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Involvement of a cadmium-induced low molecular weight protein in regulating cadmium toxicity in the diazotrophic cyanobacterium *Anabaena doliolum*

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This study demonstrated the production of a cadmium-induced low molecular weight (3.5 kDa), buthionine sulfoximine (BSO) sensitive protein in *Anabaena doliolum*. Production of this protein was accompanied by a decrease in the glutathione level of the cell. Cadmium was found to be differentially toxic to carbon fixation, O_2 evolution, ATP content, nitrate reductase, nitrogenase, alkaline phosphatase and ATPase of control (untreated), BSO, cadmium and (cadmium + BSO) pre-treated *A. doliolum*. Toxicitywas maximum in BSO-grown cells followed be control (untreated), cadmium + BSO and least in cadmium-grown *A. doliolum*. Cadmium and (cadmium + BSO)-grown cells registered an increased lipid production, reduced metal uptake and low K^+, Na^+ loss. In spite of equal cadmium uptake rates, a significant difference in toxicity between cadmium-grown and (cadmium + BSO)-grown cultures was, however, noticed. Better performance of physiological and biochemical variables of cadmium-grown *A. doliolum* and its tolerance to cadmium could be due to the synthesis of low molecular weight cadmium binding protein (presumably phytochelatin) as well as an increased production of lipid.

Keywords: cadmium-inducible low molecular weight protein, *Anabaena doliolum*, glutathione, ATPase, alkaline phosphatase, nitrogenase, buthionine sulfoximine

Introduction

The ecological adaptation of plant communities to chronically metal contaminated habitats presents a classical example of rapid evolution under extreme selection pressure (Stokes, 1975). Although metals have a direct bearing on various physiological and biochemical processes, including reduction in growth, photosynthesis, chlorophyll *a* content, inhibition of enzyme activities and degradation of chloroplasts, some species posses unique abilities to adapt rapidly and develop tolerance to such adverse conditions. Survival of organisms in metaliferous environments has been suggested to be due to exclusion of metals (Foster 1977, Rai *et al.* 1991b); compartmentalization of metals in vacuoles, cells walls and polyphosphate bodies (Turner 1970, Jensen *et al.* 1982); evolution of metal tolerant enzymes (Wainwright & Woolhouse 1975); and production of metal binding protein (Grill *et al.* 1988, Gekeler *et al.* 1988, Reddy & Prasad 1992). The latter has, however, gained considerable attention during the last few years. Although production of a low molecular weight (3–10 kDa) cysteine rich peptide capable of binding metal ions via thiolate coordination has been found to be conserved from orchidales, the most advanced group of higher plants, to red, green and brown algae (Gekeler *et al.* 1988), cadmium has been found to be most efficient in inducing the production of such metal binding proteins (Steffenis, 1990).

Phytochelatin [class III metallothionin (MT); Fowler *et al.* 1987, Robinson 1989] biosynthesis is a highly regulated phenomenon. It has recently been demonstrated that the formation of phytochelatin is catalyzed by a specific γ-glutamylcysteine dipeptidyl transpeptidase called phytochelatin synthase, which is activated in the presence of metal ions and uses glutathione (GSH) as substrate (Grill *et al.* 1989). Cadmium-induced phytochelatin synthesis is known to be associated with rapid depletion of the GSH pool in *Datura innoxia* cell suspension (Delhaise *et al.* 1989), and roots and shoots of maize seedlings (Meuwly & Rauser, 1992). GSH is a well known antioxidant involved

