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Research Article

ISOZYME PATTERN OF ANTIOXIDANT ENZYMES AND DNA DAMAGE IN *OCTOBLEPHARUM ALBIDUM* HEDW. A BRYOPHYTE IN RESPONSES TO CADMIUM AND COPPER STRESS

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ABSTRACT

Bryophytes are the amphibians of the plant world adapted morphologically and physiologically to overcome a plethora of environmental stress. The objective of the present study is to analyze isozyme pattern of antioxidant enzymes, DNA damage, pigments, ultra structural changes of chloroplast and mitochondria associated with the heavy metals cadmium and copper in *Octoblepharum albidum* a Bryophyte. Plants exposed at normal condition were treated as control whereas, experimental samples comprise moss treated with different concentrations of cadmium / copper (0.25, 0.5, 1, 1.5 and 2 mg/L in growth chambers). Variation in the pigment composition coupled with low chlorophyll to carotenoids ratio suggests the optimal photo protective adaptation in the moss. Isozymes of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol-peroxidase (POX) showed either an increase in intensity or new isozymes. Ultra structure of chloroplast remained unaltered in metal treated leaves. Meanwhile, mitochondria showed marginal deformities. DNA damage was marginal in the initial concentrations as revealed by comet assay. Thus, the results revealed antioxidant defense mechanisms in *O. albidum* against the heavy metal tolerance in terms of isozyme pattern of antioxidant enzymes for scavenging the reactive oxygen species formed in the cells; antioxidant defense mechanisms in *O. albidum* against the heavy metal tolerance.

Keywords: Antioxidant, Chlorophyll, Cadmium, Copper, Electron microscopy, Reactive oxygen species.

INTRODUCTION

Heavy metal contamination is one of the major threats to human life and the environment. Heavy metals entering the ecosystem may lead to geo-accumulation; bio accumulation, bio-magnification, leads to a negative impact on physiological activities of plants like photosynthesis, gaseous exchange and nutrient absorption which in turn reduce plant growth, dry matter accumulation and yield. Monitoring of the contamination of soil with heavy metals is of interest due to their influence on ground water and surface water and also on plants, animals and humans. Lead, cadmium and mercury are toxic even in low concentrations. Toxicity may result from the binding of metals to sulphydryl groups in proteins, DNA leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects. In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress[1]. Metal–plant interactions have been examined as a means for removal, recovery or detoxification of inorganic and organic metal or radionuclide pollutants. A wide variety of higher plants, fungi, algae and bacteria are now under study or are already in use as biosorbents of heavy metal remediation[2]. Phytoremediation is considered as an effective, low cost and preferred cleanup option for contaminated areas. Although the capacity of aquatic macrophytes to accumulate metals are well documented. No data are available for bryophytes of heavy metal effects on the mechanisms for their sensitivity or tolerance at the biochemical level, despite the importance of these organisms in a wide variety of terrestrial habitats. So far, certain species of mosses and liverworts are just known to accumulate heavy metals from polluted sites without obvious detrimental effects on vitality. By contrast, other species are relatively sensitive and only tolerate low concentrations of heavy metal ions in their tissues. It is known that bryophytes have little or no possibility of avoiding exposure and retention of heavy metals supplied from the atmosphere, water and their substratum. Thus, a main tolerance mechanism in these plants is thought to be the efficiency of cell walls and associated polysaccharides to immobilize heavy metal ions. In consequence, a good correlation was found between the content of metals with various *Sphagnum* species[3-5]. There are only limited studies on uptake of metals by mosses and their role as bio-indicators. From these evidences, the present study was designed to analyze the molecular process linked with mosses in monitoring of pollution with metals. Thus, the present study was aimed to establish the heavy metal tolerance in *Octoblepharum albidum* in terms of pigments, isozyme pattern of antioxidant enzymes, DNA damage and ultra structural changes in chloroplast and mitochondria.

MATERIALS AND METHODS

Plant material

Fresh *Octoblepharum albidum* plants were collected from lower floor of Shola forests of Ponmudi Hills, Thiruvananthapuram, Kerala, India. The identification was confirmed by comparing with the voucher specimen UC 215 at Department of Botany, University of Calicut, India.

Cadmium (Cd) and copper (Cu) treatments

Fresh *O. albidum* was fully hydrated and equilibrated in a controlled environment chamber for 48 h at 20°C and a radiant flux intensity 75 $\mu M/m^2s$. The samples were treated with different concentrations of cadmium and copper in a controlled

environment chamber using the same light and temperature regimes as described above. The selected species were subjected to five different metal treatment regimes (a) 0.25 mg/L (b) 0.5 mg/L (c) 1 mg/L (d) 1.5 mg/L and (e) 2 mg/L. Control plants were maintained in an optimal water conditions for each case during the whole experimental period.

Relative water content (RWC)

Six fresh thallus of *Octoblepharum albidum* from different concentrations of cadmium (Cd) and copper (Cu) treated group and control were randomly chosen to measure the RWC following the method of Turner[6]. RWC = (FM - DM) / (SM - DM) x 100, where FM is leaf fresh mass, DM is dry mass after drying at 85°C for different periods, and SM is the turgid mass of leaves after soaking in water for 60 min approximately 20°C.

