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BIOCHEMICAL BASIS OF HEAVY METAL INDUCED STRESS TOLERANCE IN THE N_2 FIXING CYANOBACTERIUM *ANABAENA DOLIOLUM*

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ABSTRACT

The effect of heavy metals (Cd and Cu) on the nitrogen fixing cyanbacterium, *Anabaena doliolum* was observed in the present study. To explore the survival strategy of the test cyanobacterium, Chl/CAR content, protein content, antioxidative defense system (SOD, APX and GR) as well as biochemical fractionation (carbohydrates, lipids, protein, DNA and RNA) were studied. Increasing concentrations of metals inhibited the growth and survival significantly; chlorophyll and carotenoid content were found inhibited with increase in concentration of metals. Among the antioxidative enzymes, SOD and APX were increased with the increase in concentration of both the metals, whereas Catalase and Glutathione reductase were decreased at higher dose of Cd and Cu. APX played a major role for scavenging H_2O_2 rather than CAT. Results revealed decrease in all parameters with the duration of time. The role of metal induced PC in offering tolerance to UV-B was confirmed by measuring lipid peroxidation and antioxidant defense system of the cyanobacterium treated with Cd and UV-B as well as in the Cd pretreated cells of *A.dolium* exposed to UV-B. Lipid peroxidation (measured in terms of MDA content) as well as SOD and APX were found to be less induced, thus showing less oxidative damage incase of interactive treatment when applied separately. However, CAT and GR which showed sensitivity at higher dose of both the stresses were found to be induced. Thus Cd appears to antagonize the effect of UV-B in test cyanobacterium. To know the actual reason for the antagonism, PC concentration was measured in the cells with and without BSO (a potent inhibitor of phytochelatin synthase) pretreatment. The results emphasized that the extent of antagonism was reverted in the BSO pretreated cells than the normal BSO non- treated cells. Nevertheless, the PC content was found to be more in case of Cd + UV-B than the individually treated cells, but the PC was more or less completely inhibited after BSO pretreatment in all the cases. The above finding was also visualized on the SDS-PAGE. Therefore this study showed that Cd induced PC has role in UV-B tolerance.

Introduction

Cyanobacteria are the major primary producers of the aquatic eco-system. They also account for the N_2 economy of the soil. These organisms also experience the various environmental stresses largely due to the increased heavey metal pollution. *Anabaena doliolum* is one of the potent nitrogen fixing cyanobacterium of rice fields, which faces the threat of heavey metals. Certain heavy metals are essential microelements because they are required in very low concentrations by all living organisms. However, higher concentrations of these metals cause toxic effects on the organism. Due to anthropogenic activities (industrialization, use of chemical fertilizers, coal burning, mining etc) and natural sources (volcanic eruption, weathering of rocks, combustion etc), the heavey metal concentration is

continuously increasing in the soil and water bodies. The heavy metals Cd (non-essential) and Cu (essential) were chosen for the present work because they constitute a major percentage of the pollutant heavey metals and to see the adaptation strategies of the cyanobacterium to essential and non essential metals.

The major source of contamination to agricultural fields are traffic, metal processing industries, mining and by-products of mineral fertilizers e.g. DDT, Carbofuran, Machete, (18) and pesticides (6). The effects include inhibition of growth and photosynthesis, reduction in C^{14} uptake and inhibition of nitrogenase activity (21).

The metal toxicity has a great impact on the cyanobacteria population. To survive in the stressful conditions all organisms including bacteria will have to adopt to different strategies at ecological, physiological, biochemical and molecular level. The formation of various reactive oxygen species (ROS) by different environmental stresses viz. heavy metals, drought, pesticides, invasion by pathogens has been reported (12), (10). It has been generally accepted that active oxygen produced under stress is a detrimental factor, which cause lipid peroxidation, enzyme inactivation and oxidative damage to DNA and protein. To counteract the ROS- induced damage the antioxidant defense system is stimulated. This defense system consists of several enzymatic and non-enzymatic components. Among the antioxidative enzymes, the first enzyme is superoxide dismutase (SOD) which scavenges the 0^{-2} and converts it into H_2O_2 that can be scavenged by either catalase (CAT) or ascorbate peroxidase (APX). Although H_2O_2 is less reactive than 0^{-2} but in the presence of the reduced transition metal in a chleated form resulting in occurrence of OH (Fanton reaction) which is more detiruos. Further more H_2O_2 also has the capacity of carrying out the chain reaction. Thus it becomes necessary to detoxify the ROS.