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in defense against free radicals in plants. The metal induced GSH depletion has been reported to cause oxidative stress in plants (Ric de Vos *et al.* 1992). However, this phenomenon was found to operate especially in the presence of redox cycling metals like copper. The purpose of this study was to find out (i) if phytochelatin is produced in the nitrogen-fixing cyanobacteria, (ii) if cadmium can cause such oxidative stress through GSH depletion, and (iii) how far the physiological and biochemical behavior of phytochelatin containing cyanobacterium differs from the normal strain. It is worth stating that such information does not exist in the case of nitrogen fixing cyanobacteria, although production of metal binding proteins having chromatographic properties similar to mammalian class I MT has been reported in the non-nitrogen-fixing cyanobacterium *Synechococus* (MacLean *et al.* 1972). Olafson *et al.* (1988) also characterized a prokaryotic MT in *Synechococcus* that was a class II type. It is worth mentioning that these MTs are gene products, not the secondary metabolites (Robinson *et al.* 1990, Morby *et al.* 1993, Turner *et al.* 1993).

Materials and methods

Test system

A. doliolum Bharadwaja was grown axenically in modified Allen & Arnon's medium (1955) at pH 7.5 under 72 μmol photon $m^{-2} s^{-1}$ PAR light intensity and a photoperiod of 14:10 h at 24 ± 2 °C. Cultures, only, from logarithmic phase were used for toxicity testing. A stock solution of $CdCl_2 \cdot H_2O$ was prepared in double glass-distilled water and passed through a millipore membrane filter (0.45 μm) before use. Biochemicals used were obtained from Sigma (St Louis, MO) and Glaxo (Dehli, India).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

A dense culture (0.4 OD at 663 nm) of *A. doliolum* was grown in the presence of 20 μM cadmium, 2 mM BSO and 20 μM cadmium plus 2 mM BSO for 7 days, and the cells were harvested by centrifugation. All subsequent treatments were carried out at 4 °C. Cells (3 g fresh weight) washed in 50 mM Tris–HCL (pH 6.8) were resuspended in the same buffer and disrupted by sonication. The homogenate was centrifuged for 30 min at 20000 r.p.m. and the supernatant was taken for further study. SDS–PAGE was carried out in 1.0 mm thick gels containing 15% acrylamide. The gel loaded with 20 μl sample was run at constant voltage of 220 V at 8 °C and stained with Coomassie blue as per the method of Sambrook *et al.* (1989).

Measurement of reduced GSH, photosynthesis and ATP contents

GSH was measured by the method of Anderson (1985). Carbon fixation was measured in terms of ^{14}C uptake from $NaH^{14}CO_3$ (specific activity 18.5×10^5 Bq) in a LS 7000 liquid scintillation counter (see Rai & Raizada 1986). Photosynthetic O_2 production was measured with a polarographic oxygen electrode enclosed in a 10 ml airtight reaction vessel and connected to an oxygen analyser (Rank Brothers, UK). The total ATP was measured by luciferin–luciferase assay (Larsson & Olsson 1979) using LKB 1250 luminometer.

Estimation of nitrate reductase, nitrogenase, alkaline phosphatase and ATPase

The activity of nitrate reductase was estimated by measuring the formation of nitrite from nitrate (Camm & Stein, 1974). The reagents used were sulfanilamide and *r*-(*N*-1)-naphthylethylene diamine dihydrochloride. Nitrogenase was measured by the acetylene reduction technique of Stewart *et al.* (1968) in a CIC gas chromatograph equipped with a Porapak R column and flame ionization detector. Alkaline phosphatase activity was assayed by the method of Ihlenfeldt & Gibson (1975). The specifc activity was expressed as μmol *p*-nitrophenol liberated mg $protein^{-1}$ h^{-1}. Mg^{2+}-dependent ATPase activity was measured as per the method of Ohnisi *et al.* (1975).

Cation loss, cadmium uptake and lipid content

Cyanobacterial samples incubated for 24 h in metal spiked medium were withdrawn and centrifuged. The K^+ and Na^+ present inside the cells were released by heating the washed pellets in boiling water bath. Cells were then removed by centrifugation, and amounts of K^+ and Na^+ were determined with the help of a flame photometer. Cadmium uptake by cyanobacterial cells over a 2 h incubation was measured as per the method of Martin (1979) with the help of a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer. Approximately 100 ml culture was harvested by filtration on Whatman 44 filters. Lipids were extracted from samples using chloroform:methanol (2:1, v/v) and dried under a flash of nitrogen at 40 °C. The total lipid so obtained was estimated gravimetrically.