Determination of cadmium and copper

Mosses without cadmium and copper and samples treated with Cd and Cu (0.25, 0.5, 1, 1.5 and 2 mg/L) for 24 to 120 h were washed with deionized water and dried at room temperature on absorbent towels. About 300 g (± 100 mg) of powdered and homogenized sample was treated with 5 ml of concentrated HNO_3 , 4 ml of H_2O and 1 ml of H_2O_2 (35 %). To avoid cross-contamination, digestion vessels were previously cleaned in a bath of 10 % (v/v) nitric solution for 48 h. Samples were analyzed by fame atomic absorption spectrometry using an EDL lamp. The detection limit (LOD = 0.010 µg/mL) was calculated as the concentration corresponding to signals equal to three-times the standard deviation of 10 replicates of a blank solution. The standards of Cd and Cu used were of 0.25, 0.5, 1 and 2 mg/L. A preparation with 0.05 mg/mL was used to test the calibration curve (quality control of the method). The accuracy (bias) and precision (% RSD) of the methods were tested with certified reference materials. One sample of reference materials and blanks were included in each analytical batch and three parallel measurements were made in all cases. The analytical precision and accuracy determined by quality assurance and quality control procedures using certified reference materials, a duplicate, blanks and internal standards, was better than ± 10 %.

Chlorophyll fluorescence

F_v/F_m (maximum quantum efficiency of PSII photochemistry) was quantified using an open gas exchange system connected with an integrated fluorescence chamber. Following dark adaptation for 2 h, the minimum fluorescence (F_o) was determined by measuring light of about 0.5 µmol photon/m^2s^1, and the maximum fluorescence (F_m) was determined with 0.8 s saturating flash of about 10,000 µmol photon/m^2s^1 in the dark-adapted leaves. F*v*/F*m* was calculated as (F*m*-F*o*)/F*m*[7]. Five replicates per treatment were randomly obtained from different individuals after respective concentrations of treatments.

Pigment analysis

Chlorophyll a, b and carotenoid pigments were extracted from Cd and Cu treated samples. For the determination of the CHL a and b content, 1 g of fresh leaves was homogenized with methanol using polytron. The homogenate was then filtered. The CHL a, b and carotenoid concentration in the supernatant was determined by measuring the absorbance at 470, 646.8 and 663.2 nm and calculated according to Porra *et al*[8].

Protein content

The soluble protein content in the moss leaves was analyzed from frozen leaves ground in liquid nitrogen to a fine powder and suspended in buffer containing 100 mM NaH_2PO_4/Na_2HPO_4 (pH 7.0), 1 mM phenyl methyl sulphonyl fluoride, 1 % (v/v) β-mercaptoethanol, and then centrifuged for 5 min at 9800 g. The soluble protein concentration in the supernatant was determined using BSA as standard.

Transmission electron microscopy

Leaf pieces (control and cadmium metal treated) were fixed in 2 % (w/v) paraformaldehyde and 1 % (v/v) glutaraldehyde in 100 mM PIPES buffer (pH 7.2) for 18 h, then thoroughly washed in piperazine-N, N′-bis(2-ethanesulfonic acid (PIPES) buffer and post-fixed in 1 % (v/v) osmium tetroxide (OsO_4) in 50 mM PIPES buffer (pH 7.2) for 1 h at room temperature. The excess OsO_4 was removed by rinsing the samples in PIPES buffer before being dehydrated in ethanol series (30, 50, 70, 90, 95 and 100 %) at 15 min intervals. The final dehydration step in 100 % ethanol was followed by gradually infiltrating the material in LR White resin at room temperature before embedding the samples in resin within gelatin capsules. The resin was polymerized at 60°C for 24 h and transverse sections of approximately 70 nm in thickness were cut. Sections were stained with lead citrate according to Reynolds[9] and 5 % (w/v) aqueous uranyl acetate for 20 min and then examined under transmission electron microscope.

DNA analysis

The method of single cell gel electrophoresis (Comet assay), was used to detect DNA damage in the leaves induced by cadmium and copper stress[10]. Electrophoresis was then carried at 4°C in the alkaline buffer[11]. Each slide was stained with 50 µL of 13 mg/L ethidium bromide and visualized using a Zeiss fluorescent microscope with an excitation filter of 510–560 nm and a barrier of 590 nm. The stained DNA gives a red emission. Images of the Comets were captured by a cool CCD. The following parameters were used as primary agent for DNA damage: percentage of nuclei with tails, the relative tail length, tail DNA (relative percentage of DNA in the comet tail), tail moment (TM, integrated value of density multiplied by migration distance).

Isozyme assay

Electrophoresis of crude enzyme extracts from the different treated samples was done in 10 % (SOD) or 8 % polyacrylamide for other antioxidant enzymes in slab gels at pH 8.9 under non-denaturing conditions. SOD activity was detected on gels using the photochemical procedure of Beauchamp and Fridovich[12]. Isozymes of CAT were visualized on gels by the method of Woodbury *et al*[13]. POX isoenzymes were visualized by incubating the gels in 50 mm phosphate buffer (pH 6.5) with 4 mm H_2O_2 and 2.7 mm guaiacol for 25 min in dark and the gels were stained according to Ros Barcelo *et al*[14] using a saturated solution of diaminobenzidine dissolved in dimethyl formamide and 4 mm H_2O_2 in 50 mm sodium acetate buffer (pH 5.6). The APX isoenzyme assay was initiated by first pre-running the gels with the buffer containing 3 mm ascorbate. Appearance of colorless bands against a blue background indicated APX activity[15].

