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Inhibition of Nodulation and Nitrogen Nutrition of Leguminous Crops by Selected Heavy Metals

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ABSTRACT: This work studied the effects, under greenhouse conditions, of six heavy metals (Cd, Co, Cr, Cu, Ni, and Pb) on three leguminous crops representing food, feed, and forage crops commonly grown in Egypt. Metal concentrations ranged from 0 to as high as 4.8 mmol kg^{-1} soil. Results showed that all three plant parameters measured (dry matter yield, nodulation, and N uptake) decreased significantly with increasing heavy-metal concentrations. Plots of the natural log of each parameter against metal concentration were linear within the ranges studies. From the slopes of these regression lines, the concentration of each heavy metal required to achieve 50% reduction (R_{50}) of each parameter was calculated. In general, the lowest metal concentrations for R_{50} were for Cd^{2+} and Pb^{2+} and the highest were for Cr^{3+} and Cu^{2+}. Heavy-metal additions to soils should be closely monitored because they can negatively affect nodulation and N nutrition of leguminous crops.

KEYWORDS: trace metals, N_2 fixation, broad bean, soybean, Egyptian clover

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Introduction

Heavy metals and trace elements in the environment, especially in soil and water, and their effects on plant nutrition and productivity have received much attention in recent years. The term *heavy metal* is used here to refer to elements with specific gravity of 5.0 or greater that are specifically toxic to organisms. The term *trace elements* refers to elements that are, when present in sufficient concentrations, toxic to living systems.[1] Soil pollution by heavy metals has become a critical environmental concern due to potential adverse ecological effects. The contamination of soils with heavy metals due to emissions from municipal waste incinerators, car exhausts, residues from metalliferous mining and the smelting industry, and the use of sludge or urban composts, pesticides, and fertilizers are common in many countries, especially in Egypt.[2,3] Accumulation of heavy metals in soil has the potential to restrict soil function, cause toxicity to plants, and contaminate the food chain.[4]

Contamination of soils with trace elements and heavy metals is of global concern (for review, see Kabata-Pendias).[2] A number of studies on the effects of heavy metals on N_2 fixation by leguminous crops have been reported,[5–10] but most of those studies were done on sewage-sludge-amended soils or on soils treated with heavy metals on a weight basis.[11,12] The information available also shows that the fraction of N in clover (*Trifolium repens* L.) derived from fixation varied from 0 to 88% depending on the soil.[5] This fraction was reduced by 50% at Zn concentrations of 737 mg kg^{-1} soil, Cu concentrations of 428 mg kg^{-1}, and Cd concentrations of 10 mg kg^{-1}. Other researchers indicate there is little evidence that symbiotic N_2 fixation is sensitive to heavy metals at the concentrations used in their studies.[12,13] In addition to N_2 fixation, the inhibition of plant growth, especially root growth and development, increased as the heavy-metal (eg, Cd) concentrations increased.[14] Because significant quantities of N are added to soils by symbiotic N_2 fixation by leguminous crops and because N_2 fixation is becoming increasingly important for crop production in developing countries with low nitrogen fertilizer inputs, studies on the effects of heavy metals on the growth and development of leguminous crops are needed.

Most Egyptian soils have low fertility and are heavily fertilized with fertilizers that may contain a variety of heavy metals.[15,16] In addition, leguminous crops are used for food, feed, and forage, but little information is available about the ecotoxicological effects of heavy metals on N nutrition of these crops. Therefore, the objectives of this work were (i) to assess the inhibition of nodulation by equimolar concentrations of six heavy metals (Cd, Co, Cr, Cu, Ni, and Pb), commonly found in heavily fertilized Egyptian soils, by three common leguminous crops (representing food, feed, and forage)—broad bean (*Vicia faba*, Giza 3), Egyptian clover (*Trifolium alexandrium*, Giza 6), and soybean (*Glycine max*, Giza 35); and (ii) to study the degree of inhibition of N nutrition of these representative crops. Thus, to study the above objectives, experiments were carried out, under greenhouse conditions, using the three crops on two diverse predominant types of Egyptian soils.

