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Cadmium Resistence in *Rhizobium* – Faba Bean Symbiosis. Synthesis of Cadmium-Binding Proteins

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With 3 figures

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Summary

EL-ENANY A. W. E. & ABD-ALLA M. H. 1995. Cadmium resitance in *Rhizobium* – Faba bean symbiosis. Synthesis of Cadmium-binding proteins. – Phyton (Horn, Austria) 35 (1): 45–53, 3 figures. – Englisch with German summary.

The effect of cadmium concentrations on nodulation, growth and nitrogen fixation was studied in potted faba bean plants. Cadmium concentrations upto 100 ppm had no significant effect on nodulation, nitrogenase activity, leghaemoglobin and protein contents of nodules. Dry matter accumulation of shoots and roots and total nitrogen of roots of faba bean plants were not affected at 100 ppm Cd. Higher levels of cadmium (200 ppm) had a deleterious effect on Rhizobium-faba bean symbiosis. The nodules formed Cd-binding protein complexes one of about 200 kD, which contained about 38% of the Cd complexed and a second of a molecular weight of 67 kD containing about 53% of the Cd complexed. The formation of Cd-binding proteins may be a mechanism by which *Rhizobium*-faba bean elevates resistance to Cd toxicity.

Zusammenfassung

EL-ENANY A. W. E. & ABD-ALLA M. H. 1995. Cadmium Resistenz in der Symbiose *Rhizobium* und *Vicia faba.* Synthese von Cadmium-bindenden Proteinen. – Phyton (Horn, Austria) 35 (1): 45–53, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Auswirkung von Cd-Konzentrationen auf die Knöllchenbildung, Wachstum und Stickstoffixierung wurde an getopften *Vicia faba*-Pflanzen untersucht. Cd-Konzentrationen bis 100 ppm hatten keinerlei signifikaten Einfluß auf Knöllchenbil-

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dung, Nitrogenase-Ativität, Leghaemoglobin- und Proteingehalt der Knöllchen. Trockengewichtszunahme von Sproßen und Wurzeln und Zunahme vom Gesamtstickstoff in den Wurzeln von *Vicia faba*-Pflanzen wurde bei 100 ppm Cd nicht beeinflußt. Höhere Mengen von Cd (200 ppm) hatte einen schädlichen Einfluß auf die Symbiose von *Rhizobium* und *Vicia faba*. Die Knöllchen formten Cd-bindende Proteinkomplexe, einer davon mit ungefähr 200 kD, der ungefähr 38% des komplexierten Cd enthielt. Ein zweiter mit einem Molekulargewicht von 67 kD enthielt ungefähr 53% des komplexierten Cd. Die Produktion von Cd-bindenden Proteinen dürfte einen Mechanismus darstellen, durch den das System *Rhizobium-Vicia faba* die Resistenz gegen die Cd-Toxizität erhöht.

Introduction

As a consequence of a directive of the Commission of European Communities (CEC 1986), the member states now have each set limits for permissible concentrations of the most potentially-toxic heavy metals in sewage sludge and in soil to which sewage sludge is applied. However, scientific evidence to support the selection of these limits is still scant, particularly as th sensitivity of soil microorganisms to relatively modest metal contamination was only recently discovered.

A unifying concept based on soil chemical factors that would predict heavy metal availability and biological toxicity under different soil conditions is still laking. One approach for evaluating biological toxicity has been based directly on microbiological responses (SKUJINS & al. 1986). In general, N cycling process (KABATA-PENDIAS & PENDIAS 1984), and especially nitrogen fixation have been shown to be sensitive to small cocentrations of heavy metals in the soil (BHANDAL & al 1990).

Only few reports are available dealing with the toxicity of different heavy metals on nodulation and nitrogen fixation (BHANDAL & al 1990, HUNG & al 1974, PAIVOKE 1993 a, b, YAKOLEVYA 1984). Conversely, field studies by HECKMAN & al 1984 failed to detect adverse changes in either plant growth or N_2-fixation in the sludge-amended soils. BORGES & WOLLUM 1981 added Cd salts to the soil and reported that N_2-fixation of soybean was not affected.