All the results were analyzed in terms of protein, which was estimated following the method of Lowry *et al.* (1951). The results were verified by using Students *t*-test.

Results

Figure 1 presents the SDS–PAGE protein profile of control (untreated), cadmium-grown, and cadmium (20 μM) + BSO (BSO was supplemented 2 h before the addition of cadmium)-grown *A. doliolum*. In contrast to control (lane 2) a new dense band [lane 3, low molecular weight protein (LMP)] with a molecular weight of 3.5 kDa (approximately) was noticed in cadmium-grown cells. This band was, however, not observed in cadmium-supplemented cultures pretreated with BSO (lane 1).

Figure 2 demonstrates a reduction in the glutathione level of the cells following cadmium treatment. For example, the untreated cells contained 12.6 ± 0.8 nM glutathione mg $protein^{-1}$. The GSH level was reduced in a

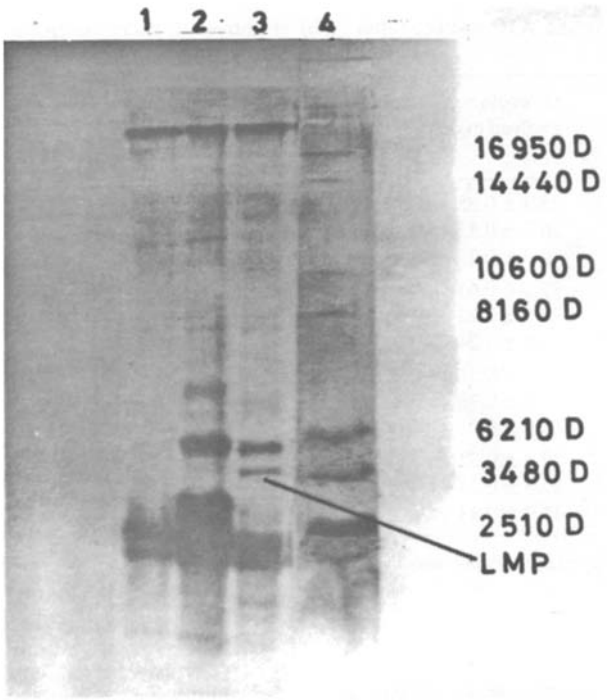

Figure 1. Gel electrophoretic study of (cadmium + BSO)-pretreated (lane 1), control (lane 2) and cadmium-treated (lane 3) *A. doliolum* after 7 days of treatment. LMP, low molecular weight protein. Lane 4, standard molecular weight markers ranging from 16950 to 25100 Da [1, myoglobin (polypeptide backbone 1–153): 16950; 2, myglobin (I + 11, 1–131): 14440; 3, myoglobin (I + III, 56–131): 10600; 4, myoglobin (1, 56–131): 8160; 5, myglobin (II, 1–55): 6210; 6, glucagon: 3480; 7, myoglobin (III, 132–153): 2510].

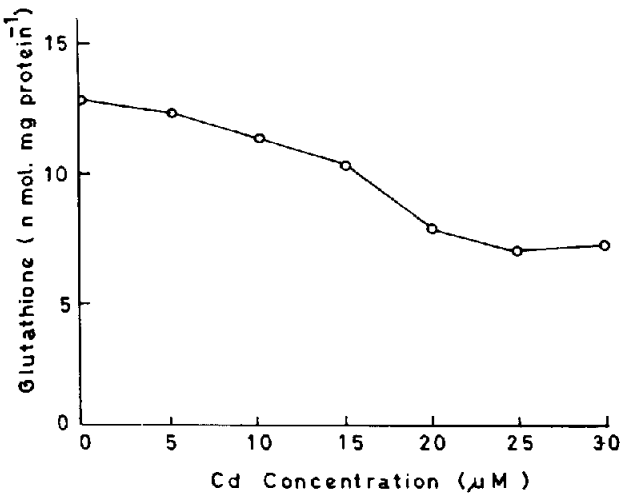

Figure 2. Effect of cadmium supplementation on GSH levels of the cell.

