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Responses of *Bacopa monniera* Cultures to Cadmium Toxicity

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Metal ions such as Cu, Mn, Zn, and Fe are essential trace nutrients taking part in vital metabolic functions such as redox reaction and electron transfer in a multitude of enzyme-catalyzed reactions and forming a structural component of several enzymes. Many other metals like Ag, Cd, Hg and Pb have no known biological functions but are highly toxic. At higher concentrations, however, all metals inhibit growth and metabolism, and may even cause death of the organism (Zenk, 1996), the extent of inhibition depending on plasmalemma-metal interaction in roots or the uptake of metal ions from soil by plants. Plants have evolved mechanisms to regulate the uptake of both essential and non-essential ions so as to survive under heavy metal stress. Cadmium is one of the potent toxins with a low natural abundance. Industrial activities, mining and mineral processing, modern agricultural practices and sewage disposal operations have contaminated the soil with high levels of Cd. Soil also receives minor amounts of Cd through phosphate fertilizers (William and David, 1973). Moreover, the bio-nondegradability of metal ions often leads to their concentration build-up despite their dissipation through leaching and sequestration in some insoluble and non-bioavailable forms. Plants grown in such soils can accumulate Cd which affects a number of metabolic activities (Iqbal and Khudsar, 2000). Cd is known to be absorbed passively and transported across the cytoplasmic membrane (Cutler and Rains, 1974). Plants can grow with toxic concentrations of Cd if a tolerance mechanism is operative. This involves production of metal binding cysteine-rich compounds, most of which belong to the phytochelatin (PC) group (de Knecht et al., 1994) and have been attributed a role in the cellular metal homeostasis (Steffens, 1990).

Bacopa monniera, commonly known as 'Brahmi', is a small creeping herb (of Scrophulariaceae) known primarily for its use as memory vitalizer. Its exuberant *in vitro* regeneration (Ali et al., 1996) prompted us to study its regeneration and growth potential under Cd-stress. We have produced the salt- and heavy-metal tolerant regenerants of this species (Ali et al., 1998, 1999). However, no systemic study of Cd stress on this plant has been carried out so far.

The present study investigates the effects of cadmium toxicity on proline and protein accumulation, morphogenic response and photosynthetic performance, and estimates the differential accumulation of cadmium on the cell wall and in the cytoplasm of the regenerants of *Bacopa monniera*.

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MATERIALS AND METHODS

Stem segments (20 mm long) of *Bacopa monniera* (L.) Wettst. of the family Scrophulariaceae were procured from the Herbal Garden at Jamia Hamdard. Five hundred stem explants were thoroughly washed under running tap water for 30 min and with 5% solution of cetrimide (Tetradecyltrimethylammonium bromide, SRL, India) for 10 min. The explants were sterilized with 10% freshly prepared aqueous sodium hypochlorite for 10 min and with 0.1% mercuric chloride for 5 min, followed by a final rinse with 70% alcohol for 2 min. The explants were then thoroughly rinsed with sterile distilled water before implantation.
Murashige and Skoog's (1962) medium (MS) without any Cd, gelled with 0.62% agar (Qualigen, India) and pH adjusted to 5.7 before autoclaving for 15 min at 121°C and 15 kg cm^2 was used throughout. MS (sucrose 3%) medium supplemented with various levels of 1-nephthaleneacetic acid (NAA, 0.1-0.2 mg L^{-1}), 6-benzylaminopurine (BAP, 0.5-5.0 mg L^{-1}) and casein hydrolysate (CH, 1000 mg L^{-1}) was used for culture initiation. Each experiment was repeated thrice with atleast 24 replicates. All the cultures were maintained at 25 ± 2°C with 55 ± 5% relative humidity in a culture room provided with a 14-hr-photoperiod through 40 W cool white fluorescent tubes and incandescent bulb (total intensity being 100 μmole m^{-2} s^{-1}).

Though shoots could be obtained on all combinations of NAA/BAP, the regenerants were maintained on MS (sucrose 2%) medium supplemented with NAA (0.2 mg L^{-1}), BAP (0.5 mg L^{-1}) and glutamine (50 mg L^{-1}). Four-wk-old cultures with five 20 mm long regenerants (500 mg fresh mass) were transferred to culture vials each containing the above medium amended with different concentrations (25, 50, 75 and 100 μM) of $CdCl_2$. The morphogenic response (number of shoots per culture, height of shoots, increase in the total fresh mass of regenerants and the root formation) was recorded every four weeks.

The samples taken in triplicate (1000 mg each) from cultures grown on MS medium without Cd and with various concentrations of Cd, were homogenized with 1 mL of 1*M* Tris HCl buffer (pH 7) in a chilled mortar and pestle. The crude extract was centrifuged at 8714 g for 5 min and separated; the supernatant was taken as cytoplasmic fraction and the debris as cell wall fraction in a crucible (Ali et al., 1998, 1999). Both were dried separately in an oven for 4 hr at 65°C, and incinerated in a muffle furnace at 500°C until a colourless or light-grey ash was obtained (Chakravarty and Srivastava, 1997a). The ash was dissolved in 1*N* HCl and the volume was made up to 25 mL with distilled water. Cadmium content in the two fractions was estimated by atomic absorption spectrophotometer (Perkin Elmer 3110, Norwalk, USA).

