Bull. Environ. Contam. Toxicol. (1996) 56:505-512

Bulletin of Environmental Contamination and Toxicology

Induction of Phytochelatins in *Hydrilla verticillata* (l.f.) Royle under Cadmium Stress

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Received: 1 June 1995/Accepted: 30 August 1995

Plants tolerate Cd by sequestering them through synthesizing phytochelatins with the general structure (γ -Glu-cys)n-gly where n= 2-11 (Grill *et al.* 1987, 1989) depending upon the species from which these peptides are isolated. Recent biochemical evidence suggests that these peptides are synthesized via post-translationally activated, metal-dependent enzymatic pathways from the precursor glutathione (Grill et al. 1987, 1989). However, most of these studies are confined to terrestrial species and only a few studies have been made on higher aquatic plants (Fujita 1985; Fujita & Kawanishi 1986; Gupta *et al.* 1995).

Recently *H. verticillata* and other aquatic higher plants have been reported to be hyperaccumulators of Cd and have demonstrated the ability to remove many toxic metals, including Cd, from wastewater (Rai *et al.* 1995a). It is hypothesized that cadmium hyper-accumulating ability of the macrophyte is associated with induction of the metal chelating peptides, the phytochelatins (PCs), to copeup with high cellular Cd levels. In view of this, it was considered worthwhile to examine the induction of phytochelatins and changes in levels of glutathione and related metabolites in *H. verticillata* under Cd stress.

MATERIALS AND METHODS

Plants of *H. verticillata,* collected from an unpolluted water body, were grown in large hydroponic tubs for six months in tap water. New shoots (ca 25-30 cm long) of acclimatized plants were separated from mother plant and cultured in 5% mineral medium (Tatsuyama *et* al. 1977) under laboratory conditions (115 μ mol $m^{-2}s^{-}l$ light intensity by day fluorescent lamps for 14 hr per d at 25± 2°C). The addition of salts of Cu and Zn to

NBRI Research Publication No. 433 (NS)

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the nutrient medium were omitted due to the possibility of induction of PCs by these metal ions. After one week, the plants were washed with deionized water and 2.5 and 10.0 µM Cd were supplied for 24, 72 and 168 hr.

For cysteine estimation, plant material (500 mg FW) was extracted in 5% chilled perchloric acid ($HClO_4$), centrifuged at 10,000 x g for 20 min. Cysteine content was measured in the supernatant using acid-ninhydrin reagent (Gaitonde 1967) at 560 nm in a Spectronic 1201 Milton Roy Spectrophotometer.

Acid-soluble thiol (AS -SH) and total glutathione were measured in plant tissue (500 mg FW) frozen in liquid nitrogen and stored at -80°C until assayed. For -SH, tissue was homogenized in 6.67% 5-sulfosalicylic acid and kept in ice for 10 min. The mixture was centrifuged at 13,000 x g at 4°C for 10 min. The supernatant was diluted 10 to 20 times with buffer (final concentration: 120 mM Na phosphate (pH, 7.5), 5 mM EDTA, 0.6 mM 5,5-dithiobis (2-nitrobenzoic acid) and absorbance was measured at 412 nm (Ellman 1959).

For total glutathione (GSH & GSSG), frozen tissue was homogenized in Na-phosphate EDTA buffer (pH, 7.5) and 25% metaphosphoric acid (HPO_3) which was used as a protein precipitant and centrifuged at 17,000 g for 20 min. Determination of GSH and GSSG was performed fluorometrically using o-phthaldialdehyde (OPT) as the fluorogenic agent by the method of Hissin & Hilf (1976) and fluorescence intensity was recorded at 420 nm after excitation at 350 nm on a fluorescence spectrophotometer (Hitachi model no. 650-60).

HPLC assay for PCs was performed following the method of Grill *et al.* (1991). Plant tissue (500 mg FW) frozen in liquid N_2 was homogenized with 0.5 mL 1N NaOH containing 1 mg/ml $NaBH_4$ (freshly prepared solution). After centrifugation at 11,000 x g at 4°C for 15 min the supernatant was acidified by adding 3.6 N HCl in the ratio of 5:l and left on ice for 15 min. Precipitated protein was removed by centrifugation and 20µl aliquot of the supernatant was used for HPLC analysis. Phytochelatin peptides were separated on a reverse phase column (µ Bondapack RP 4µm C-18) with a gradient of 0-20% acetonitrile in 0.1% trifluoroaceticacid (TFA) at a flow rate of 0.5 ml/min by using HPLC (Applied Biosystems, model no.783A). Detection of the PCs were performed at 220 nm. Peaks were quantified considering peak areas of standard PC samples run under similar conditions and -SH contents in the eluents were measured as described.

Experiments were run in triplicate and repeated twice.