Materials and Methods

Soils and their properties. The two soils used had different chemical and physical properties (Table 1). The soil samples were surface soils (0–15 cm) from unfertilized fields representing typical soils in Egypt. The samples were a clay loam soil obtained from the Experimental Farm of the Faculty of Agriculture and a sandy soil obtained from the Shousha zone, Agricultural Research Center, Minia University, El-Minia. Each sample was air-dried, mixed, and passed through a 2-mm sieve. In the analyses reported in Table 1, pH was determined by a combination electrode (soil:water or 0.01 M $CaCl_2$ ratio of 1:2.5), particle-size distribution was determined by a pipette method,[17] total N was determined by a semimicro-Kjeldahl method,[18] organic C was determined by the Mebius[19] method, and calcium carbonate equivalent was determined by a back-titration procedure.[20] Available P and SO_4-S were determined as described by Olsen and Dean[21] and by Bardsley and Lancaster,[22] respectively.

Table 1. Properties of the soils used in the study of heavy-metal effects on plant growth parameters.

PROPERTY	PROPERTIES OF THE SOIL SPECIFIED	
	CLAY LOAM	SANDY
pH	6.8 (6.5)[a]	8.0 (7.7)[a]
Organic C (g kg^{-1})	17.4	0.6
$CaCO_3$ equivalent (g kg^{-1})	18	139
Total N (g kg^{-1})	1.8	0.08
Available P (mg kg^{-1})	12.3	2.4
Available SO_4-S (mg kg^{-1})	20.4	12.2
Clay (g kg^{-1})	400	30
Sand (g kg^{-1})	290	890
Total metal (mg kg^{-1} soil)		
Cd	0.5	0[b]
Co	8.0	0
Cr	36.0	9.0
Cu	26.3	4.4
Ni	10.0	2.4
Pb	25.8	7.9

Notes: [a]Figures in parentheses are pH values obtained for soil: 0.01 M $CaCl_2$ ratio of 1:2.5. [b]Below detection limit.

Heavy metals. Six heavy metals representing those commonly found in fertilizers[15,16] and industrial wastes in Egypt were used in this study. The salts of the heavy metals were cadmium sulfate ($CdSO_4{\cdot}8H_2O$), cobalt sulfate ($CoSO_4{\cdot}7H_2O$), copper sulfate ($CuSO_4$), chromium sulfate [$Cr_2(SO_4)_3{\cdot}12H_2O$], lead acetate [$Pb(CH_3COO)_2$], and nickel sulfate ($NiSO_4{\cdot}6H_2O$). The heavy metals used were Fisher-certified and of reagent-grade (Thermo Fisher Scientific, Waltham, MA, USA).

The total concentrations of the six metals in soils were determined in digests prepared by using the method of Akagi and Nishimura.[23] In this method, 1 g of soil sample (<180 µm) was placed in a 50-mL Erlenmeyer flask, treated with 14 mL of a reagent containing HNO_3, H_2SO_4, and $HClO_4$ at a ratio of 1:5:1 and placed on a sand bath adjusted to 220°C. The flask was covered with a watch glass after 1 h and digested for 2 h. After digestion, the sample was removed from the sand bath and cooled and 70 mL of deionized water was added. Because of exothermic reactions, the sample was again allowed to cool to room temperature, filtered through a Whatman No. 42 filter paper into a 100-mL volumetric flask, and made up to volume with distilled water. The flask was stoppered and mixed thoroughly. The digest was then analyzed for the six heavy metals by inductively coupled plasma atomic emission spectroscopy (Optima 8300, PerkinElmer, Waltham, MA, USA).

Greenhouse experiments. To study the effect of heavy metals on crop growth, Egyptian clover, broad bean, and soybean were evaluated in a pot study in the greenhouse in a randomized block design with two soils × six heavy metals × six rates with three replications. The six rates of heavy metals were 0, 5, 25, 50, 100, and 250 mg kg^{-1} soil. Because heavy metals have different atomic masses, these concentrations were converted to a mole basis. Expressed in mmol kg^{-1} soil in parentheses, the concentrations ranged from 0 to maxima as follows: Cd (2.2), Co (4.2), Cr (4.8), Cu (3.9), Ni (4.3), and Pb (1.2). In each experiment, 2.5 kg of soil was placed in plastic pots (30-cm diameter), treated with 1 L of deionized water containing one heavy metal at the desired concentration, and the moisture content was adjusted to 60% of the water-holding capacity by using deionized water. The plants were grown (five seeds per pot, which were thinned to three plants after 10 days) for

50 days. Before planting, the seeds were treated with specific *Rhizobium* or *Bradyrhizobium* inoculants containing a minimum of 3×10^9 viable cells mL^{-1}, supplied by the Agriculture Genetic Engineering Research Institute (Cairo, Egypt). The rhizobia were added in a sucrose solution (200 g in 900 mL of deionized water) to aid adhesion of the inoculants to the seeds. The soil moisture level of all pots was kept at ca. 60% of water-holding capacity during plant growth by randomly weighing the pots and adding deionized water as needed.