In this study, it was intended to investigate growth, nodulation and nitrogen fixation of faba bean cultivated in soils amended with cadmium. The mechanism of resistance to Cd was also investigated.

Materials and Methods

Plant culture and experimental conditions:

Surface-sterilized faba bean seeds (*Vicia faba* cv. Giza 3) were inoculated with 5 ml *Rhizobium leguminosarum* biovar *viceae* strain TAL 1402 per five seeds and were planted into plastic pots (17 cm diameter) containing 3 kg autoclaved clay soil. The soil was treated with different levels of cadmium (50, 100 and 200 ppm), by adding about 500 ml of cadmium chloride solution per 3 kg soil.

The soil was thoroughly mixed and allowed to equilibrate for a week. The treated soil was then put in the pots. The pots were irrigated with tap water on al-

ternate days and with nitrogen-free Hoagland's solution after 10 days. Seedlings were thinned to two per pot after five days. The plants were maintained in a wire proof greenhouse. Plants that did not receive cadmium solution were used as a control.

Treatments were arranged in a randomized block design with three replicates each. plants were harvested 58 days after planting. Within this time period large enough samples of nodules had been produced for chemical analysis. Nodule number, nodule fresh weight, shoot and root dry wight were recorded. Nitrogenase activity was determined on detached root systems. Assays were conducted in a closed system (ABD-ALLA 1992 a) using a spectrophotometrically method (LARUE & KURZ 1973).

Protein contents of nodules (cytosol and bacteroids) were determined according to LOWRY & al. 1951 after nodule fractionation and bacteroid isolation (ABD-ALLA 1992 b). Leghaemoglobin content was measured colorimetrically as described by JOHNSON & HUME 1973. Photosynthetic pigments (chl. a, chl. b and carotenoids) were determined as recommended by METZNER & al. 1965. Total nitrogen of shoots and roots was determined using the Kjeldahl technique (BLACK & al. 1965), total carbohydrates with the anthrone sulphuric acid method (FALES 1951).

Isolation of Cd-binding protein:

Two grams of frozen nodules were homogenated with buffer A (10 mM Tris-HCl, 1% mercaptoethanol and 1 g polyclar T pH = 8.0). The homogenate passed through three layers of cheeseclothes and centrifuged 15 min. at 15,000 rpm at 4 C. The supernatant was heated to 60° C for 5 minutes, cooled immediately, centrifuged for 30 minutes at 15,000 rpm and lyophilized (GRILL & al. 1985).

Purfication of Cd-binding protein:

The lyophilized protein extracted was centrifuged and the supernatant applied to a sephadex anion exchanger column (1.5 * 20 cm) equilibrated with buffer A. Bound protein was eluted (0.5 M NaCl in buffer A) and collected in fractions of 5 ml volume. The fractions containing protein were collected. Ultrafiltration cell (Sartorious, Ultrasart cell 10 SM 16666) with an Amicon Diaflo ultrafiltration membrane (Type Ycos > 500 MW) was used for desalsation and concentration of the cadmium-binding protein.

Fractionation of Cd-binding protein:

The Cd-binding protein was fractionated on a column (2 * 60 cm) with bed volume 160 ml, packed with sephadex G-100 equilibrated with buffer A (pH = 8.0, flow rate 0.5 ml/minue, fraction volume 5 ml). The column was calibrated with the following proteins (Bovine serum albumin 67 kD, Myoglobin 17,2 kD, Myoglobin 1+11 14,6 kD and Myoglobin 1 8,2 kD). In each fraction the absorbance was detected at 254 nm with UV detector (Holochrome unit, Gilson) (Figure 1) and cadmium was measured by Perkin Elmer Atomic Absorption Spectrophotometer Model 2380.