Student's t-test was performed to determine the level of significance (Schefler 1969).

RESULTS AND DISCUSSION

Cysteine content increased in response to Cd concentration and treatment durations. The maximum increase was noticed at the 10 µM concentration after 72 hr. Similarly, the initial level of acid-soluble thiols enhanced considerably (ca 285%) at 10 µM Cd after 72 hr. After 168 hr of treatment duration, this enhancement was almost constant. On the other hand, Cd treatment resulted in the reduction of glutathione levels. The decline in glutathione level was more pronounced at 10 µM Cd (ca 52% reduction) than 2.5 µM Cd (ca 32% reduction) after 72 hr of exposure period. This level continued even after 168 hr (Table 1).

The effect of BSO on accumulation of AS-SH and GSH decline in response to Cd were evaluated (Fig. 1). Both AS-SH and GSH content declined inside the cell at both concentrations. Cycloheximide treatment for 24 hr to the Cd treated plants resulted in decreased levels of acid soluble thiols, however, no change was observed at the 4- hr exposure period (Fig. 2).

Table 1. Cysteine, AS -SH and glutathione levels in *H. verticillata* as a function of Cd concentration and treatment durations.

Cd conc.(µM)	24 hr	72 hr	168 hr
		Cysteine (n mol/g FW)	
0.0	49.02±1.42	49.52±1.33	50.55±1.38
2.5	70.50±2.05^{b}	93.18±2.43^{c}	105.70±3.67^{c}
10.0	116.10±3.89^{b}	134.50±4.13^{c}	129.09±4.05^{c}
		AS-SH (µ mol / g FW)	
0.0	0.90±0.03	1.05±0.04	1.04±0.04
2.5	1.86±0.09^{c}	2.01±0.12^{b}	2.42±0.11^{c}
10.0	2.44±0.09^{c}	2.84±0.10^{c}	2.80±0.12^{c}
		Glutathione (µ mol / g FW)	
0.0	0.55±0.02	0.56±0.02	0.56±0.03
2.5	0.37±0.02^{b}	0.38±0.018^{a}	0.38±0.012^{a}
10.0	0.28±0.01^{c}	0.26±0.01^{c}	0.24±0.01^{c}

Mean value ±S.E. (n=3); a= P<0.025,b= P<0.01,c=P< 0.005 (t-test, one tailed).

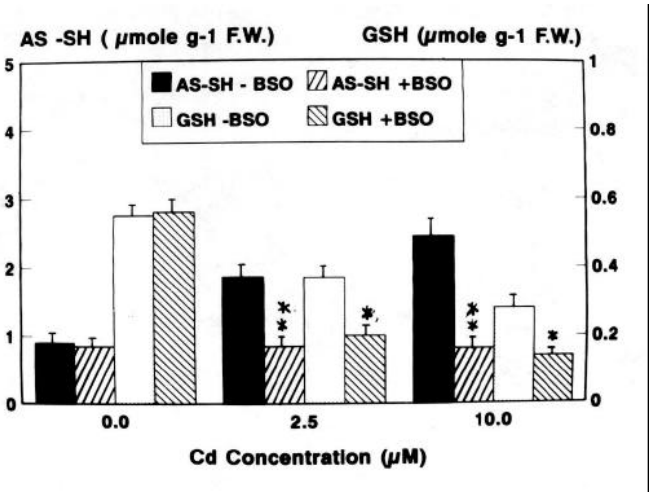

Figure 1. Effect of BSO (500 μM) on depletion of GSH (a) and accumulation of AS-SH (b) under-24 hr Cd treatment in *H. verticillata.* Bars indicate ±S.E. (n=3); *=P < 0.025, **=P<0.005.(t-test, one tailed).

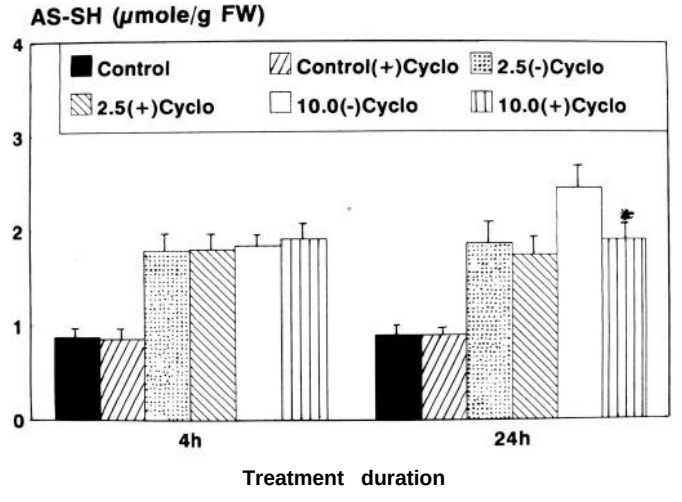

Figure 2. Effect of cycloheximide (2.5 μg/ml) on accumulation of AS-SH under 4 and 24- hr Cd treatment in *H. verticillata.* Bars indicate ±S.E. (n=3); *=P<0.025, (t-test, one tailed).