After 50 days of growth, the plants were carefully uprooted and the roots gently washed with deionized water. The number of nodules per plant was counted and the total plant material was oven dried at 65°C for 72 hours, weighed, ground to pass a 0.15-mm mesh sieve, and analyzed for total N by the Kjeldahl method described by Piper.[24] From the plant weight and percentage N in the plant, the yield of N was calculated.

Statistical analysis. To calculate the concentration of each heavy metal required to achieve 50% reduction (we define it here as R_{50}) in the plant parameters studied, we used a first-order kinetics equation by plotting the natural log of the specific parameter against heavy-metal concentration (expressed in mmol kg^{-1} soil). From the slope (k) of the linear relationship obtained, we calculated the R_{50} for the decrease in dry matter weight, number of nodules formed per plant, and N yield as described by Ajwa and Tabatabai[25] for calculation of the half-lives of decomposition of different organic materials in soils.

$$R_{50} = 0.693/k \qquad (1)$$

Statistical analysis, including analysis of variance (ANOVA), contrast comparison, and separation of means by least significant differences were performed by the general linear models procedure of the SAS program[26] for the combined experiments.

Results and Discussion

Effect of heavy metals on plant yield. The plant parameters (plant and N yields) of the three leguminous crops studied decreased as metal concentrations increased. The results for Cr^{3+} and Pb^{2+} added to the two soils are reported in Figures 1 and 2, respectively. Similar plots were obtained for the other metals studied. The extent to which concentration of the heavy metals affected plant parameters differed somewhat among the three crops. For example, broad bean and soybean parameters decreased as Cr^{3+} concentrations increased, especially at the concentration range from 0 to 2.0 mmol kg^{-1} soil. A similar