Statistical analysis:

The data were analysed by one-way analysis of variance (PC-state computer program) and least significant difference (LSD) was used to test the significant between treatments.

Results and Discussion

Nodulation and nitrogen fixation:

The influence of cadmium concentrations on nodulation and nitrogen fixation is presented in Table 1. Cadmium concentration upto 100 ppm had no significant effect on nodulation. The results obtained for nodule fresh weight showed similar trends. Calculation of nodule number per gram root dry weight shows that the treatment had an effect on growth rahter than on nodule formation.

Table 1

Effect of cadmium chloride on nodulation and nodule activity of *Vicia faba* cv. Giza 3 inoculated with *Rhizobium leguminosarum* biova *viceae* TAL 1402.

Treatment Cd ppm	Nodules/Plant No.	Fw (g)	Nodules/g dry wt root	Acytelene reduction (µmol/h) per plant	per g Nodule	Leghaem. Protein content (mg/g nodule f. wt) Cytosol	Bacter.	Cytosol
00	113.5	1.24	55.36	6.01	4.81	2.37	3.29	5.69
50	118.5	1.31	61.45	6.45	4.91	2.48	3.32	5.71
100	116.5	1.27	70.60	6.31	4.95	2.30	3.34	5.55
200	84.5	0.83	53.82	3.94	4.74	2.34	3.24	5.40
L.S.D at 5%	39.34	0.41	1.09	1.75	0.32	0.19	0.10	0.20
L.S.D at 1%	65.25	0.68	1.08	2.91	0.53	0.32	0.16	0.33

* Each values represents the mean of three replicates.

Cadmium at 100 ppm had no significant effect on absolute (per plant) and specific (per gram fresh weight nodules) nitrogenase activity of faba bean plants. Leghaemoglobin and protein contents of nodule cytosol and bacteroids showed similar patterns. These results are in agreement with the data of BORGES & WOLLUM 1981 and HECKMAN & al. 1984 who found that addition of cadmium to soil had no significant effect on growth, nodulation and nitrogen fixation of soybean. However, BHANDAL & al. 1990 reported that low levels of cadmium inhibited growth, nodulation and nitrogen fixation of *Vigna radiata.*

Higher concentration of cadmium (200 ppm) significantly reduced nodule number, nodule fresh wight and absolute nitrogenase activity. The decrease in the number of nodules per plant may be due to the deleterious effect of cadmium on growth of *Rhizobium.* As root growth was inhibited by 200 ppm cadmium (Table 2), it may also be assumed that fewer sites were available for the infection process. Our findings corroborate those of

YOKOLEVA 1984 who found that cadmium oxide affected the infection process in clover by itself rather than the growth of *Rhizobium*.

In this study, cadmium at 200 ppm had no effect on specific nitrogenase activity. This could be attributed to the resistance of protein and leghaemoglobin of the nodules to cadmium (Table 1).

Plant growth and nitrogen content:

An inhibitory effect of cadmium on the dry weight of shoots and roots was apparent at cadmium concentration of 200 ppm (Table 2). Low levels of cadmium (100 ppm) had an slightly affect on faba bean dry matter accumulation. Calculation of shoot/root ratio on dry weight basis show that the dry matter decrement as the result of Cd treatment is equally partitioned between shoot and root up to 100 ppm Cd. At 200 ppm an adverse effect on shoot rather on root was observed.

Table 2

Effect of cadmium chloride on growth, pigment content, total carbohydrate and total nitrogen contents of *Vicia faba* plants nodulated with *Rhizobium leguminosarum* biovar *viceae* strain TAL 1402.