The total buffer soluble proteins in the regenerants were estimated as described by Ali et al. (1999). Proline content of the regenerants was determined according to Bates et al. (1973). LI-6200 Portable Photosynthesis System (LI-COR, Inc., Lincoln, USA) was used for automatic measurements of net rate of

photosynthesis, stomatal conductance and internal CO_2 concentrations in the various samples.
The data were analysed using the Student's t-test so as to determine whether the changes observed in treated samples over the control values are significant.

RESULTS AND DISCUSSION

The *B. monniera* cultures grown on control (devoid of Cd) medium, gradually gained in fresh mass (FM), and in number and height of shoots. Growth and the rooting potential of cultures were adversely affected on media containing Cd. The cultures grown on MS medium supplemented with 50 μM Cd experienced a gradual decline in number and height of shoots and FM of cultures up to 12 weeks. When transferred back to 25 μM Cd after 12 weeks, these cultures showed a gradual recovery four weeks after the transfer (Table 1). The regenerants maintained on Cd (25 μM), when transferred after 8 weeks to higher concentrations (50, 75 μM) of cadmium (data not presented), showed no adverse effect on growth up to 20 weeks. At higher levels of Cd (50-100 μM), however, root elongation was inhibited (Table 2). Four-week-old cultures on 50 μM, when transferred to 75 μM Cd, succumbed by the 4th week of the transfer. The cultures grown directly on 75 and 100 μM Cd failed to survive beyond 4 weeks and 2 weeks, respectively (Table 1).

In the cultures grown on 25 and 50 μM Cd, accumulation of Cd, more in cell wall fraction than in cytoplasm, was detected (Table 2). Proline accumulation enhanced in the cultures grown under Cd stress and was correlative to the level of cadmium in the medium (Table 3). The rate of photosynthesis, stomatal conductance and internal CO_2 concentrations in Cd-stressed regenerants declined in comparison to control. The total protein content, however, considerably increased in response to the enhanced Cd levels (Table 3).

Thus, the present investigation demonstrates that regenerants of *B. monniera* are able to grow on Cd up to 50 μM for 12 weeks, and that the plantlets can sustain growth at 75 and 100 μM Cd only for 4 weeks and 2 weeks, respectively. Normal growth could be resumed when the regenerants were brought on to lower concentrations (25 μM) for 8 weeks and transferred back to higher concentrations (50-75 μM) of Cd (data not presented). These cultures survived considerably longer (20-25 weeks). Moreover, neither direct nor step-wise transfer of cultures on 100 μM Cd and beyond could promote growth after two weeks. The regenerants could not survive when transferred to Cd-free media, thus indicating that they had become metallophilic. Calcium is critical to proper root development, to the extent that an acclimated tolerant plant establishes regulatory balance in the presence of cadmium; and placing the plant in a substantially lower cadmium environment could disrupt the equilibrium controls of the Ca-calmodulin system, even to the point of being lethal. It is evident that regenerants acquired through gradual exposure to higher concentrations of Cd are more promising than those through direct transfer. Root growth was hampered by $CdCl_2$. Root

Table 1. Effect of $CdCl_2$ on morphogenic response (number and height of shoots, fresh mass of cultures) in *Bacopa monniera*.

$CdCl_2$ (μM)	After 4 weeks			After 8 weeks			After 12 weeks			After 16 weeks		
	No.	Height	FM	No.	Height	FM	No.	Height	FM	No.	Height	FM
00	16.0 ±0.82	5.6 ±0.19	1.6 ±0.06	17.0 ±0.45	5.7 ±0.14	1.7 ±0.05	18.0 ±0.31	6.0 ±0.15	1.8 ±0.06	18.0 ±0.48	6.2 ±0.13	1.8 ±0.08
25	11.0 ±0.92*	2.5 ±0.19*	1.3 ±0.08*	11.0 ±0.17*	2.8 ±0.11*	1.3 ±0.05*	12.0 ±0.65*	3.0 ±0.15*	1.3 ±0.09*	13.0 ±0.46*	3.9 ±0.12*	1.4 ±0.08*
50	9.0 ±0.78*	2.1 ±0.14*	0.93 ±0.04*	7.0 ±0.62*	1.5 ±0.05*	0.63 ±0.04*	5.0 ±0.38*	1.3 ±0.09*	0.55 ±0.04*	succumbed		
75	5.0 ±0.11*	1.5 ±0.04*	0.62 ±0.02*	succumbed								
100	succumbed											
Transferred after 12 weeks from 50 to 25 μM Cd												
25	10.0 ±0.64*	2.3 ±0.13*	1.1 ±0.08*	11.0 ±0.72*	2.5 ±0.18*	1.2 ±0.03*						
Transferred after 4 weeks from 75 to 25 μM Cd												
25	7.0 ±0.35*	1.8 ±0.03*	0.92 ±0.05*	7.0 ±0.43*	2.2 ±0.09*	1.0 ±0.02*						

Mean ± S.E. based on 24 replicates; No = Number of shoots per culture, Height = shoot height (cm), FM = Fresh mass of culture (g)
* = Significant at 1% level

Table 2. Root differentiation, root length and accumulation of cadmium (in cell wall and cytoplasmic fractions) in 16-wk-old regenerants grown on various concentrations of $CdCl_2$.