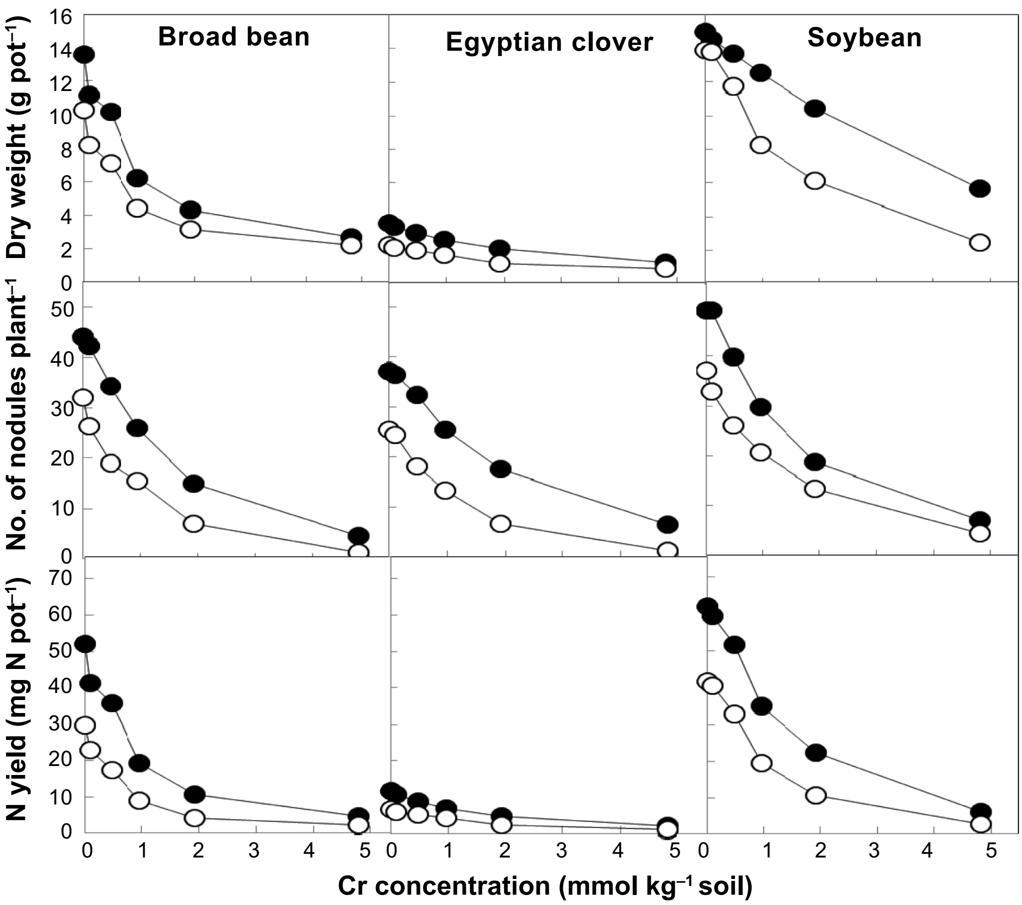

Figure 1. Dry matter yields, number of nodules, and N yields produced in the two soils as a function of Cr^{3+} concentration added to soils. At all data points, the differences among the triplicate values were smaller than the point size. Soils: ●, clay loam; ○, sandy.

