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Responses to Cadmium Toxicity during *In Vitro* Growth in *Arachis hypogaea*

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Toxic effects of increasing concentration of cadmium (Cd) in the environment due to rapid industrialization has become a major environmental concern. Cadmium is readily taken up by the plant system which retards the growth and yield of crops (Shrivastava and Singh 1989). It has been implicated in inhibition of the photosynthetic mechanism (Bhardwaj and Mascarenhas 1989) as well as nodulation, nitrogen fixation, and consequently the growth of leguminous plants (Sawhney *et al.* 1990). Cell cultures are convenient systems for the study of mechanism of metal toxicity, as they eliminate the interfering processes of translocation and organ-specific trapping of metal ions (Wajda *et al.* 1989) Genetic manipulations for metal tolerance can improve the responsiveness to the environmental stress and promote productivity of plants (Boyer 1982).

To determine the extent of tolerance and raise resistant callus lines, cultures of *Arachis hypogaea* (groundnut), an important oil-yielding crop, were subjected to different concentrations of Cd in the media. The callus growth during the different passages as well as the percentage of dividing cells and mitotic abnormalities was recorded. Also, the protein profiles, quantitative and qualitative, were studied to detect whether synthesis of Cd-binding protein occurs.

MATERIALS AND METHODS

Callus was raised from hypocotyl explants of *Arachis hypogaea* L. var. NFG-7 (seeds procured from the Indian Agricultural Research Institute, New Delhi, India). The seeds were germinated on Murashige and Skoog's (1962) basal medium (MS) without hormones. For callusing, MS medium (containing in $mg.l^{-1}$: 0.50 each of nicotinic acid and pyridoxine HCl, 0.10 thiamine HCl, 100 inositol, and 2.00 glycine) was supplemented with 1 $mg.l^{-1}$ each of napthaleneacetic acid (NAA) and benzylaminopurine (BAP). Cadmium was supplied as cadmium chloride ($CdCl_2$) in final concentrations of 100, 250, 500, and 1000 μM. pH of the medium was adjusted to 5.7 before autoclaving at 15 lb/sq inch for fifteen minutes. Cultures were maintained at 27°C under a 16/8 hours light/dark regime.

For each subculture, 500 mg of callus was transferred to a fresh medium after every 30 days. One set of callus was maintained on the medium containing $CdCl_2$ and another transferred after each subculture to a medium without cadmium. Callus cultures without any Cd in the medium served as control. Twelve replicates were used

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for each treatment. For growth measurements callus pieces were weighed at the time of inoculation and at the end of the growth period that lasted 30 days. Fresh weight (FW) of callus was taken after carefully blotting out the adhering media and moisture. Dry weight (DW) was measured after overnight drying in an oven at 60°C.

Pieces of callus were fixed in Carnoy's fixative, six days after transfer to the fresh medium for cytological studies. The tissue was stained in 2% acetorcein (2 g Orcein powder dissolved in 45% acetic acid), squashed in 45% acetic acid and observed under the microscope. The percentage of dividing cells and mitotic abnormalities were scored. Quantitative estimation of protein was carried out after homogenizing the callus tissues in a mortar and pestle on ice with tris-glycine buffer (pH 8.3). Total buffer soluble proteins were estimated by Bradford's (1976) method using bovine serum albumin as standard.

For qualitative estimation of protein the protein profiles of callus lines were prepared by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Buffer-soluble proteins from 1 g fresh weight (FW) of callus were extracted in 5ml of tris-glycine buffer (pH 8.3), centrifuged, and the supernatant was boiled with 0.3% SDS. The samples were run on 11.1% polyacrylamide slab gels using the method of Weber and Osborne (1975). The gels were stained in 0.05% Coomassie blue and bands were observed after destaining. As markers, standard proteins of known molecular weights (BSA, 68 kd, lysozyme, 14.3 kd) were run simultaneously. For statistical analysis, the standard deviation (S.D.) and standard errors (S.E.) of Means was calculated,

$$S.E.= \frac{S.D.}{\sqrt{n}}$$ where n is the number of observations.