Statistical analysis

All experimental were carried in three independent trials and in triplicates. Values are represented as mean ± SD. Significance was recorded $P < 0.005$.

Table 1: Cadmium Level (Cd) (mg/g) in the Thallus of *O. albidum* from 24 to 120 h Duration against Different Concentrations of Cadmium Treated (0.25 to 2 mg/l)

Cd concentration ($mg \cdot L^{-1}$)	0 h	24 h	48 h	72 h	96 h	120 h
0.25	0.03 ± 0.002	0.08 ± 0.001	0.09 ± 0.005	0.1 ± 0.003	0.12 ± 0.002	0.15 ± 0.003
0.5	0.049 ± 0.001	0.1 ± 0.001	0.14 ± 0.003	0.19 ± 0.001	0.22 ± 0.003	0.29 ± 0.003
1	0.055 ± 0.002	0.14 ± 0.003	0.2 ± 0.003	0.38 ± 0.002	0.45 ± 0.002	0.63 ± 0.007
1.5	0.09 ± 0.002	0.19 ± 0.001	0.28 ± 0.005	0.39 ± 0.023	0.68 ± 0.054	1.1 ± 0.36
2	0.12 ± 0.04	0.29 ± 0.07	0.48 ± 0.06	0.74 ± 0.08	1.18 ± 0.22	1.46 ± 0.28

Table 2: Copper Level (Cu) (mg/g) in the Thallus of *O. albidum* from 24 to 120 h Duration against Different Concentrations of Cadmium Treated (0.25 to 2 mg/l)

Cu concentration ($mg \cdot L^{-1}$)	0 h	24 h	48 h	72 h	96 h	120 h
0.25	0.05 ± 0.001	0.1 ± 0.002	0.12 ± 0.004	0.14 ± 0.002	0.18 ± 0.003	0.2 ± 0.005
0.5	0.05 ± 0.002	0.13 ± 0.001	0.17 ± 0.003	0.24 ± 0.006	0.29 ± 0.05	0.36 ± 0.02
1	0.06 ± 0.02	0.17 ± 0.004	0.27 ± 0.06	0.47 ± 0.08	0.69 ± 0.09	0.81 ± 0.12
1.5	0.09 ± 0.01	0.28 ± 0.05	0.38 ± 0.08	0.56 ± 0.27	0.83 ± 0.04	1.3 ± 0.03
2	0.13 ± 0.03	0.39 ± 0.12	0.67 ± 0.23	0.89 ± 0.31	1.56 ± 0.32	1.77 ± 0.08

Table 3: Pigment Profile ($mg \cdot g^{-1}$) of *O. albidum* in Control and Different Concentrations of Cadmium and Copper Stress (1- Control, 2- 0.25 mg/l, 3- 0.5 mg/l, 4- 1 mg/l, 5- 1.5 mg/l and 6- 2 mg/l)

Pigments ($mg \cdot g^{-1}$)		Control	0.25 $mg \cdot L^{-1}$	0.5 $mg \cdot L^{-1}$	1 $mg \cdot L^{-1}$	1.5 $mg \cdot L^{-1}$	2 $mg \cdot L^{-1}$
CHL a	Cd	0.87 ± 0.1	0.91 ± 0.2	0.79 ± 0.08	0.68 ± 0.04	0.6 ± 0.07	0.59 ± 0.01
	Cu	0.87 ± 0.05	0.93 ± 0.03	0.94 ± 0.07	0.89 ± 0.01	0.88 ± 0.03	0.88 ± 0.02
CHL b	Cd	0.68 ± 0.09	0.62 ± 0.05	0.6 ± 0.01	0.58 ± 0.05	0.51 ± 0.03	0.49 ± 0.02
	Cu	0.71 ± 0.09	0.74 ± 0.05	0.78 ± 0.01	0.70 ± 0.05	0.69 ± 0.03	0.71 ± 0.02
CHL	Cd	1.55 ± 0.23	1.53 ± 0.16	1.39 ± 0.17	1.26 ± 0.28	1.11 ± 0.35	1.08 ± 0.41
	Cu	1.58 ± 0.03	1.67 ± 0.27	1.72 ± 0.33	1.59 ± 0.13	1.57 ± 0.16	1.59 ± 0.03
CHL a/b	Cd	1.27 ± 0.01	1.47 ± 0.22	1.32 ± 0.24	1.2 ± 0.14	1.2 ± 0.23	1.2 ± 0.15
	Cu	1.22 ± 0.05	1.26 ± 0.09	1.21 ± 0.02	1.27 ± 0.14	1.28 ± 0.16	1.24 ± 0.22
CAR	Cd	0.62 ± 0.03	0.77 ± 0.02	0.81 ± 0.11	0.88 ± 0.23	0.89 ± 0.08	0.87 ± 0.14
	Cu	0.68 ± 0.05	0.75 ± 0.13	0.78 ± 0.01	0.73 ± 0.03	0.7 ± 0.04	0.69 ± 0.04
CAR/ CHL	Cd	0.4 ± 0.01	0.5 ± 0.03	0.58 ± 0.07	0.69 ± 0.03	0.80 ± 0.03	0.80 ± 0.05
	Cu	0.43 ± 0.09	0.45 ± 0.03	0.45 ± 0.03	0.46 ± 0.02	0.45 ± 0.01	0.43 ± 0.01