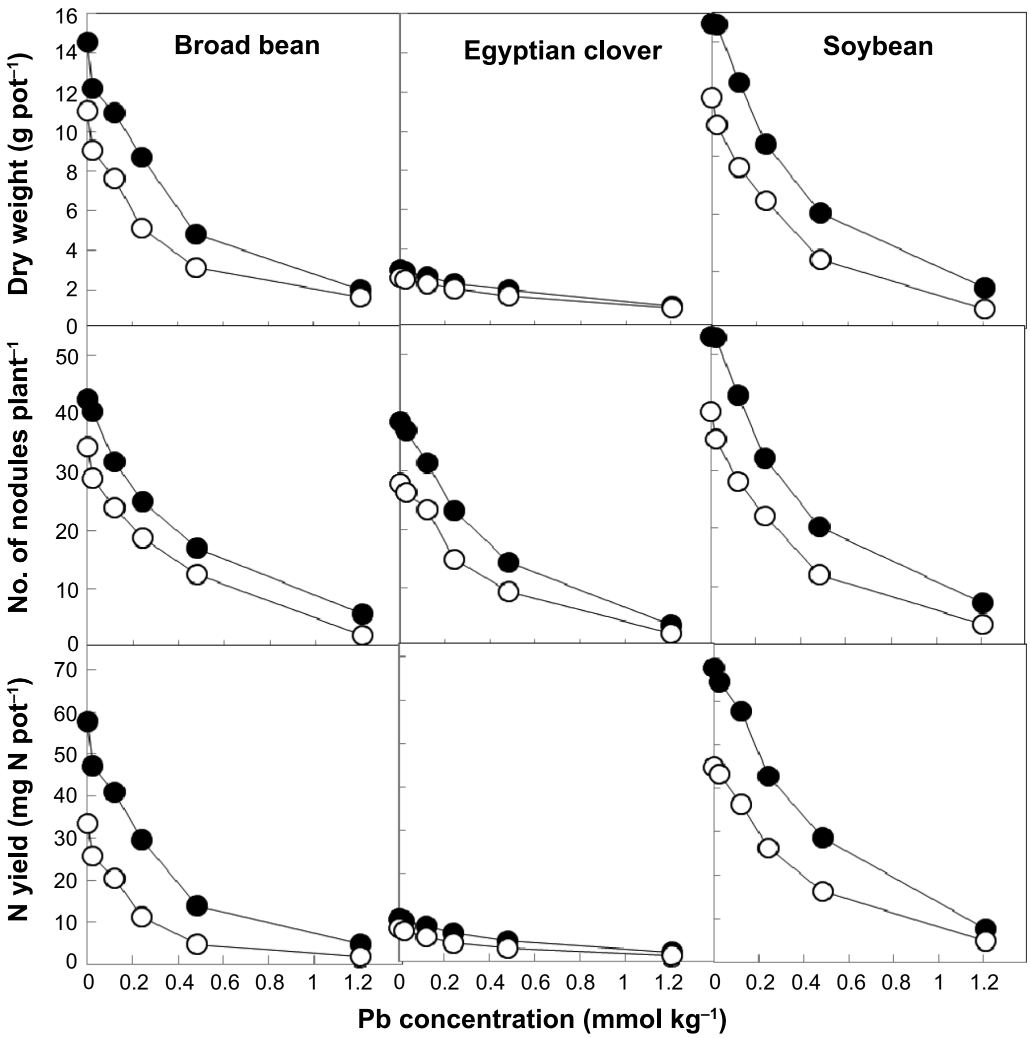

Figure 2. Dry matter yields, number of nodules, and N yields produced in the two soils as a function of Pb^{2+} concentration added to soils. At all data points, the differences among the triplicate values were smaller than the point size. Soils: ●, clay loam; ○, sandy.

reduction occurred when the Pb^{2+} concentration increased from 0 to 0.4 mmol kg^{-1} soil; ie, Pb^{2+} was a more effective inhibitor of dry matter and N yields than was Cr^{3+}. The effects of the heavy metals on the growth parameters of Egyptian clover were greater than on the other two crops studied (Figs. 1 and 2); this was especially true for nodule formation. The results obtained with Cr^{3+} support results reported by Stephen and Craig,[27] who showed that increasing the Cd^{2+} concentration in soils significantly reduced nodule number, dry weight, and N_2 fixation of soybeans. The finding that metal ions have different effects on the parameters studied was expected because the reactions of the ions involve a number of chemical and biochemical reactions that influence their solubility and plant availability.[2] The effect of metal ions on enzyme reactions in soils, including nodule formation and N_2 fixation, has been reported by others.[27,28] ANOVA showed that the type and concentration of metal ions significantly affected each of the plant parameters studied (Table 2).

Effect of heavy metals on nodulation. The number of nodules per plant decreased sharply as the metal concentration increased. This is demonstrated in Figures 1 and 2 for Cr^{3+} and Pb^{2+}, respectively. Other heavy metals followed similar patterns. At low concentrations, heavy metals did not significantly affect the number of nodules, but at high concentrations, nodulation decreased significantly once the concentration reached >0.5 mmol kg^{-1} soil. At low concentrations, the clay and organic matter, especially in the clay loam soil, presumably complexed the heavy metal, decreasing its concentration in solution. At higher levels of heavy metals, however, the nodulation process was almost completely inhibited. This was likely due to the heavy metals' availability to inhibit the biological and biochemical processes involved in root growth, development, and nodule formation. The sandy soil consistently produced poorer growth and nodulation at a given concentration of heavy metal than the clay loam soil. Rother et al[28] examined nodulation and N_2 fixation (acetylene reduction) in white clover growing on mine spoils with up to 216 mg (1.9 mmol) of Cd kg^{-1}, 20,000 mg (306 mmol) of Zn kg^{-1}, and 30,000 mg (145 mmol) of Pb kg^{-1} soil and reported only slight decreases at the most contaminated sites. Results reported by others[13] showed that the numbers of

Table 2. ANOVA of effects of six heavy metals and their concentrations on dry matter yields, number of nodules, and N yields of leguminous crops (broad bean, Egyptian clover, and soybean) grown in soils for 50 days.

SOURCE	DRY MATTER YIELD[a]	NO. OF NODULES[a]	N YIELD[a]
Plant (P)	**	**	**
Soil (S)	**	**	**
P × S	**	*	**
Heavy Metal (HM)	**	**	**
P × HM	**	**	*
S × HM	ns	**	**
P × S × HM	ns	**	ns
Concentration (C)	**	**	**
P × C	**	**	**
S × C	**	**	**
P × S × C	**	**	**
HM × C	**	**	**
P × HM × C	ns	**	ns
S × HM × C	ns	**	ns
P × S × HM × C	ns	**	ns

Notes: Significant at *$p < 0.05$; significant at **$p < 0.01$.
Abbreviation: ns, not significant.