$CdCl_2$ (μM)	Rooting (per cent)	Root length (cm)	Cadmium accumulation ($\mu g\ L^{-1}$ DM)	
			Cell Wall fraction	Cytoplasmic fraction
00	100	2.5 ± 0.16	Nil	Nil
25	92 ± 2.11^{NS}	1.8 ± 0.12*	0.55 ± 0.013	0.45 ± 0.018
50	42 ± 2.32^{NS}	0.7 ± 0.15*	1.27 ± 0.019	0.57 ± 0.012

Values represent mean ± S.E. based on six replicates; * = Significant at 1% level
NS = Non-significant

elongation or inhibition rate is widely used as a reliable indicator for varietal differences in metal tolerance. Effect of pH is probably most critical in root cap and root apical meristem (Hanson and Kamprath, 1979). Bennet et al. (1987) have hypothesized that the primary site of aluminium injury is in the root cap and that effects on cell division and differentiation in the root meristem are mediated by hormones produced in the root cap. This may also hold for cadmium. Further, Cd-interference with the calcium-calmodulin regulatory pathway might affect root growth in *B. monniera* regenerants.

There are numerous explanations to account for the mechanism of metal tolerance in plants. Earlier studies indicate that to prevent supra-optimal metal concentrations from harming the cells, about 80-90% of the accumulated metal may bind to the cell wall (Jackson et al., 1984). Correlation between growth inhibition and Cd uptake in *B. monniera* indicates that the amount of Cd accumulated by cell wall fraction was considerably higher than in the cytoplasm fraction. Thus, the poor growth of plants at higher concentrations of Cd may be correlated to the saturation of binding sites at the cell wall (Chakravarty and Srivastava, 1997b). Delauney and Verma (1993) have argued that proline biosynthesis is controlled by an enzyme pyrroline-5-carboxylate synthetase (P5-CS) and that regulation is lost under water stress. This may account for high accumulation rates of proline under stress. Cadmium ions are known to affect integrity of membranes (Reddy and Prasad, 1992), altering their permeability that can cause water stress-like conditions and enhance proline levels (Pesci and Reggiani, 1992). In *Bacopa* regenerants, proline content increased but the soluble protein content decreased with increasing Cd level (Table 3). This agrees with earlier findings on various species against different metals (Costa and Spitz, 1997). Presence of Cd was thought by Hirt et al. (1989) as a stimulating factor for mRNA synthesis, leading to increase in total proteins.

Table 3. Photosynthetic activity and the proline and soluble protein contents in 16-wk-old cultures grown on various concentrations of $CdCl_2$ (μM)

$CdCl_2$	Stomatal conductance (mmol CO_2 m^{-2} s^{-1})	Internal CO_2 (μL L^{-1})	Net photosynthetic rate (μmol CO_2 m^{-2} s^{-1})	Proline content (μg/g FM)	Protein content (μg/g FM)
00	0.384 ± 0.04	424 ± 12.5	15.30 ± 0.23	21.87 ± 0.12	6.2 ± 0.19
25	0.335 ± 0.02^{NS}	342 ± 17.2*	12.20 ± 0.16*	43.90 ± 0.17*	19.2 ± 0.12*
50	0.312 ± 0.07^{NS}	318 ± 11.9*	11.30 ± 0.13*	48.62 ± 0.13*	24.2 ± 0.15*

Mean ± S.E. based on three replicates; * = Significant at 1% level; NS = Non-significant

Photosynthesis is inhibited by heavy metals due to decrease in chlorophyll biosynthesis and transpiration (Bhardwaj and Mascarenhas, 1989; Skorzynska-Polit and Baszynski, 1997). Miller et al. (1973) have demonstrated Cd-related inhibition in the mitochondrial electron transport in dark. Photosynthetic rate of *B. monniera* regenerants declined with increased concentrations of cadmium. Possibly, the smaller leaf size under stress reduced the total photosynthetic area of the plants. Stomatal conductance and internal CO_2 concentrations were reduced at all levels of cadmium as noted earlier with a variety of stresses (Costa and Spitz, 1997).

Thus, *B. monniera* exhibits a varying response to varied concentrations of cadmium. The proline and protein accumulations seem to be useful stress-indicators as they exhibit a direct correlation with the degree of stress. This study suggests that the regenerants of *B. monniera* can be grown successfully on cadmium by step-wise exposure or by keeping the regenerants on cadmium-supplemented medium for longer durations.

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