Table 4: DNA Damage Accessed by Tailed Nuclei, Relative Tail Length of DNA, Tail Moment of DNA due to Cadmium (Cd) and Copper (Cu) Stress in *O. albidum* in Control and Different Concentrations (0.25 to 2 $mg \cdot l^{-1}$)

Treatments ($mg \cdot L^{-1}$)	Tailed nuclei (%)		Mean tail length		Tail movement	
	Cd	Cu	Cd	Cu	Cd	Cu
control	9 ± 0.35	9 ± 0.12	8 ± 0.09	8 ± 0.05	9 ± 0.05	9 ± 0.06
0.25	16 ± 0.22	10 ± 0.09	17 ± 0.26	12 ± 0.02	15 ± 0.02	10 ± 0.04
0.5	23 ± 0.18	13 ± 0.0.3	21 ± 0.43	14 ± 0.05	20 ± 0.01	17 ± 0.03
1	30 ± 0.41	19 ± 0.01	27 ± 0.37	17 ± 0.07	29 ± 0.03	22 ± 0.04
1.5	38 ± 0.56	21 ± 0.06	41 ± 0.87	20 ± 0.08	42 ± 0.02	28 ± 0.04
2	45 ± 0.87	26 ± 0.09	50 ± 0.99	24 ± 0.07	51 ± 0.05	31 ± 0.01
F ratio	7.98**	8.7**	6.48*	7.8**	7.01*	8.4**

The Values are Means of Three Independent Replicates and the Values are Represented Mean ± SD

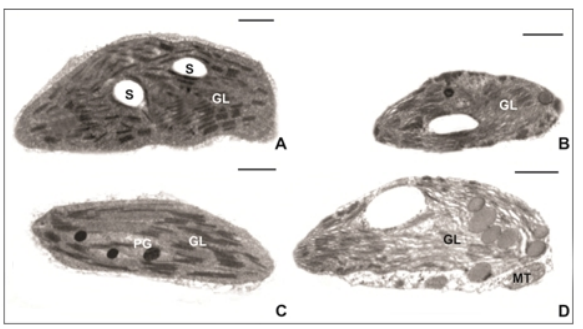

Figure 1: Transmission electron micrographs of chloroplast under cadmium (Cd) and copper (Cu) stress A-Control B-Cu1.5 mg. L^{-1}, C-Cd1.5 mg. L^{-1}, D- Cd 2 mg.L^{-1}

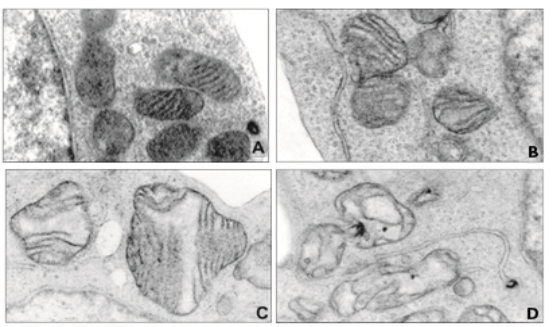

Figure 2: Transmission electron micrographs of mitochondria under cadmium stress (Cd) and copper (Cu) A-Control B-Cu1 mg. L^{-1}, C-1.5 mg. L^{-1}, D- Cd 2 mg. L^{-1}

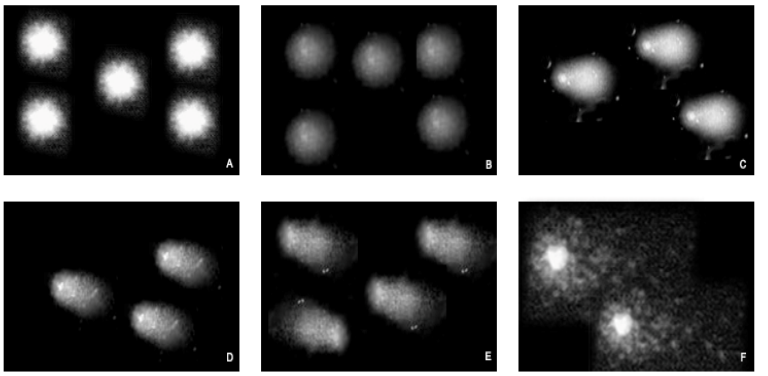

Figure 3: DNA damage induced by cadmium and copper treated thalli of *O. albidum* detected by Comet assay (A- control, B-Cu 1.5 mg. L^{-1}, C- Cu 2 mg. L^{-1}, D- Cd 1 mg. L^{-1}, E- Cd 1.5mg. L^{-1} and F- Cd 2 mg. L^{-1})

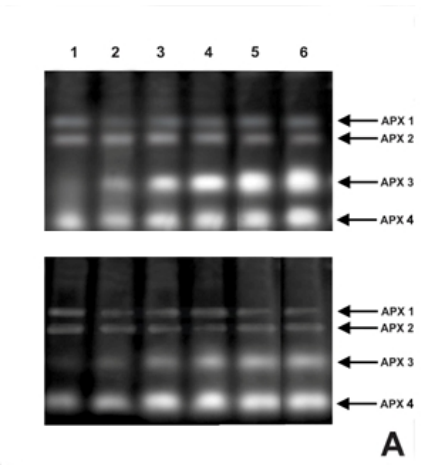

Figure 4 A: Isozyme patterns of Ascorbate peroxidase (APX) in *O. albidum*

1- control, 2- Cd/Cu 0.25 mg·L^{-1}, 3-Cd/Cu - 0.5 mg·L^{-1}, 4-Cd/Cu – 1mg·L^{-1}, 5- Cd/Cu -1.5mg·$L^{-1}$and 6- Cd/ Cu- 2 mg·L^{-1}cadmium / copper treated thalli (upper panel represents APX from Cd thalli and lower panel copper treated)