The most important protein induced during heavy metal stress is the metalothionine (MT) which is low molecular weight protein and peptide. Algae, plant and some fungi also produce a class of metal binding peptides different from metalothionines (23). The effect of heavy metal on test organisms, the cellular damage in terms of lipid peroxidation, enzymatic anti oxidant-SOD,APX,GR and CAT (total –SH and GSH) and change in the protein profile were studied. The main objectives of the present study were to observe the growth behavior, chlorophyll, caretnoid and protein content in the test cyanobacterium exposed to Cu and Cd and to study the biochemical fractionation of the cells to know the effect of Cu and Cd on DNA and RNA and protein, lipid and carbohydrate content.

Materials and Methods

In order to study metal toxicity, the nitrogen fixing cyanobacterium *Anabaena doliolum* was selected and grown on Allen Arnon's media. The media was supplemented with Fe-EDTA as iron source and prepared according to (13), K_2HPO_4 and Fe-EDTA were autoclaved separately and added to cold sterilized culture media. The pH of the media was maintained at 7.5 for Allen Arnon's media and to avoid any alteration in pH, the medium was buffered with 2.0 mm Tris (Hydroxymethyl) amine –Hcl. The test cyanobacterium, *A. dololium* was grown exenically on modified Allen and Arnon's media (1) buffered with tris/Hcl at 24± 2°C under 72 micro mol photon m^{-2} S^{-1} PAR light intensity with a photoperiod of 14:10 hrs at a pH of 7.5. The cultures were shaken by hand 2-4 times daily. Stock solutions of $CuCl_2.2H_2o$ (100ugml^{-1}) and $CdCI_2$ were prepared

in distilled water and sterilized by passing through a Millipore membrane 0.22mm.

Measurement of survival and growth

To measure the survival of *A. dololium* against Cu and Cd, cells were treated with different concentrations of Cu (0-25µm) and Cd (0-0.2µm). The LC_{50} and lethal doses for both Cu and Cd were determined by the plate colony count method. Approx. 50 and 0 % survival of the test cyanobacterium was observed after 8.2µm of Cu1 and 20um Cu2 respectively of Cu exposure. The LC_{50} and lethal concentration for Cd used in this study were 0.02µm (Cd1) and 1.0 µm (Cd2). Both the treatments were given for 15 days, growth was estimated by measuring the culture density of the bacterium at 663nm in a Baush and Lomb spectronic-20 colorimeter every day upto15th day using reference blank of basal culture medium.

Pigment Extraction and Estimation

For extraction of pigments a known volume of culture was suspended in a desired amount of acetone (80%). After overnight incubation at 4^{0}c it was again centrifuged and the resulting supernatant was used for measuring chlorophyll and caretenoid content. The optical densities (OD) were recorded with the help of Baush and Lomb "spectronic-20" spectro-colorimeter at 663 and 480nm for chlorophyll and cartenoid content respectively. Chlorophyll was calculated as per Mackinney (14) and phycocyanin as per Broody . The total amount of cartenoid was calculated using the specific absorption coefficient described by Myers and Kratz (16).

Lipid peroxidation

Oxidative damage of lipid was measured in terms of the total content of thio barbituric acid (TBA). reactive substances and expressed as equivalent of Melonildialdehyde (MDA) as per Cakmark and Horst (5) with minor modification. Reactive substances from cell culture were extracted in 3ml of 1 %(w/v) Trichloro acetic acid (TCA) at 4^{0}c and centrifuged at 13000xg for 2 minutes. An aliquot of 0.5ml from the supernatant was added to 1.5ml TBA (0.5% in TSA). Samples were incubated at 100xg for 5 minutes. Specific growth rate was calculated by using the equation, M= In (N2-N1)/ T2-T1 where M is specific growth, Ni and N2 are absorbance of culture suspension at given time interval and T1 and T2 stand for final and initial respectively. The results of final yield (expressed in terms of optical density) and specific growth rate were converted to percentage of control to permit comparison. After heating, the reaction was stopped using ice baths. The concentration of MDA was calculated for its extraction coefficient ($155mM^{-1}\ cm^{-1}$).