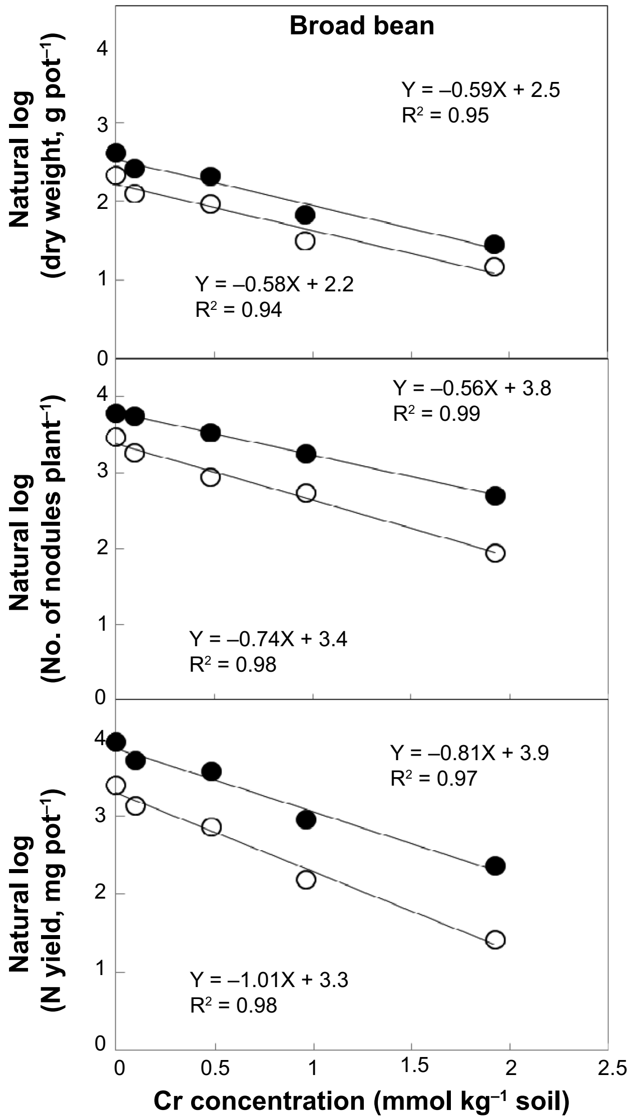

Figure 3. Natural log of dry matter yields, number of nodules, or N yields produced in the two soils as a function of Cr^{3+} concentration added to soils. At all data points, the differences among the triplicate values were smaller than the point size. Soils: ●, clay loam; ○, sandy.

Rhizobium present in the soils were greatly reduced in the most contaminated treatments and absent in soils under very acid conditions.

R_{50} of the selected heavy metals. To normalize the results among the three crops and the three plant parameters studied, we calculated the concentration of each heavy metal required to achieve 50% reduction (Equation 1). Graphs were prepared by plotting the natural log values of dry weight, number of nodules, and N yield of each leguminous crop versus concentration (mmol kg^{-1}) of the heavy metal. For illustration, the results obtained for plant parameters of the three crops for Cd^{2+}, Cr^{3+}, and Pb^{2+} in the two soils are shown in Figures 3–5, respectively. Similar figures were obtained for the concentrations of the other heavy metals.

The R_{50} values differed among the heavy metals, crops, and the soils studied (Table 3). Expressed in mmol kg^{-1} soil, R_{50} values for dry matter yields of the three crops in the two soils ranged from 0.46 to 1.18 for Cd, from 0.99 to 2.89 for Co, from 1.18 to 2.57 for Cr, from 1.82 to 3.47 for Cu, from 1.05 to 2.04 for Ni, and from 0.28 to 0.94 for Pb. The corresponding R_{50} ranges for nodulation were 0.20–0.69, 0.77–1.47, 0.94–1.73, 0.95–1.87, 0.39–1.44, and 0.33–0.41, respectively. The corresponding values for N yields were 0.29–0.63, 0.61–1.54, 0.69–1.44, 1.07–1.98, 0.63–1.22, and 0.18–0.58, respectively. In general, the lowest metal concentrations for R_{50} were for Cd^{2+} and Pb^{2+} and the highest were for Cr^{3+} and Cu^{2+}. ANOVA for the results obtained from the experiments showed that the concentration and type of heavy metal significantly ($P < 0.05$ or 0.01) affected dry matter, N uptake, and the number of nodules per plant. The chemical and physical properties of the soil, such as organic matter content, kind and amount of clay, and soil pH, presumably influenced the toxic effect of heavy metals. This is likely because these properties affect the solubility and reactivity of the metal with the active sites of the enzymes involved. As evident from the results reported at each concentration of heavy metal added to the two soils, the metal was more effective in inhibiting nodulation and plant growth in the sandy soil than in the clay loamy soil. This presumably was because a greater proportion of the heavy metal added reacted with the organic matter and clay of the clay loam soil than the sandy soil, thus allowing less soluble heavy metal in