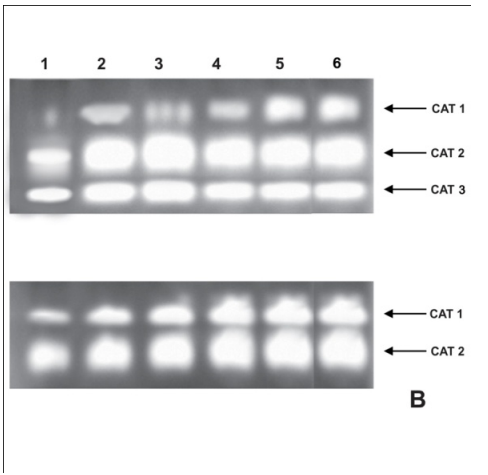

Figure 4 B: Isozyme patterns of Catalase (CAT) in *O. albidum*

1- control, 2- Cd/Cu 0.25 mg·L^{-1}, 3-Cd/Cu - 0.5 mg·L^{-1}, 4-Cd/Cu – 1mg·L^{-1}, 5- Cd/Cu -1.5mg·$L^{-1}$and 6- Cd/ Cu- 2 mg·L^{-1}cadmium / copper treated thalli (upper panel represents CAT from Cd thalli and lower panel copper treated)

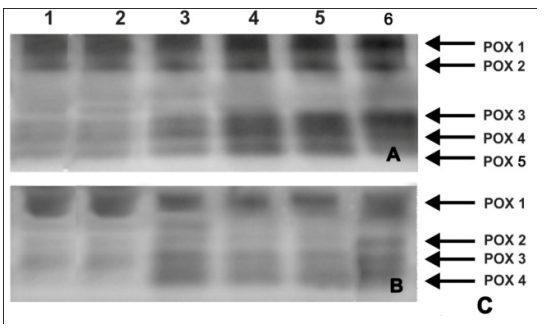

Figure 4 C: Isozyme patterns of Peroxidase (POX) in *O. albidum*

1- control, 2- Cd/Cu 0.25 mg·L^{-1}, 3-Cd/Cu - 0.5 mg·L^{-1}, 4-Cd/Cu – 1mg·L^{-1}, 5- Cd/Cu -1.5mg·$L^{-1}$and 6- Cd/ Cu- 2 mg·L^{-1}cadmium / copper treated thalli (upper panel represents POX from Cd thalli and lower panel copper treated)

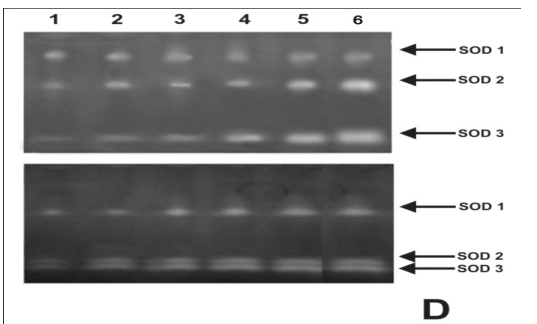

Figure 4 D: Isozyme patterns of Superoxide dismutase (SOD) in *O. albidum*

1- control, 2- Cd/Cu 0.25 mg·L^{-1}, 3-Cd/Cu - 0.5 mg L^{-1}, 4-Cd/Cu – 1mg·L^{-1}, 5- Cd/Cu - 1.5mg·$L^{-1}$and 6- Cd/ Cu- 2 mg·L^{-1}cadmium / copper treated thalli (upper panel represents SOD from Cd thalli and lower panel copper treated)

RESULT AND DISCUSSION

Estimation of Cd and copper in *O. albidum*

The concentration of Cd measured in dried *O. albidum*, increased from 0.03 mg/g at 0 h of the experiment (0.25 mg/L treated thalli) to 1.46 mg/g at 120 h (2 mg/L treated thalli). However, at 120 h the amount of Cd accumulated in the thalli was approximately 50 % of the treated concentrations. Meanwhile, copper displayed more accumulation in the treated thalli i.e., 0.05 to 1.77 mg/g at 24 to 120 h durations of treatments (concentration ranged from 0.25 to 2 mg/L). Since, cadmium is not an essential element for plants; the increase of Cd (II) during the course of the experiment (Table 1 and 2) must be associated with the bio-accumulation of this element. While, copper is a minor nutrient, its accumulation may be related with active physiology of the plant. The present results suggest the capacity of mosses to sequester heavy metals when they are in high concentration in the environment. The 50 % Cd (II) concentration observed on 120 h of the experiment is counter to the expected bio-accumulation, which suggests a possible effect of loss of efficiency or saturation of the enzymatic bio-accumulation machinery. As with any enzyme kinetics, the bio-accumulation of heavy metals has a saturation point. Two bryophyte species *Asterella angusta* and *Cyathodium tuberosum* growing across different regions of Koyana wildlife sanctuary have been used as indicators of metal pollution. Ghatge *et al*[3] analyzed the effect of heavy metals like Cu, Zn, Pb, Ni, Cd and Cr against these two bryophyte species using atomic absorption spectroscopy. The reported accumulation of cadmium by *O. albidum* was more commendable than *Asterella angusta* and *Cyathodium tuberosum*.