Enzyme assay

Pellets collected from exponentially growing cultures of *A. dololium* were suspended in cell lysis buffer (pH 7.0) and sonicated in ice cold condition. The buffer contained 1mM EDTA and 1% poly vinyl pyrolidone (PRP) with the addition of 1mM ASC in the case of APX assay. The sonicated sample was centrifuged at 15000xg for 30 minutes at 4^{O}c. Total SOD activity was assayed by monitoring the inhibition of nitro blue tetrazolium (NBT) according to the method of Gianopolids and Ries (11). A 3ml of reaction mixture containing 50mM potassium phosphate buffer (pH 8.0), 133mM methionine, 75um NBT, 2um riboflavin, 0.1 mM EDTA and 100µl of enzyme extract, the reaction mixture was illuminated for 20 minutes at a light intensity of $500m^{-2}S^{-2}$ PAR. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction. CAT activity was determined by measuring the consumption of

H_2O_2, (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 3min. (1). The reaction mixture containing 50mM potassium phosphate buffer (pH 7.0), 10mM H_2O_2 and 200ul of enzyme extract, GR activity was determined by measuring the oxidation of H_2O_2, (extinction co-efficient, 6.2mM^{-1} cm^{-1}) for 3 min in 2ml assay mixture containing 50mM potassium phosphate buffer (pH 7.8) 2mM Na_2 EDTA, 0.15mM NADPH, 0.5mM GSSG and 200µl of enzyme extract. The reaction was initiated by adding NADPH, correction were made for the background absorbance at 340nm without NADPH (20). APX activity was determined by measuring the decrease in absorbance at 290 nm (extinction coefficient 2.8mM cm^{-1}) for 1 min in 1ml reaction mixture containing 50mM potassium phosphate buffer (pH 7), 0.5mM, ASC, 0.1mM.$H_2O_2$and 200µl of enzyme extract the corrections were made for low , non enzymatic oxidation of H_2O_2.

Biochemical fractionation

To a culture of 0.5 OD, 5ml per chloric acid was added and kept in ice for about 20 mins, centrifuged at 5000 rpm for 10 min. Pellet so obtained was dissolved in 3:1 (methanol : ethanol) and heated to 65^{0}C. The supernatant was taken for lipid estimation whereas the pellet was dissolved in 5% TCA warmed for 20 mins and centrifuged at 5000 rpm. The supernatant obtained is used for DNA and RNA estimation and the pellet for protein estimation.

Phytochelation Estimation:-

Total thiol was estimated according to method of Ellman (1959). The total GSH content was estimated by the 5,5 dithiobis 2, nitrobenzoic acid (DNTB) glutathione reductase coupled assay as described by Anderson (3), Protein content was measured by using formula (total –thiol-GSH). All the above parameters were measured in the control as well as BSO (2mM) pretreated cells.

Protein extraction and analysis

Cu and Cd treated cells of *A.dololium* were used for extraction of total cell protein. SDS – PAGE was carried out in 1.0 mm thick gels of 15% acrylamide . The gel loaded with 20ul sample as well as molecular weight marker (Sigma chemical Co.USA) were run at constant voltage of 220 V at 8^{o}c and stained with coomassie blue as per the method of Sambrook and Russel (24).

Results were stastically analyzed using a one way ANOVA, followed by Duncan's multiple range tests and corelationary coefficients (r). The number of independent replicates for each experiment were three.