concentration dependent manner upto 20 μM cadmium. However, at 25 and 30 μM cadmium no significant change in its level was noticed as compared with 20 μM cadmium.

To compare the physiological and biochemical behavior of control (untreated), cadmium-grown, BSO-grown and (cadmium + BSO)-grown *A. doliolum* under cadmium stress, the test cyanobacterium was precultured for 7 days separately in the presence of cadmium, BSO and cadmium + BSO before undertaking the toxicity study. The untreated culture of each type was designated as its respective control. Table 1 shows the effect of cadmium on carbon fixation and O_2 evolution of *A. doliolum*. About 58 and 82% reduction in carbon fixation was noticed following supplementation of 0.5 and 1.0 μM cadmium to the control culture. In BSO and (cadmium + BSO) precultured cells the percent inhibition was, respectively, 84 and 97, and 52 and 71 in the above order. Cells pretreated with cadmium, when exposed to cadmium again for toxicity measurement, registered a significant reduction in toxicity, i.e. only 15 and 39% inhibition was noticed, respectively, at 0.5 and 1.0 μM cadmium. The cadmium-induced O_2 evolution also followed a trend similar to carbon fixation with inhibition percentages of 53 and 71 for control, 90 and 100 for BSO-grown, 44 and 62 for (cadmium + BSO)-precultured, and 14 and 38 for cadmium-precultured cells, respectively, at 0.5 and 1.0 μM cadmium. It is also clear from Table 1 that 0.5 and 1.0 μM cadmium reduced the ATP pool of the untreated (control) *A. doliolum*, respectively, by 60 and 83%. The inhibition of ATP followed the trend of inhibition of O_2 evolution. Cadmium-grown cells showed a significant reduction in toxicity (20 and 30%) as compared with (cadmium + BSO)-grown (41 and 61%) cells. In BSO-treated cells the percent inhibitions were about 82 and 93% in the above order.

Effects of cadmium on nitrate reductase, nitrogenase, alkaline phosphatase and ATPase are summarized in Table 2. An appreciable difference in toxicity between control, cadmium-grown, BSO-grown and (cadmium + BSO)-grown cultures was noticed. Untreated *A. doliolum* showed an inhibition of 57, 61, 58 and 53%, respectively, for nitrate reductase, nitrogenase, alkaline phosphatase and ATPase at 0.5 μM cadmium. A further increase in percent inhibition was noticed at 1.0 μM cadmium. However, only 18, 9, 9 and 11, and 31, 20, 27 and 32% inhibition was observed, respectively, at 0.5 and 1.0 μM cadmium in cells pre-grown in the presence of cadmium. In contrast to this, the BSO-grown cells showed a percent inhibition of 68, 77, 79 and 83, and 83, 92, 89 and 89 in the above order. The untreated (control) cells when supplemented with 0.5 and 1.0 μM cadmium registered an uptake of only 0.015 and 0.023 mg cadmium mg protein^{-1} (Table 3) after 2 h. BSO-grown cells showed almost same uptake level. The uptake values were, however, 0.012 and 0.018 μg cadmium mg protein^{-1} for cadmium-pregrown cells. No significant difference in cadmium uptake was noticed between cadmium- and (cadmium + BSO)-precultured cells.