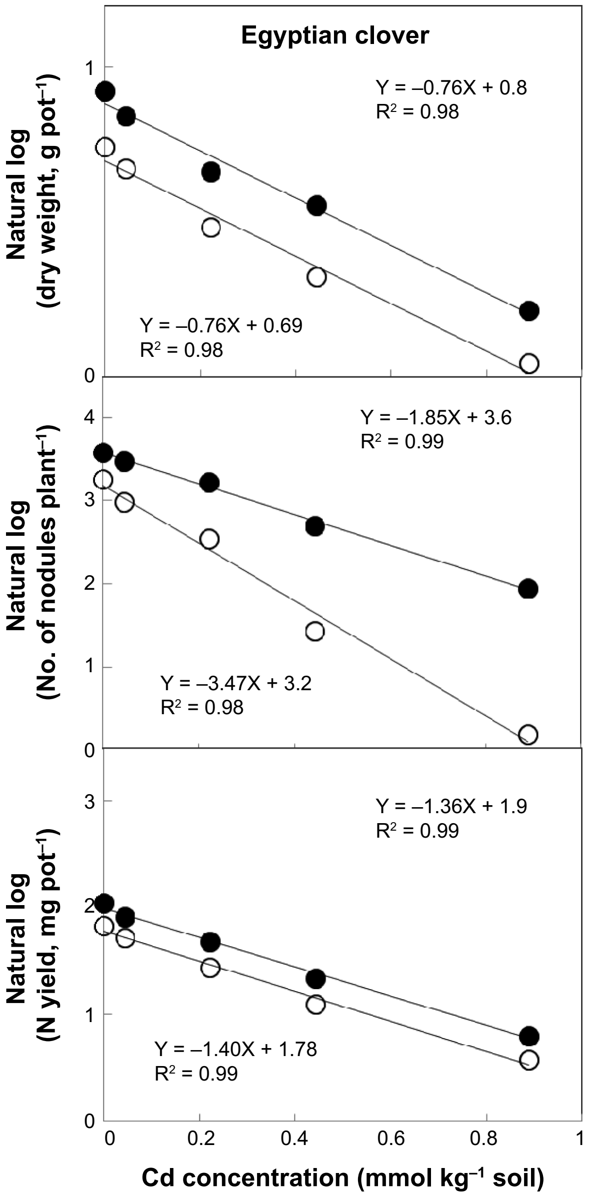

Figure 4. Natural log of dry matter yields, number of nodules, or N yields produced in the two soils as a function of Cd^{2+} concentration added to soils. At all data points, the differences among the triplicate values were smaller than the point size. Soils: ●, clay loam; ○, sandy.