Relative water content (RWC)

Cadmium stress remarkably reduced RWC of all treatments. Cadmium induced reduction in RWC occurred to a greater extent during 1.5 and 2 mg/L treatments (58, 69 % respectively), and to a lesser degree at 0.25, 0.5 and 1 mg/L[1] treatments (13, 22, 28 % respectively). Copper accumulation does not effectively bring any change in the RWC of the thalli (5.6 to 11. 3 %).

Chlorophyll fluorescence, pigment and total protein content

After respective doses of treatments, the values of F*v*/F*m* marginally decreased under severe cadmium stress at 1.5 mg/L and 2 mg/L (0.68 ± 0.002; 0.69 ± 0.006) than during 0.25 to 1.25 mg/L (0.68 ± 0.001; 0.67 ± 0.009; 0.65 ± 0.005). The control value was 0.64 ± 0.001 ($P < 0.005$). Copper treated thalli showed no variation in F*v*/F*m* ratio compared to respective controls. Total chlorophyll (CHL), CHL a, CHL b, CHL a/b ratio and CAR/CHL ratio content showed significant changes under cadmium stress, compared to controls ($P < 0.005$). Contradictorily, the total CAR concentration was significantly higher. During 0.25 to 1.5 mg/L treatments the leafy thallus showed decrease in CHL a and b content compared to the control condition (Table 3). Regression equations and correlation coefficient displayed significant positive correlation between RWC and total CHL. In copper treated thalli CHL a (0.87 to 0.94 mg/g), b (0.71 to 0.78 mg/g) and CAR (0.68 to 0.78 mg/g) level increased along the concentrations from 0.25 to 0.5 mg/L but at higher concentrations it showed a marginal decrease. Cadmium stress can affect the chlorophylls and carotenoids content in the leaf tissues. Fatoba *et al*[16] correlated chlorophyll and carotenoid content under lead, copper, cadmium, iron and vanadium stress with leaf area, a defensive response to reduce the harmful effects of drought stress. The total chlorophyll content significantly decreased under metal stress, but not during the initial concentration of Cd (0.25 mg/L). The regression analysis and correlation coefficient between RWC and CHL a, b are significant ($r = 0.887^{**}$, 0.769^{*} respectively). Increased level of CAR contents in neem, mango and oleander leaves growing in Pithampur industrial area was supporting the present data. Heavy metal tolerance with more carotenoids is an osmotic regulation reported in many metal tolerant crops[17]. The present study showed a positive correlation between CAR content and RWC ($r = 0.745^{*}$). CARs have a potential role as photo-protective by quenching triplet chlorophyll II and singlet oxygen radicals derived from higher light intensity, thus protecting photo oxidative damage. Decrease of pigments content in leaves may be as a result of either slow synthesis or increased break down of chlorophyll by chlorophyllase, has been considered as a physiological maker of oxidative stress[18]. Under mild Cd stress, the decrease of pigments content (CHL a + b) did not cause any decrease of F*v*/F*m* which suggests that breakdown of pigments was not accomplished by the decreasing of the maximum photochemical efficiency. This may be considered as stress protective adaptation through decreasing light absorbance by reducing the pigments concentration. Higher ratio of CHLa/CHLb was also considered as a decreased emphasis on light collection in relation to the rates of PSII photochemistry[19]. Reduction of photosynthetic pigments is one of the responses to heavy metal exposure. Baycu *et al*[20] reported reduction of chlorophyll and carotenoid contents in plants due to heavy metals such as Cu, Zn, Pb and Cd cause (For example, 15-30 % decreases of chlorophyll content were observed in plants grown in a Cu-Ni-polluted area). Similarly, 43-66 % decreases were reported for urban tree leaves compared to control. It was reported that reduction in photosynthetic pigment content by Cu, Pb, Zn and Cd exposure was due to damage to the protein protochlorophyllide system. Thus, heavy metal accumulation in soil can reduce the chlorophyll content of plants, planted in polluted area. The changes in the green coloration and the distortion of the foliar cells and nuclei of *Barbula lambarenensis* as observed by Fatoba *et al*[16] is a manifestation of the detrimental effects of the heavy metals used. This fact is hinged on the maintenance of the bright green coloration, and intact cells of the control experiment. Sergio[21] indicated pollutants such as SO_2 and heavy metals in the disappearance and changes in the flora around urban and industrial areas. Martin and Coughtrey[22] reported that metals vary in their toxicity and degree of toxicity is proportional to their concentration. Brown and Whitehead[23] observed that low concentration of mercury inhibits photosynthesis temporarily, increase respiration, reduces chlorophylls a and b concentrations and also causes substantial loss of intracellular potassium from *Rhytidiadephous squarrosus.* The reduction of chlorophylls a and b and total chlorophylls observed in this study agreed with the findings of Brown and Whitehead[23]. Copper contaminated soils (l0 µg/g) were found to inhibit the protonemal growth of *Funaria hygrometrica*[24]. He further stated that SO_2 progressively changed green coloration of lichens and mosses to whitish brown as one moved from mildly to the highly polluted zone. A resultant color change was observed in *Fontinalis squamosa* which absorbed metal ions from contaminated water[25]. Interestingly, copper treated thalli showed no significant decrease in pigmental content suggesting that, the moss is effectively bio-accumulate the heavy metal without affecting the cellular metabolism. During cadmium stress, the accumulation of total proteins exhibited different responses to Cd concentrations i.e., exhibiting higher values under moderate drought stress (0.25 to 1 mg/L) when compared to control, but decreased under severe stress treatments (1.5 to 2 mg/L) (1.68 ± 0.34; 2.09 ± 0.25; 2.25 ± 0.11; 2.4 ± 0.3; 1.65 ± 0.27; 1.4 ± 0.68 respectively; $P < 0.005$). Copper stress also displayed more or less similar results (1.49 ± 0.21; 1.89 ± 0.09; 2.3 ± 0.65; 2.5 ± 0.15; 1.5 ± 0.02; 1.3 ± 0.03 respectively; $P < 0.005$). Boudet *et al*[26] studied zinc, copper, cadmium and mercury impact in *Schoenoplectus californicus* and *Ricciocarpus natans* in terms of