	Dry wt (g/plant)		Shoot/ Root	Pigment contents (mg/g f. wt. leaves)			T. Carbohyd. (mg/g dry wt)		T. Nitrogen (mg/g dry wt)	
Cd ppm	Root	Shoot	ratio	Chl.a	Chl.b	Carot	Root	Shoot	Root	Shoot
00	2.05	4.80	1.52	2.30	0.57	0.36	100.1	254.9	74.1	331
50	1.92	4.22	1.07	2.20	0.42	0.48	86.4	210.8	76.9	119
100	1.65	3.85	0.87	2.30	0.32	0.39	73.7	195.9	72.1	103
200	1.57	2.65	0.54	1.70	0.08	0.27	50.3	151.8	31.9	75.1
L.S.D at 5%	0.46	1.02	–	0.18	0.01	0.01	7.2	14.9	12.2	10.3
L.S.D at 1%	0.77	1.70	–	0.31	0.11	0.11	11.9	24.8	20.3	17.1

* Each value represents the mean of three replicates.

The reduction of plant dry weight correlated with the effect of cadmium on several aspects of plant metabolism (PAGE & al. 1973, HAGHIRI 1973 and GREGER & LINDBERG 1986). Inhibition of pigments synthesis at 200 ppm Cd resulted in much lower root and shoot carbohydrate contents (Table 2). This reduction may have been at least partially responsible for the inhibition of the nodulation and absolute nitrogenase activity (Table 2).

Total nitrogen content of the roots significantly decreased at 200 ppm Cd. This decline may be a result of the general inhibition of nodulation and nitrogen fixation. The nitrogen content of the shoots was significantly reduced as the Cd level increased. This decline may be attributed to an interference of Cd with the translocation of fixed nitrogen from roots to

shoots. JARVIS & al. 1976 recorded nearly no transport from roots to shoots after a long duration exposure to cadmium. This affect may be due to alterations of the root function by Cd treatment (BARCELEO & al. 1988).

Cadmium contents of roots, shoots and nodules are shown in figure 2. The contents in plant organs increased gradually as cadmium level increased in soil. Roots accumulated cadmium at a higher extent than shoots, while nodules accumulated cadmium at a higher extent than both, roots and shoots. These results are consistent with previously published observations (ROOT & al. 1975, JARVIS & al. 1976, RAUSER & GLOVER 1986). DOMAZLICKA & OPATRNY 1989 found that about 42% of cadmium was retained in roots of potato. Apparently, roots act as a barrier restricting the transport of cadmium to the shoots (JARVIS & JONES 1978).

Metalloprotein (Cd-binding protein) was isolated from nodules of faba bean, grown for 58 days at 100 ppm Cd and 00 Cd, respectively by fractionation on a sephadex G-100 (Figure 3 a, b). The major cadmium containing protein eluted with a molecular weight higher than 67 kD, close to the void volume of the column (200 kD). This protein constituted 38% of the total Cd-binding protein (metallothioneins). A second peak was noticed in protein fractions of a molecular weight of about 67 KD. This fraction contained about 53% of Cd-binding protein. A very small amount of cadmium (ca. 3.4%) was eluted with proteins of 8–17 kD.

Figure 3 b shows the fractionation of proteins isolated from nodules of *Vicia faba* grown without addition of cadmium. This protein profile revelaed that the proteins of a molecular weight of 67 kD were present only in very low amounts. Apparantly, the cadmium-binding protein of 67 kD was synthesised only in the presence of appreciable cadmium concentrations. These resuls are in agreement wth those obtained by YOSHIDA 1986,

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Figure 1: Calibration curve of sephadex G-100 column (2 * 50 cm) with bovine serium albumin 67 kD, Myoglobin 17,2 kD, Myoglobin 1+11 14,6 kD and Myoglobin 1 8,24 kG. The molecular weight plotted versus the elution volume.

Figure 2: Cadmium contents of roots, shoots and nodules of faba bean plants grown in different concentration of cadmium (00,50, 100, 50 and 100 ppm) for 58 days.