The loss of cations from the cell is given in Table 3. About 67 and 80% Na^+ loss was noticed, respectively, at 0.5 and 1.0 μM cadmium in control cultures. These percentage were, however, 85 and 93 in BSO-grown cells. It is interesting to note that K^+ loss was more pronounced than Na^+. Comparatively low loss of cations was noticed in cadmium-grown and (cadmium + BSO)-grown cells.

Table 1. Cadmium toxicity to carbon fixation, O_2 evolution (after 2 h) and ATP content (after 72 h) of untreated, cadmium-grown, BSO-grown and (cadmium + BSO)-grown *A. doliolum*

Type	Treatment (μM)	Carbon fixation (c.p.m × 10^6 mg protein^{-1} h^{-1})	O_2 evolution (μmol O_2 evolved mg protein h^{-1})	ATP content (μg ATP mg protein^{-1})
Untreated	control	0.413 ± 0.06 (−)	35.3 ± 0.35 (−)	1.21 ± 0.03 (−)
	0.5	0.173 ± 0.03 (58)	16.8 ± 0.53 (53)	0.48 ± 0.02 (60)
	1.0	0.074 ± 0.05 (82)	8.3 ± 0.23 (77)	0.21 ± 0.04 (83)
Cadmium-grown	control	0.434 ± 0.05 (−)	32.1 ± 0.61 (−)	1.11 ± 0.02 (−)
	0.5	0.370 ± 0.03 (15)*	26.5 ± 0.22 (14)	0.89 ± 0.04 (20)
	1.0	0.264 ± 0.03 (39)	19.9 ± 0.24 (38)	0.78 ± 0.01 (30)
BSO-grown	control	0.411 ± 0.04 (−)	30.2 ± 0.51 (−)	1.03 ± 0.04 (−)
	0.5	0.115 ± 0.02 (72)	3.1 ± 0.33 (90)	0.19 ± 0.03 (82)
	1.0	0.062 ± 0.02 (85)	0 ± 0 (100)	0.07 ± 1.02 (93)
(Cadmium + BSO)-grown	control	0.415 ± 0.05 (−)	30.8 ± 0.32 (−)	1.09 ± 0.02 (−)
	0.5	0.199 ± 0.02 (52)	17.3 ± 0.24 (44)	0.65 ± 0.01 (41)
	1.0	0.120 ± 0.03 (71)	11.7 ± 0.43 (62)	0.43 ± 0.03 (61)

Data in parentheses denote percent inhibition; t significant at $P < 0.05$, $^*P < 0.2$; 20 μM cadmium and 2 mM BSO were used in all the experiments and organism was precultured for 7 days.

Table 2. Effect of cadmium on nitrate reductase, nitrogenase, alkaline phosphatase and ATPase activities of control, cadmium-grown, BSO-grown and (cadmium + BSO)-grown *A. doliolum* after 24 h of treatment

Types	Treatment (μM)	Nitrate reductase (μg NO_2 mg protein^{-1} h^{-1})	Nitrogenase (nmol C_2H_4 mg protein^{-1} h^{-1})	Alkaline phosphatase (μmole PNP mg protein^{-1} h^{-1})	ATPase (μg PO_4^{3-} liberated mg protein h^{-1})
Untreated	control	0.49 ± 0.08 (−)	5.8 ± 0.05 (−)	6.91 ± 0.04 (−)	0.735 ± 0.05 (−)
	0.5	0.21 ± 0.03 (57)	2.3 ± 0.03 (61)	2.3 ± 0.03 (58)	0.345 ± 0.02 (53)
	1.0	0.13 ± 0.05 (74)	1.5 ± 0.04 (74)	1.8 ± 0.02 (74)	0.198 ± 0.03 (73)
Cadmium-grown	control	0.45 ± 0.03 (−)	5.6 ± 0.04 (−)	7.5 ± 0.02 (−)	0.733 ± 0.02 (−)
	0.5	0.37 ± 0.02 (18)	5.1 ± 0.02 (09)	6.8 ± 0.04 (09)	0.649 ± 0.02 (11)
	1.0	0.31 ± 0.05 (31)	4.5 ± 0.03 (20)	5.5 ± 0.02 (27)	0.497 ± 0.03 (32)
BSO-grown	control	0.43 ± 0.02 (−)	4.9 ± 0.05 (−)	6.9 ± 0.4 (−)	0.682 ± 0.03 (−)
	0.5	0.13 ± 0.02 (68)	1.1 ± 0.03 (77)	1.4 ± 0.02 (79)	0.116 ± 0.02 (83)
	1.0	0.07 ± 0.01 (83)	0.4 ± 0.02 (92)	0.8 ± 0.02 (89)	0.075 ± 0.03 (89)
(Cadmium + BSO)-grown	control	0.45 ± 0.03 (−)	5.3 ± 0.02 (−)	7.8 ± 0.04 (−)	0.729 ± 0.02 (−)
	0.5	0.28 ± 0.01 (38)	3.9 ± 0.02 (27)	3.7 ± 0.02 (53)	0.423 ± 0.3 (42)
	1.0	0.14 ± 0.03 (69)	3.1 ± 0.03 (42)	2.8 ± 0.04 (64)	0.343 ± 0.02 (53)