Results

Inhibition of growth rate was consistent with the increase of metal concentration, Maximum inhibition of -298.8% was shown at 50um followed by 124,129 and 170% at 4.2 µm, 8.2µm, and 15µm Cu respectively. In case of Cd, maximum inhibition of 32.64% was observed at 0.1µM concentration followed by 29.33, 28.92. 27.14 and 11.57% at 0.01µm, 0.04µm, 0.06µm and 0.08µm respectively. A simultaneous inhibition of pigment was noticed with increase of metal concentration. An inhibition of -41.74, -144.26,-125.24 and -80.78% chlorophyll and -156.02, -132.46, 129.6 and 92.73% cartenoid was observed at 50µm, 152µm, 8.2µm and 4.2um Cu respectively where as in case of Cd, an inhibition of 64.80, 62.01, 157.26, 46.08 and 44.41% of chlorophyll and 65.09, 45.19, 53.16, 47.67 and 31.43% of cartenoid at 0.1, 0.04, 0.06, 0.08, and 0.01µm Cd respectively as compared to control. For DNA estimation , 2ml of supernatant was mixed with 1ml of DPA reagent

(diphenylamine) and boiled for 10 min. The colour so developed was measured by recording OD at 595nm. For RNA estimation, 2ml of supernatant was mixed with 3ml of orcinol reagent and boiled for 10 minutes. The optical density of the mixture was recorded at 665nm. To estimate the protein concentration, A 10ul sample was mixed with 90µl cell lysis buffer and 1ml Bradford reagent, OD was measured at 595nm (4). To estimate the carbohydrate content, 1ml of algal sample was mixed with 1ml of phenol and kept for 15 minutes as such, 5ml of conc. .H_2SO_4 was mixed and boiled followed by cooling. The colour so developed was measured by recording the OD at (8). For lipid estimation, the supernatant was used for estimation of lipid through TLC. Approximately 100ml culture was harvested, lipids were extracted from samples using chloroform: methanol (2:1 v/v) and dried under flash of nitrogen at 4^{o}c. The total lipid obtained were estimated gravimetrically and stored at -10^{0}c in deep freezer. The quantitative estimation of lipid were performed by TLC on 200mm plates impregnated with silica gel using chloroform :methanol: acetic acid and water (85:15:10:3) as solvent. Plates were activated for 20 mins at 120°C pooled with appropriate lipid extract and visualized by iodine vapours Table b. summarize the toxicity of Cu and table e, the toxicity of Cd on chlorophyll and cartenoid content of *A. dololium*. Simultaneous inhibition of pigment was noticed with increase of metal concentration. An inhibition of -41.74,-144.26,-125.24 and -80.78% chlorophyll and -156.02,-132.46,129.6 and 92.73% cartenoid was observed at 50um, 152um, 8.2 um and 4.2um. Cu respectively, whereas in case of Cd, an inhibition of 64.80, 62.01, 57.26, 46.08 and 44.41% of chlorophyll and 65.04,45.19,53.16,47.67 and 31.43% of cartenoid at 0.1, 0.06, 0.08 and 0.01um respectively as compared to control was observed. A marked reduction in the protein content of the organism was observed with increasing concentration of Cu and Cd in the medium. A decline in the protein content was found to be 99.28, 72.04, 68.28 and 247.04, 56.56, 45.20, 37.07, 33.81 and 24.21% at 50, 15, 8.2, and 4.8um of Cu and 0.1, 0.08, 0.04 and 0.01um of Cd respectively as compared to control.

The impact of different doses of Cd and Cu on the carbohydrate and protein content of *A. dololium* has been incorporated in the tables (1-a) and (1-d). Carbohydrate and protein are found to be sensitive to metal and continuous decline was observed at different doses of these metals. An inhibition of 284.6, 638.4 and 762.7% of carbohydrate and 1208.6, 435.6, 1440.7 and 315.4% of protein at 8.2, 20 Cu, 0.02 and 0.1 Cd doses respectively as compared to control. However the table (1-b and (1-c) encompasses the data on the DNA and RNA in context of the organism. A simultaneous decrease in the DNA and RNA content was registered after the Cu and Cd treatment with the passage of time. From the first to fifteenth day, an inhibition of 1208.1, 435.6, 1440.7 and 315.4% of DNA and 1372.7, 331.9, 1424.0 and 257.3 % of RNA content was noticed at Cu 8.2, Cu 20, Cd 0.02 and Cd 0.1uM concentrations respectively over the control. On the other hand the lipid content was also found to be affected and showed a remarkable decline after the metal treatment.