Figure 3a: Elution profile of Cd-binding protein of *Rhizobium*-faba bean nodules, grown in 100 ppm Cd for 58 days, applied to sephadex G-100 column (2 * 60 cm), equilibrated with Tris-Hcl (pH = 8.0). Proteins were eluted with the same buffer at flow rate of 0.5 ml/min; fraction volume was 5 ml. Cadmium was measured by Atomic Absorption Spectrometry. – nm 254, . . . Cd.

Figure 3b: Elution profile of Cd-binding protein of *Rhizobium*-faba bean nodules, grown in 00 ppm Cd for 58 days, applied to sephadex G-100 column (2 * 60 cm), equilibrated with Tris-Hcl (pH = 8.0). Proteins were eluted with the same buffer at flow rate of 0.5 ml/min; fraction was 5 ml. Cadmium was measured by Atomic Absorption Spectrometry. -nm 254, . . . Cd.

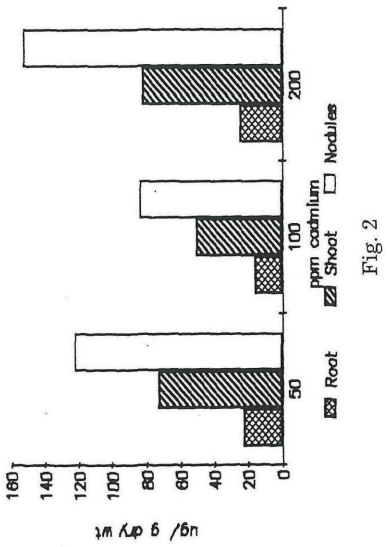

Fig. 2

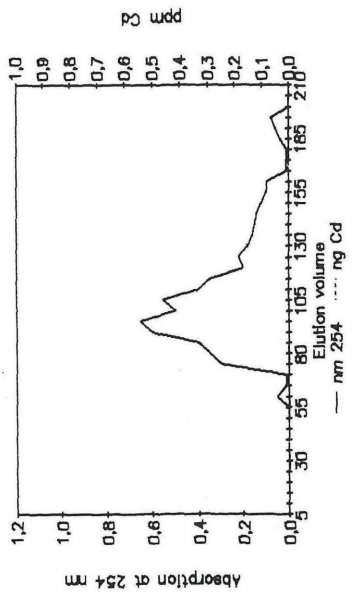

Fig. 3 b

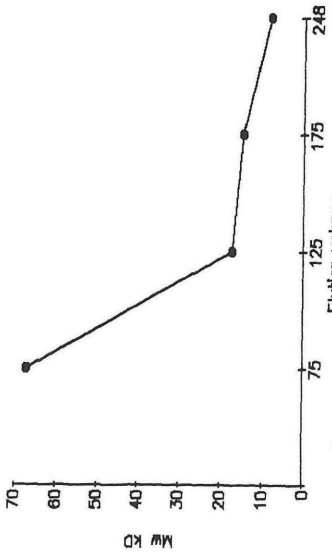

Fig. 1

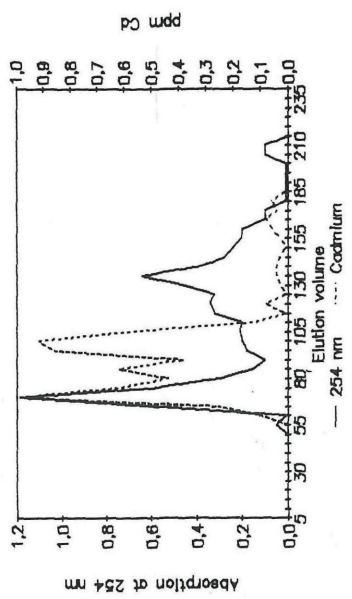

Fig. 3 a

working with soybeans. The author found that the major cadmium containing is protein had molecular weights of 50 kD and 10 kD.

From these data it appears that Cd-binding proteins play an important role in the detoxification of excess Cd and determining resistance of faba bean-*Rhizobium* symbiosis to cadmium toxicity.

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