Data in parentheses denote percent inhibition; t significant at $P < 0.05$; cells were preculture for 7 days.

The lipid content of control, cadmium-grown, BSO-grown and (cadmium + BSO)-grown *A. doliolum* is summarized in Table 3. The control cells were found to contain 0.25 mg lipid mg protein^{-1} of cells. Likewise the BSO-treated cells contained almost the same amount (0.26 mg protein^{-1}) of lipid. About 48 and 52% increase in lipid content was noticed, respectively, in cadmium-grown and (cadmium + BSO)-grown cells as compared to control.

Discussion

The appearance of a new protein band with a molecular weight of 3.5 kDa in a 15% polyacrylamide gel of cadmium-treated cells (Figure 1) amply demonstrated the potential of *A. doliolum* to synthesize a new protein under cadmium stress. The disappearance of this band from cells treated with BSO, a potent inhibitor of phytochelatin synthase (a precursor of phytochelatin synthesis), indicated that the new protein band could be of phytochelatin. The enzyme phytochelatin synthase (r-glutamylcysteine dipeptidyl transpeptidase) transfers r-glutaminecysteine from GSH to another GSH molecule, thereby forming the primary structure of phytochelatin (Grill *et al.* 1989). Hence, a reduction in GSH levels of the cell following cadmium treatment also gave indirect proof to the phytochelatin nature of the new protein.

A concentration-dependent cadmium-induced inhibition of the ATP pool, photosynthesis and enzyme (nitrate reductase, alkaline phosphatase, ATPase, nitrogenase) activities (Tables 1 and 2) agrees well with the findings of Rai *et al.* (1991a), who demonstrated that inhibition of

Table 3. Cadmium induced changes in cellular Na^+ and K^+, lipid content and uptake of cadmium by untreated, cadmium-grown, BSO-grown and (cadmium + BSO)-grown cells of *A. doliolum*