RESULTS AND DISCUSSION

Callus growing on media with 250, 500, and 1000 μM of Cd showed rapid loss in weight after the first passage of culture, became brown and ultimately succumbed after 30 days. Favorable growth, however, was observed only on 100 μM Cd. Therefore, further subcultures were maintained only on 100 μM of Cd. An earlier report has demonstrated that 100 μM Cd stimulates growth of cell cultures of tobacco (Hirt *et al.* 1989). In our experiments with *A. hypogaea*, the fresh weight of the callus on 100 μM Cd, though much lower than the control, remained appreciable after the first two subcultures. After the third subculture, a decline in fresh-weight was observed. The callus growth, however, recovered after the fourth subculture on Cd. Callus transferred back to control medium after each subculture showed a revival in fresh weight, (Fig 1) indicating growth inhibition due to presence of Cd. Toxic effects of Cd have been reported earlier (Wajda *et al.* 1989). Growth inhibition due to Cd, increases with passages in culture until some threshold concentration is reached, at which point inhibition is maximum (third subculture). Surprisingly, the sudden decrease in fresh weight after the third subculture on Cd, did not correspond to a decrease in the dry weight (Fig.1). The callus growing on Cd was compact, hard, and green in contrast to the pale-yellow and friable callus growing on the control medium. Since only a decrease in FW and not in the DW of callus was noted, Cd is possibly influencing the total water uptake of the cells by increasing the permeability of the membrane (Reddy and Prasad 1992).

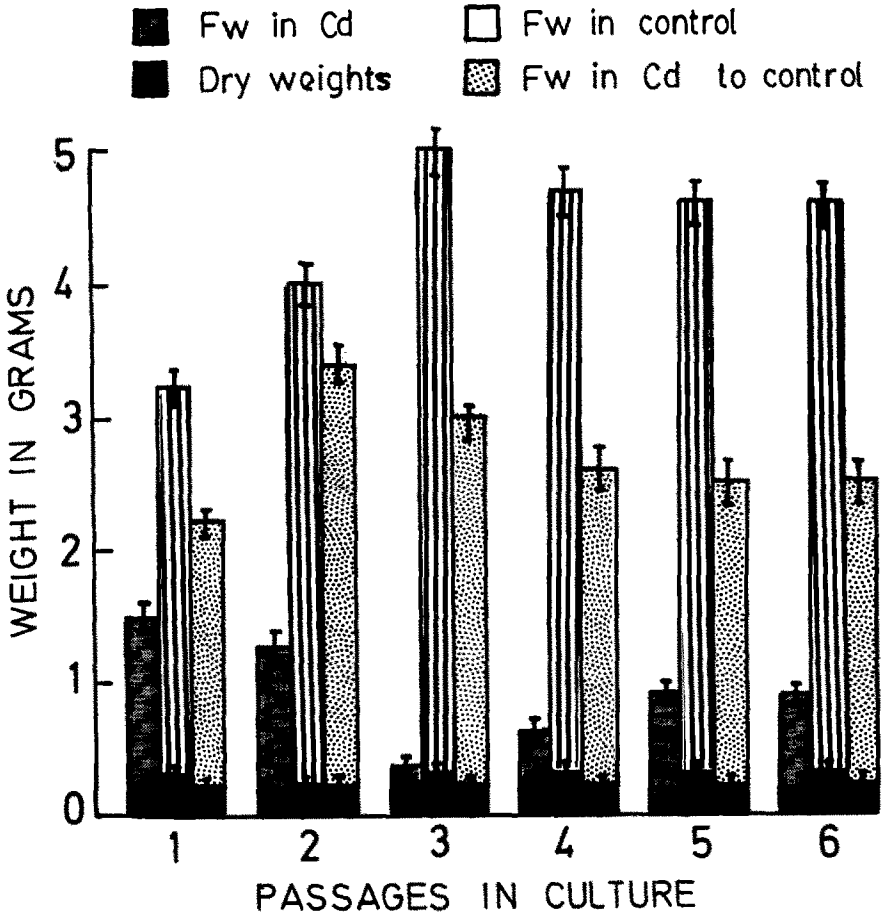

Figure 1. Effect of 100 μM Cd on fresh weight and dry weight of callus of *A. hypogaea* in different passages of culture.

Cytological study of the callus tissues growing on Cd showed that after the first two subcultures, the mitotic index of cells was very similar to the control (Table 1). But after the third subculture, a decline in division rate was noted, although it revived again after the fourth subculture and thereafter remained steady. The mitotic abnormalities of cells growing in Cd showed a constant increase with successive passage in culture (Table 1). During the first two passages, the cells mostly showed stickiness of chromosomes and formation of anaphase bridges (Table 2). After the second and third subcultures, chromosome breaks, clumping of nuclei, micronucleus formation and polyploidy were also observed. Cytological studies showed similarities in behavior of dividing cells and growth pattern of callus, indicating that growth is due to a corresponding increase in the mitotic division of cells. The mitotic abnormalities observed had no bearing on the callus growth, since revival in growth was observed after the fourth subculture which had higher mitotic abnormalities. Chromosomal instability and mitotic abnormalities due to prolonged growth in culture is a common phenomenon (Larkin and Scowcroft 1981). In the present study,

Table 1. Cytological analysis of callus cells of *A. hypogaea* growing with and without cadmium (100 μM) to determine the division rate and total mitotic abnormalities.