Table (2-a, b, c and d) compiles data on the impact of different doses of Cu and Cd on the enzymatic antioxidant activities (SOD, CAT, APX and GR) of *A. dololium*. The SOD activity showed enhancement from 23.8, 61.9, 4.78 and 33.3% to 24.8, 90.5, 205.9 and 368.9 fold after the treatment of 8.2, 20µm Cu ,0.02 and 1.0µm Cd respectively as compared to control. Thus SOD activity was found to increase more in case of Cd 0.1µm treatment. According to Pearson correlation

coefficient SOD activity was significantly correlated with APX activity and negatively with CAT. ($P<0.01$). However, CAT appeared to be metal sensitive and registered a decline in the activity after Cu and Cd treatment. In contrast to SOD activity, CAT showed decrease with the passage of time. activity was decreased by -42.2, -51.80 and 44.66 following the treatment with 20uM Cu, 0.02 and 0.1μM Cd on thee fifteenth day as compared to control where as in case of Cu 8.2, CAT was found to be increased by 75.2% on the fifteenth day. CAT is negatively correlated with APX. ($P<0.01$) but highly significant with the GR in the early days of treatment. The APX activity registered an increase of 84, 141.8, 64.0 and 104.2 fold for Cu 8.2, Cu 20, Cd 0.02 and Cd 0.1μm respectively as compared to control. However the magnitude of increase differed significantly between the two treatments of Cu and Cd (Duncan's multiple range test). During the initial days of metal stress, AP was non significantly related to GR but it showed a highly significant correlation in the late phase. Further the APX activity was significantly correlated with the SOD activity ($P<0.01$). A further enhancement was observed in case of GR activity after exposure of Cu and Cd. However, the enhancement was more at the lower concentration as compared to higher concentration of metal. A 6.2 and 8.7 fold enhancement in case of 8.2μm Cu and .02μm Cd was observed whereas 2.5 and 4.6 fold enhancement in case of Cu 20 and Cd1 as noticed, GR was highly correlated with APX.

Table I (a) Effect of different treatments on the carbohydrate content of *A. dololium* over a time course of 15 days. The values in the parenthesis denote the percent increase or decrease.

Treatments	1st day	3rd day	6th day	9th day	12th day
Control	0.171±0.006	0.108±0.013	0.130±0.006	0.195±0.30	0.251±0.003
Cu (8.2μM)	0.143±0.004 (-15.9)	0.324±0.052 (362.2)	0.602±0.007 (362.2)	0.557±0.310 (185.6)	0.996±0.018 (284.6)
Cu(20μM)	0.100±0.004 (-41.2)	0.155±0.012 (42.8)	0.458±0.003 (251.7)	0.465±0.044 (138.9)	1.51±0.018 (501.50
Cd(0.002μM)	0.127±0.022 (-25.5)	0.233±0.019 (113.9)	0.373±0.019 (168.0)	1.63±0.028 (738.6)	1.85±0.006 (638.41)
Cd(0.1 μM)	0.061±0.004 (-64.0)	0.389±0.025 (257.2)	0.504±0.008 (290.6)	1.19±0.013 (510.51)	2.16±0.009 (762.7)

Table I (b) Effect of different treatments on the DNA content of *A. dololium* over a time course of 15 days. The values in the parenthesis denote the percent increase or decrease.

Treatments	1st day	3rd day	6th day	9th day	12th day
Control	3.p0±0.0020	3.72±0.005	9.07±0.002	8.87±0.003	8.05±0.003
Cu (8.2µM)	7.90±0.003 (102.71)	7.46±0.005 (100.5)	28.1±0.003 (210.4)	60.2±0.004 (579.4)	11.2±0.004 (120.86)
Cu(20µM)	6.55±0.003 (68.0)	4.22±0.004 (13.4)	23.3±0.001 (157.6)	56.1±0.016 (533.2	45.5±0.00 (435.6)
Cd(0.002µM)	5.46±0.002	6.07±0.001 (63.2)	24.1±0.076 (165.9)	58.9±0.056 (565.0)	130.9±0.002 1440.7)
Cd(0.lµM))	4.06±0.003 (4.1)	4.23±0.001 (13.9)	19.2±0.003 (11.5)	26.1±0.006 (195.1)	35.5±0.003 (315.4)

* All values are mean + SD of three replicates. Values in parenthesis show % increase over control and those with a negative sign indicate percent inhibition.