Types	Treatment (μM)	Na^+ in the cell (mg Na^+ mg protein^{-1})	K^+ in the cell (mg K^+ mg protein^{-1})	Lipid content (mg mg protein^{-1})	Cadmium uptake (mg cadmium mg protein 2 h^{-1})
Untreated	control	0.15 ± 0.05 (−)	0.13 ± 0.06 (−)	0.25 ± 0.05 (−)	−
	0.5	0.05 ± 0.04 (67)	0.04 ± 0.02 (69)	0.13 ± 0.03 (48)	0.015 ± 0.003
	1.0	0.03 ± 0.02 (80)	0.02 ± 0.02 (85)	0.05 ± 0.02 (80)	0.023 ± 0.002
Cadmium-grown	control	0.14 ± 0.08 (−)	0.14 ± 0.05 (−)	0.37 ± 0.03 (−)	−
	0.5	0.12 ± 0.03 (14)**	0.12 ± 0.04 (14)**	0.29 ± 0.02 (22)	0.012 ± 0.002
	1.0	0.09 ± 0.03 (36)	0.08 ± 0.04 (43)*	0.26 ± 0.04 (30)	0.018 ± 0.003
BSO-grown	control	0.13 ± 0.05 (−)	0.12 ± 0.03 (−)	0.26 ± 0.05 (−)	−
	0.5	0.02 ± 0.05 (85)	0.01 ± 0.02 (92)	0.11 ± 0.03 (58)	0.015 ± 0.003
	1.0	0.01 ± 0.02 (93)	ND	0.03 ± 0.01 (89)	0.022 ± 0.004
(Cadmium + BSO)-grown	control	0.15 ± 0.06 (−)	0.14 ± 0.03 (−)	0.38 ± 0.03 (−)	−
	0.5	0.08 ± 0.03 (47)*	0.07 ± 0.02 (50)	0.20 ± 0.02 (47)	0.012 ± 0.004
	1.0	0.04 ± 0.02 (74)	0.03 ± 0.05 (79)	0.13 ± 0.02 (66)	0.018 ± 0.002

Data in parentheses denote percent inhibition; t significant at $P < 0.1$, *$P < 0.2$, **$P <$ not significant; ND, not detectable; cells precultured for 7 days.

ATP generation due to disruption of photosynthetic electron transport by mercury and zinc was the major cause for reduction of all metabolic activities. The cells pretreated with BSO showed greater susceptibility to cadmium as compared with control (Tables 1 and 2). The cadmium uptake rates were, however, equal in both the cases (Table 3). Hence, the increased toxicity in BSO-pretreated cells might be due to the absence of GSH as well as inhibition of phytochelatin biosynthesis by BSO, as both GSH and phytochelatin biosynthesis are known to share a common enzymatic pathway (Steffenis 1990).

In spite of the absence of GSH and phytochelatins in BSO-pretreated cells, the (cadmium + BSO)-grown cells depicted reduced cadmium toxicity. This is ascribed to the reduced cadmium uptake in (cadmium + BSO)-grown cells (Table 3). A further study revealed an increased production of lipid, which might have changed the permeability of the plasma membrane, thereby hindering the cadmium uptake and resulting in low toxicity. A low Na^+ and K^+ efflux from (cadmium + BSO)-pretreated cells (Table 3) as compared with untreated ones confirmed an alteration in the permeability of the plasma membrane of *A. doliolum* cells when grown under cadmium stress.

Although cadmium-grown and (cadmium + BSO)-grown cells showed equal metal uptake rates, the former depicted a low sensitivity to cadmium. In other words the toxicity was high in (cadmium + BSO)-grown cells. Thus it is difficult to pin point if the difference in toxicity was due either to production of phytochelatins in cadmium-pretreated cells or inhibition of GSH synthesis in (cadmium + BSO)-grown *A. doliolum*. Ric de Vos *et al.* (1992), in the case of *Silene cucubalus*, demonstrated that copper tolerance does not depend on the production of phytochelatins but is related to the plant's ability to prevent GSH depletion by restricting copper uptake. However, a comparison of control (untreated) and cadmium-pretreated cells in our study revealed that a 20–22% reduction in cadmium uptake resulted in a 40–54% decrease in cadmium toxicity of all the metabolic processes of cadmium-pretreated cells. This suggests that the cadmium taken up by cadmium-pretreated cells might have been detoxified internally through binding with newly synthesized protein. On the basis of the above observations it is concluded that a difference in metabolic behavior of cadmium-pretreated and control cells and a greater tolerance of pretreated cells was due to production of a low molecular weight protein. In addition to this the increased production of lipid also played a very important role in the differential toxicity of cadmium in the test cyanobacterium.

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