Passages in culture	Percentage of dividing cells		Percentage of total mitotic abnormalities	
	without Cd	with Cd	without Cd	with Cd
I.	1.45 ± 0.03	1.14 ± 0.02	2.51 ± 0.08	21.88 ± 0.17
II.	1.57 ± 0.03	1.02 ± 0.02	2.89 ± 0.09	36.53 ± 0.19
III.	1.22 ± 0.03	0.43 ± 0.03	3.17 ± 0.14	51.27 ± 0.21
IV.	1.06 ± 0.02	0.47 ± 0.02	4.24 ± 0.19	58.32 ± 0.23
V.	0.92 ± 0.02	0.52 ± 0.02	5.63 ± 0.18	65.18 ± 0.21
VI.	1.17 ± 0.01	0.84 ± 0.02	6.42 ± 0.18	69.75 ± 0.22

The readings indicate mean values of 1000 cells ± standard error.

Table 2. Types of mitotic abnormalities in callus cells of *A. hypogaea* growing on cadmium (100 μM) medium.

Passages in Culture	Percentage of dividing cells showing abnormalities					
	Stickiness	Anaphase bridges	Chromosome breaks	Clumping	Micronuclei	Polyploidy
I.	4.25 ± 0.16	17.63 ± 0.17	-	-	-	-
II.	10.41 ± 0.19	21.20 ± 0.19	-	-	3.40 ± 0.12	1.52 ± 0.08
III.	12.62 ± 0.17	24.23 ± 0.16	0.69 ± 0.09	2.73 ± 0.12	8.64 ± 0.14	2.36 ± 0.13
IV.	12.89 ± 0.19	25.16 ± 0.21	0.73 ± 0.08	4.91 ± 0.11	10.04 ± 0.16	4.59 ± 0.15
V.	13.36 ± 0.19	26.93 ± 0.22	1.03 ± 0.08	6.22 ± 0.14	11.34 ± 0.18	7.30 ± 0.19
VI.	13.78 ± 0.20	27.14 ± 0.21	1.21 ± 0.09	8.36 ± 0.13	12.16 ± 0.17	9.10 ± 0.18

The readings indicate mean values of 1000 cells ± standard error.

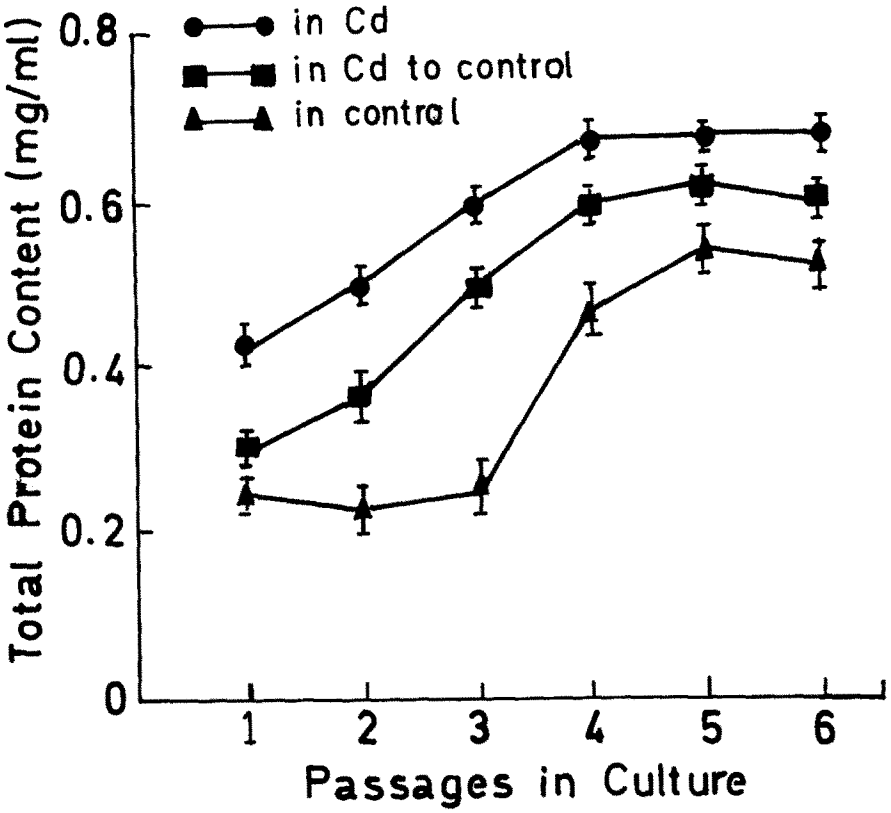

Figure 2. Total buffer-soluble proteins (mg/ml of extraction buffer) in callus lines of *A. hypogaea* during different passages of culture.