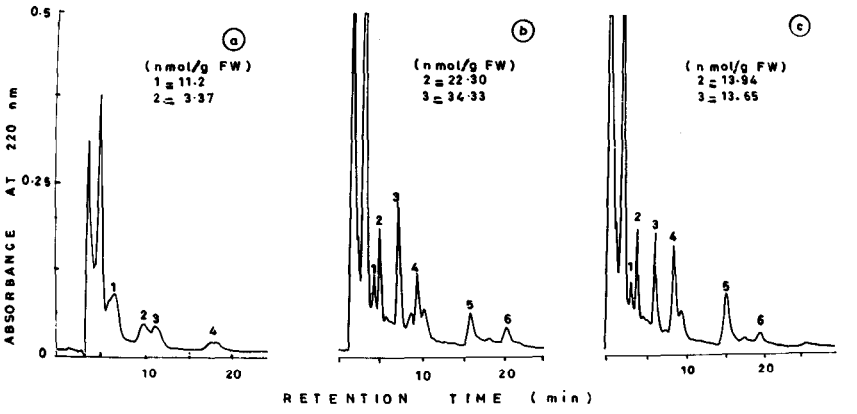

Figure 3. HPLC profiles of PCs in crude extracts of H. *verticillata* exposed to 2.5 (b, peak No. 2 & 3 are PC_2 & PC_3) and 10.0 µM Cd (c, peak NO. 2 & 3 are PC_2 & PC_3) Cd for 72 hr in comparison to control (a, peak No. 1 & 2 are PC_2 & PC_3).

Various Cd concentrations resulted in the induction of phytochelatins at both concentrations that exhibited positive thiol reactions. Some of the peaks were identified as PC_2 and PC_3, while others were designated as unidentified thiols (Fig. 3). The amount of PC_2 and PC_3 were more at 2.5 µM than at 10 µM Cd. However, PC_3 predominated over PC_2 at this concentration. Control plants grown without Cd also showed lesser amount of these peptides.

Potential of *H. verticillata* for bioamelioration of wastewater containing the toxic metals Cr, Cd, Mn, Hg, Pb, Fe and Cu has been demonstrated (Chandra *et al.* 1993; Gupta & Chandra 1994; Rai *et al.*1995 a,b). These studies have shown high accumulation and tolerance of the macrophyte to elevated metal concentrations. However, the mechanism responsible for such a high metal accumulation and tolerance potential remains unexplored. We recently have demonstrated that *H. verticillata* has a cellular mechanism for Pb detoxification (Gupta *et al.* 1995).The results presented in this report show that intracellular Cd

content has affected the cellular levels of cysteine, glutathione, acid-soluble thiols and induced synthesis of metal-binding peptides.

The increase in cysteine level under Cd stress may be due to enhanced levels of ATP sulfurylase and adenosine 5'-phosphosulfate sulfotransferase as reported in maize (Nussbaum *et al.* 1988). The extent of decline in GSH less in absence and more in the presence of BSO reflects the involvement of GSH in PC synthesis. That PCs are synthesized using glutathione as a precursor is further evidenced by more depletion of AS-SH accumulation in the presence of BSO than in its absence in Cd-treated *H. verticillata.* BSO is an established inhibitor of GSH biosynthesis (Earnshaw & Johnson 1985). Our findings are in agreement with earlier observations (Scheller *et al.* 1987; Gupta *et al.* 1995).

Treatment of cycloheximide showed no change in AS-SH levels up to 4 hr, however, it is reduced after 24 hr at higher Cd concentrations, suggesting PC synthesis is not dependent on early *de novo* cytoplasmic protein synthesis. Further, results showed a constitutive nature of phytochelatin synthase required for PC synthesis (Grill *et al.* 1989), as evidenced by the presence of PCs in lesser amounts in normal control plants. Barring more content of PC_3 at 2.5 µM cd, like other plants in this case PC_2 also predominated over PC_3 (Tukendorf & Rauser 1990; Gupta et *al* 1995).

It seems that *H. verticillata* has a cellular detoxification mechanism for Cd, via synthesizing PCs utilizing glutathione as substrate.

Acknowledgments. We thank Dr. P. V. Sane, Director, NBRI, Lucknow (India) for his interest in this work and to Prof. M.H. Zenk, Lehrstuhl fur Pharmazeutische Biologie, Karlstrasse, Munchen (Germany) for a gift of standard PCs. Financial assistance to one of us (M.G.) from CSIR, New Delhi is gratefully acknowledged.

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