a 50% inhibition by cadmium.

The second part, deals with the addition of some chemical compounds to the heavy metal stressed *Rhizobium*. Some of these compounds mimic the natural occurring compounds, found naturally in the soil. Their effectiveness in a *Rhizobium* culture system was tested. Their lowest and most effective concentrations were shown in Table 3.

In aired and low pH soils, calcium chloride was added to rescue the survival of *Rhizobium*. Calcium is known to be a common intracellular second-messenger molecule in euokaryotic cells, which modulate many cellular

processes. Therefore, present study tested the ability of added extracellualr calcium (in the form of $CaCl_2$) in regaining the normal growth. Results from Table 3, shows that recovery of growth was achieved by 88% by adding 7.79 mM $CaCl_2$ and this recovery percentage is diminished by using higher concentration of calcium. Moreover, the presence of calcium was further studied by using calcium ionophore. This study used the calcium ionophore A23187, which transverses the lipid bilayer and introduces Ca^{2+} into the cell and into intracellular organelles. As a result, cytosolic calcium is raised and this causes the enhancement of secretion[26]. Therefore attempts, in this study, were made to use this calcium ionophore, in order to investigate its effect in a bacterial system. Furthermore, the role of cytosolic calcium was shown by Slmon and Downie[27] in formulating the mutualistic relationships between pro- and eukaryotic cells during nitrogen-fixing *Legume rhizobia* symbiosis. This occurs through the selective transfer of metabolites and ion transport, Ca^{2+} in particular, across the peribacteroid membrane. Moreover, the treatment with calcium channel blockers, as verapamil, caused a considerable calcium depletion of symbiosomes in the infected nodule cells of *Vicia faba*[28].

Surprisingly, our results show that normal *Rhizobium* growth was recovered by 84% in presence of cadmium, zinc or lead, due to the presence of the calcium ionophore. The explanation for this effect might be due to the symbiotic interaction between the *Rhizobium* and the leguminous plants, causing the production of root nodule, which in turn infected the bacteria via infection thread, which leads to the process of nitrogen fixation. This signaling mechanism between the *Rhizobium* and the host plant occur through the secretion of the host plant root to flavonoid compounds[29]. These compounds induce the transcription of *Rhizobium* node genes[30] which is responsible for the secretion of lipo-oligopolysaccharide. Therefore, we studied the effect of dihydoxyflavone on the recovery of *Rhizobium* growth. By increasing the concentration of dihydoxyflavone from 0.34 to 0.69 mM, the growth recovery was increased from 58 to 83%. Also thiamine was tested, since it is a vitamin, so it's activation of growth was expected. High concentration (1.9 mM) was less effective, while lower concentration (0.96 mM) was more effective with 90% recovery.

Finally, the most effective activator in alleviating heavy metal toxicity was due to the addition of 214 mM Mannitol + 42 mM Glutamate. This maximum recovery of *Rhizobium* growth was 97%. This result could be explained, based on its ability to produce EPS, as noted earlier[7] with the exception that the previous study was performed in absence of heavy metals or any other contaminant.

The drawback of this study lies in the following possibility: since the inhibitory effect of each heavy metal was treated separately, so different result might be obtained if they were present together and with other heavy metals (as in soil). On the other hand, this research has an immediate application in being able to treat heavy metal highly contaminated soil with one of these experimented compounds. The treatment of such soil, specifically, with calcium chloride, since it is the cheapest among the tested compounds, might help in restoring the normal *Rhizobium* activity. Moreover, this present study opened a new era of research to elucidate the mechanism, by which each of these compounds acted upon the intracellular organelles. Is it through sequestering the toxic heavy metal, activating the protein synthetic machinery through activating certain enzymes which were pre-inhibited by the heavy metals or by many other mechanisms. The addition of these activators, which had a mystery unknown mechanism, had led to restoring the normal bacterial generation time during incubation with the heavy metal. More experimental investigations at the intracellular level are required to be carried out in order to evaluate the positive results of the above-mentioned tested compounds.

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