growth and development. Copper and zinc were positively correlated with total organic matter whereas cadmium was not, suggesting low affinity of Cd. *R. natans* presented the highest metal levels among all studied matrix, with the presence of mercury. These findings showed the capacity of both *S. californicus* and *R. natans* to accumulate and remove heavy metals from sediments and water, which might be useful for phyto-remediation programs.

Ultra structural analysis

Fine structure of chloroplast was studied by transmission electron microscopy exhibited an ellipsoid shape with distinct membrane and packed thylakoids system in *O. albidum* (control) (Figure 1A). However, in Cu treated thallus (1.5 mg/L), cells suffered marginal damage with intact grana in structure and shape (Figure 1B). Chloroplast becomes smaller in contrast to control with many osmophilic plastoglobuli. The integrity of the outer membrane and the internal network of thylakoid membranes (expanded) were lost with increasing Cd stress from 1.5 and 2 mg/L (Figure 1C). However, cells lost their original form and shape marginally from control plants including thylakoids (Figure 1D). Copper treated thalli 1.5 mg/L showed marginal deformity in mitochondria compared to control (Figure 2 A-B). Meanwhile, the membranes of the mitochondria remained intact, although the integrity of the cristae lost at 1.5 mg/L concentrations of Cd treated cells (Figure 2 C-D). The integrity of other organelles appeared to be retained in the metal stressed state, i.e., the nuclear membranes, ribosomes, endoplasmic reticulum and lipid bodies. The number of vacuoles becomes reduced considerably during low dosage. The responses of cell organelles and the ultra structures, suggested that metal stress caused the moss to retain the physiological function at the expense of cellular morphological integrity, when the moss was subjected to 1 .5 mg/L dose of Cd or Cu treatments. Contraction of the membrane during stress reduces osmotic stress on the internal components of the organelles. Choudhury and Panda[27] observed oxidative stress and ultra structural changes in moss *Taxithelium nepalense*, under lead and arsenic phytotoxicity. The present results in *O. albidum* showed no shrinkage in the initial doses (1 mg/L) (data not shown). However, at 1.5 mg/L Cd concentration, chloroplast and mitochondria displayed marginal shrinkage. The ultra structure of *O. albidum* appears somewhat different from *Bryum* sp.[28]; *Atrichum angustatum, Polytrichum commune, P. juniperinum, P. ohioense*[29] and *T. ruralis*[30]. Iljin[31] suggested that small sized vacuoles in small cells would cause less stress on the protoplasm during stress than by large vacuoles. *O. albidum* possesses small vacuoles so more tolerant to stress. This is in direct contradiction to the theory proposed by Iljin[31]. Electron micrographs showed the typical grana stacks in *O. albidum* as seen in higher vascular plants. Miodzianowski[32] and Tucker *et al*[30] showed moss chloroplasts without such stacks and thus suggested that mosses could be considered primitive. Condensation of the protoplasm in dried *T. ruralis* occurred at the proximal and distal cell walls, resulting in an empty center[30]. No such deformities noticed in *O. albidum* during Cd treatments. Further, the species retained chlorophyll, as indicated by the green chloroplasts. In the present study, the thylakoid stacks are less disturbed in the swollen chloroplasts, and this may be related to the increase in number and size of plastoglobuli as lipids which protect thylakoids from degradation[33]. Gametophytes of *O. albidum* treated plants retained the photosynthetic capacities; this was in turn reflecting the degree of chloroplast integrity observed in the electron micrographs of chloroplast[34]. The integrity of the cristae in mitochondria of metal treated *O. albidum* suggests the respiratory efficiency of plants[35] (Figure 2 A–D). Ribosomes observed in the cytoplasm reflecting the potential level of protein synthesis in the species[36]. Quantitative studies of the total proteins further support the integrity of ribosomes[37]. A large number of lipid bodies were observed in the cells of *O. albidum.* The lipid bodies may provide the necessary level of short carbon chain compounds, after reaction with enzymes possibly produced by micro bodies. These biochemical phenomena have yet to be proved related to cellular repair mechanisms in lower non vascular plants. The higher frequency of lipid bodies in the cells of the more stress tolerant moss and their absence or infrequent occurrence in the susceptible species may be of major importance when explaining the responses to stress.