mitotic abnormalities were observed both with and without cadmium, although the percentage was much higher in cells growing with Cd than without (Table 1). This implicates Cd in inducing mitotic abnormalities, and types of abnormalities due to Cd *in vitro* were similar to those observed *in vivo* as reported earlier (Chakravarty and Srivastava 1992).

As shown in Figure 2, the total buffer-soluble protein content of callus growing on Cd showed a progressive increase with subcultures. On control medium, the increase in the protein content with age in culture was less marked. Callus transferred from Cd to control medium after each subculture showed a decline in the total protein content. The highest increase in protein was noted in callus lines growing on Cd followed by those which were transferred from Cd to control medium, the lowest being in callus on control medium. The differences in the protein contents of these three callus lines became less evident after the fourth subculture and variation thereafter was not significant. The qualitative analysis of peptide bands separated by SDS-PAGE showed nearly 30 bands with 2 or 3 prominent bands in the mid-region (Fig.3). In successive subcultures on Cd medium, no new band could be detected during the first three subcultures but after the fourth subculture, a peptide band of low molecular weight increased in intensity (Fig. 3).

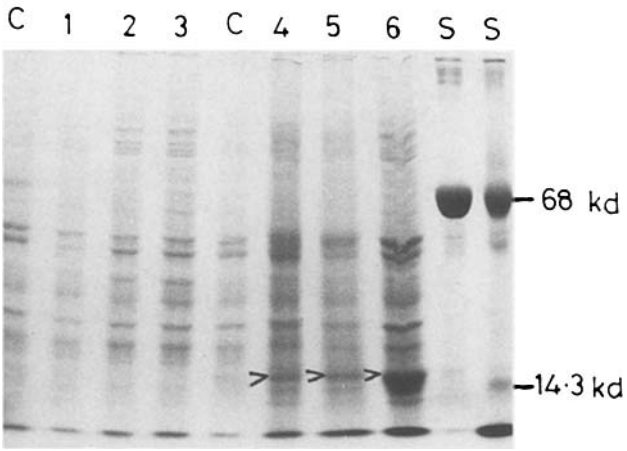

Figure 3. SDS-PAGE protein profiles of callus of *A. hypogaea* growing in Cd in different passages of culture (1 to 6). C : the control line growing on untreated medium, S : the standard proteins [BSA mol. wt. 68 kd and lysozyme 14.3 kd]. Prominent protein band after fourth subculture denoted by arrow (>).

Thus, SDS-PAGE analysis of total protein has revealed that extra protein may be synthesized during culture on Cd.

Transfer of callus from Cd to control medium showed no difference in protein profiles for the first three subcultures. The prominent peptide band observed after fourth subculture on Cd also remained visible in subsequent transfers to control media. The control line of callus growing without Cd did not show any change in protein profiles during culture. This shows that cadmium tolerance which could be conferred by the formation of this protein due to repeated subcultures on Cd is not lost even on transfer to the control medium and appears to be a stable character. In the present report, the gradual tolerance to Cd during *in vitro* growth has been recorded which could be correlated with the increasing prominence of a protein band. Whether the existing protein has increased in amount or a new protein is being formed is yet to be ascertained.

The decrease in protein content of the callus on transfer from Cd to control medium was more marked in early subcultures in contrast to the later subcultures. This indicates that early subcultures are subjected to more toxic effects of Cd whereas after fourth subculture the callus gradually develops tolerance to Cd. Whether this accommodation to Cd is due to the synthesis of Cd-binding protein (Cd BP) as reported earlier (Jackson *et al.* 1987; Robinson *et al.* 1988) is currently under investigation. The low molecular weight of the protein band observed (Fig.3) suggests similarity with a Cd-binding complex reported earlier (Wagner 1984). Further studies on this aspect are in progress. Cd-tolerant callus lines developed here could become amenable to successful regeneration of Cd-tolerant plants which would help in understanding the mechanism and expression of this phenotype, and also in the reclamation of wasteland.

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