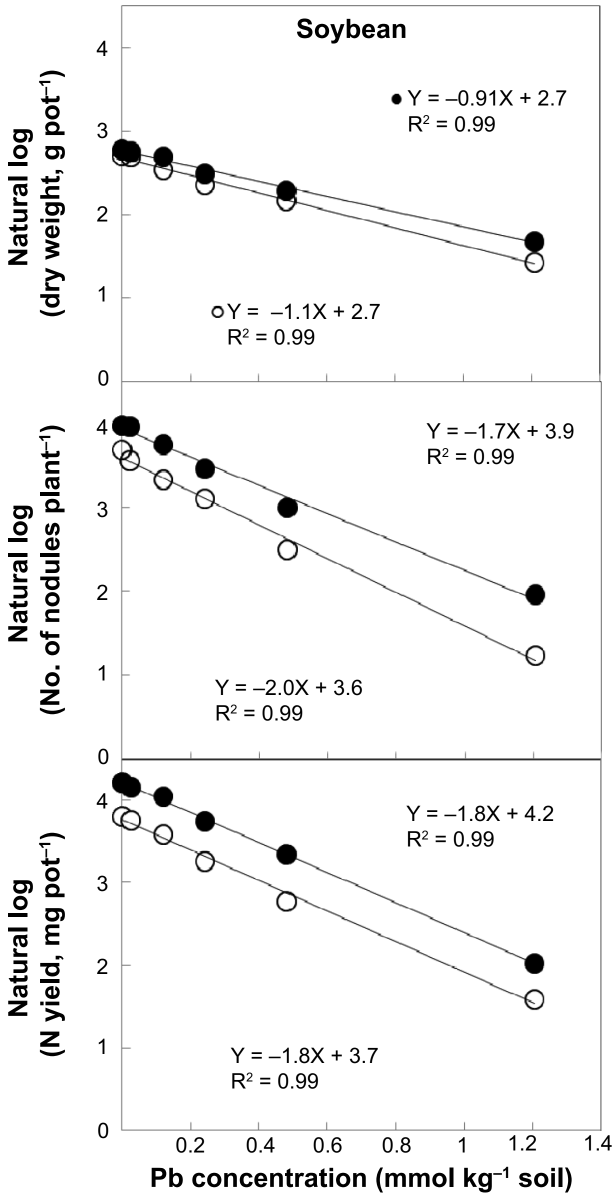

Figure 5. Natural log of dry matter yields, number of nodules, or N yields produced in the two soils as a function of Pb^{2+} concentration added to soils. At all data points, the differences among the triplicate values were smaller than the point size. Soils: ●, clay loam; ○, sandy.

the soil solution for reactions in the biochemical processes involved.

Conclusions

Dry matter yield, nodulation, and N uptake by broad bean, Egyptian clover, and soybean decreased as the concentration of six heavy-metal ions (Cd, Co, Cr, Cu, Ni, and Pb) increased from 0 to as high as 4.8 mmol kg^{-1} soil in two predominant types of Egyptian soils. The concentration of each heavy metal required to achieve 50% reduction (R_{50}) varied among the soils, crops, and the crop parameters studied. Those values were, in general, greater for the clay loam soil than for the sandy soil. R_{50} values showed the urgent need for experimentation beyond simple laboratory studies to provide a good understanding of the effects of heavy-metal toxicity to microorganisms in soils, and how we can use these results to protect our soils.

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Author Contributions

Conceived and designed the experiments: SAH, AAA. Analyzed the data: SAH, AAA, MAT. Wrote the first draft of the manuscript: SAH. Contributed to the writing of the

Table 3. R_{50} values of broad bean, Egyptian clover, and soybean plants, defined as the concentrations (mmol kg^{-1} soil) at which plant parameters were reduced by 50% (value was calculated from the equation $R_{50} = 0.693/k$) for the six heavy metals specified.

	Cd^{2+}		Co^{2+}		Cr^{3+}		Cu^{2+}		Ni^{2+}		Pb^{2+}	
	CLAY LOAM	SANDY	CLAY LOAM	SANDY	CLAY LOAM	SANDY	CLAY LOAM	SANDY	CLAY LOAM	SANDY	CLAY LOAM	SANDY
R_{50} values for dry matter yields												
Broad bean	0.52	0.46	1.10	0.99	1.18	1.19	1.82	1.82	1.05	1.07	0.32	0.28
Egyptian clover	0.91	0.91	2.89	2.77	2.57	2.17	2.77	3.47	1.61	1.65	0.88	0.94
Soybean	1.18	0.95	2.48	1.98	2.39	1.93	2.29	1.69	2.04	1.98	0.76	0.63
R_{50} values for No. of nodules per plant												
Broad bean	0.43	0.24	1.47	0.87	1.24	0.94	1.41	1.10	0.95	0.67	0.36	0.35
Egyptian clover	0.36	0.20	1.31	0.77	1.73	0.98	1.87	0.95	0.89	0.39	0.36	0.33
Soybean	0.69	0.58	1.47	1.44	1.69	1.65	1.51	1.33	1.44	1.28	0.41	0.35
R_{50} values for N yields												
Broad bean	0.35	0.29	0.75	0.61	0.86	0.69	1.31	1.07	0.79	0.63	0.25	0.18
Egyptian clover	0.51	0.50	1.54	1.36	1.44	1.26	1.69	1.98	1.00	1.02	0.58	0.50
Soybean	0.63	0.58	1.31	1.14	1.41	1.22	1.20	1.12	1.18	1.22	0.39	0.39

manuscript: MAT, TEL. Agree with manuscript results and conclusions: SAH, MAT, TEL. Jointly developed the structure and arguments for the paper: SAH, MAT. Made critical revisions and approved final version: MAT, TEL. Reviewed and approved of the final manuscript: SAH, MAT, TEL.

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