Comet assay and DNA damage

In Comet assay the DNA breakages migrated in the electric field towards the anode showing a picture like a Comet and the DNA migrated from the nuclei could be used to assess the DNA damage. In the Comet assay, only slight DNA migration from the nuclei was observed under the treatment 1.5 to 2 mg/L Cu treatments compared with control (Figure 3 A, B, C). The DNA migration was remarkable during Cd treated thalli i.e., 1 to 2 mg/L (Figure 3 D, E, F). DNA migration was found induced by increasing concentration of treatments. Marginal observed malformations of the interphasic nuclei i.e. the elongation and the fragmentation of nuclei (Figure 3) can be interpreted as the early symptoms of cell death of treated cells. The increase in % of tailed nuclei was significant ($p < 0.01$) with respect to concentration of Cd (Table 4). Highest % of tailed nuclei was associated with Cd treated thalli (2 mg/L). It was seen that, the mean comet tail length also showed a concentration dependent relationship. Tail DNA appeared to be the most pertaining parameter of DNA damage. Similarly, tail moment also increased with concentration. However, copper treated thalli showed a range of 9 to 26 (tailed nuclei), 8-24 (tail length) and 9-31 (tail movement). Comparatively, the copper is not much affecting the DNA of the cell. Results of the present study suggest that the enhancement in DNA damages associated with Cd treatments may be due to the oxidative stress induced in the leaves of *O. albidum*. The ROS formation in the tissues caused DNA damage. Metal stress may damage the cell nucleus through purine and pyrimidine bases or nucleoproteins or may denature spindle. Although, the antioxidant machinery was stimulated by the metal stress, the DNA damage increased in the leaves under higher Cd exposure, pointed out the enhanced SOD activity with decreased CAT and POX activities. SOD dismutase superoxide anions into H_2O_2 which may cause DNA damage in the plant[38].

Isozyme analysis

APX an antioxidant enzyme scavenge H_2O_2 into H_2O via ascorbate glutathione cycle. Four isozymes were observed with Cd and Cu treated thalli. APX 3 and 4 isoenzymes were hyperactive from 0.5 mg/L treatment onwards than control thalli (Figure 4A). Control showed only 3 prominent bands (APX 1, 2 and 4). Similarly 3 CAT isozymes were noticed in the Cd treated thalli compared to control (CAT 2 and 3 only). CAT isozyme 2 and 3 was intensified (more active) concomitant with increase in Cd concentration (Figure 4B). Copper treated thalli showed only two isozymes of CAT with increased intensities at par with concentrations. Five POX isozymes activities were detected on native gels using benzidine (Figure 4C). The intensities of POX 3 and 4 isoperoxidases were intensified in thalli treated from 1 mg/L Cd or Cu onwards compared with 0.25 mg/L treated thalli. Three distinct SOD isoenzymes were detected (Figure 4D) in Cd or Cu treated thalli. The presence of these isoenzymes generally was consistent in respect with Cd or Cu treatments (0.25 to 1 mg/L). However, during increased Cd or Cu dosage (1.5 and mg/L) SOD isozyme 1 and 2 showed an increased activity suggesting the more formation of H_2O_2 in

consequence with oxidative stress by the impact of heavy metals. Study of isoenzymes of antioxidant enzymes in relation to metal stress suggests that the presence/ absence of specific isoenzymes may play a role in stress tolerance or susceptibility in the selected bryophyte. The evidence supporting this hypothesis is strongest for most of the isoenzymes. Usually, the changes of staining intensities of isozymes in plants strongly depended on genotypes. These findings in the present study suggest that, different APX, CAT and SOD isozymes might be regulated by Cd or Cu stress and is involved in distinct physiological processes to eliminate ROSs formed in consequence to stress. The present findings could also be used as a basis for elucidating the mechanisms of the levels of isozyme transcripts induced in response to abiotic stress. POX isozymes are known to occur in a variety of plant tissues. The expression pattern of isoenzymes varies in different tissues of plants and is developmentally regulated or influenced by environmental factors[39]. To determine whether there were developmental or stress mediated differences among individual POX isozymes, POX activity assays were performed by activity staining. These isozymes showed different staining intensities with individual Cd or Cu treatments in the taxa. The regulatory mechanism of POX isozymes against Cd or Cu stress might be complicated, and the relationship of the genes to these enzymes requires further analysis. In addition, the changes in the staining intensities of these isoenzymes showed a similar trend compared to the quantitative changes of *in vitro* POX activity in the plant (data not shown). These results suggest that the increased POX activity could contribute to the antioxidant machinery of bryophyta against higher Cd or Cu stress. High catalase activity was coincident with an appearing of new molecular form of the enzyme. Garnczarska *et al*[40] showed different expression of CAT gene during lupine seed development to tolerate desiccation. In summary, *O. albidum* treated with heavy metal cadmium or copper displayed varied defense response of tolerance, which underlines the usability of bryophyta for genomic analysis for identifying stress related genes. With the present results, it can be concluded that Cd or Cu stress does not produce sound impacts in the plant. The RWC, chlorophyll, carotenoid, isozyme patterns of antioxidant enzymes, DNA analysis by comet assay and ultra structure of chloroplast and mitochondria add structural efficiency of the plant against metal stress. Further studies are warranted at molecular level to identify the metal stress tolerant genes and proteins in the lower vascular plant as adaptive mechanism and this will contribute to identify useful traits for improving abiotic tolerance in future breeding programmes.

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