Table I(c) Effect of different treatments on the DNA content of *A. dololium* over a time course of 15 days. The values in the parenthesis denote the percent increase or decrease.

Treatments	1st day	3rd day	6th day	9th day	12th day
Control	24.84±0.005	26.0±0.006	22.7±0.005	20.2±0.002	20.3±0.001
Cu (8.2µM)	49.8±0.001 (100.2)	67.1±0.002 (157.6)	108.0±0.002 (370/8)	146.8±0.003 (624.0)	299.4±0.003 (1372.7)
Cu(20µM)	35.2±0.005 (41.7)	53.5±0.003 (104.7)	58.2±0.002 (155.41)	45.1±0.001 (122.5)	87.8±0.003 (331.9)
Cd(0.002µM)	21.0±0.002 (-19.5)	54.3±0.005 (108.5)	72.0±0.060 (238.2)	173.5±0.014 (756.1)	309.9±0.003 (1424.0)
Cd(0.lµM))	12.1±0.003 (-51.4)	79.1±0.003 (203.5)	100.4±0.006 (340.7)	55.8±0.003 (175.5)	72.6±0.002 (259.3)

Table I (d): Effect of different treatments on the protein concentration of *A. dololium* over a time course of 15 days. The values in the parenthesis denote the percent increase or decrease.

Treatments	1st day	3rd day	6th day	9th day	12th day
Control	7.91±0.007	9.20±0.003	9.58±0.008	10.2±0.008	10.6±0.009
Cu (8.2µM)	3.66±0.004 (-53.7)	2.97±0.006 (-67.7)	1.92±0.010 (-79.9)	1.37±0.004 (-86.7)	0.059±0.002 (-94.4)
Cu(20µM)	3.48±0.011 (-56.0)	2.99±0.006 (-67.5)	1.78±0.015 (-81.4)	1.36±0.005 (-86.6)	0.379±0.007 (-96.4)
Cd(0.002µM)	4.06±0.024 (-48.6)	3.20±0.025 (-65.2)	2.32±0.015 (-81.4)	1.17±0.008 (-88.5)	0.068±0.002 (-93.5)
Cd(0.1µM))	3.49±0.017 (55.8)	1.96±0.024 (-78.6)	1.701±0.021 (-82.2)	1.06±0.003 (89.6)	0.024±0.001 (-95.9)

* All values are mean + SD of three replicates. Values in parenthesis show % increase over control and those with a negative sign indicate percent inhibition.

Table2 (a): Effect of the different treatments on the SOD activity of *A. dololium* over a time course of 15 days.

Treatments	1st day	3rd day	6th day	8th day	12th day	15th dqy
Control	0.251± 0.002	0.190± 0.004	0.157± 0.003	0.188± 0.006	0.011± 0.004=5	0.152± 0.003
Cu(8.2um)	0.253± 0.004 (0.049)	0.433± 0.004 (127.8)	0.528± 0.003 (235.5)	0.829± 0.002 (391..2)	0.945± 0.003 (821..9	2.51± 0.003 (1249.2)
Cu (20um)	0.250±0.006 (0.385)	0.423±0.003 (122.7)	0.461±0.002 (193.2)	0.496±0.004 (194.3)	0.1o5±0.002 (831.6)	2.183±0.005 (1249.2)
Cd (0.02uM)	0.255±0.006 (1.48)	0.412±0.003 (116.5)	0.479±0.005 ((204.5)	0.696±0.004 (9312.4)	1.16±0.002 (1011.7)	2.183±0.005 (1335.7)
Cd(0.1uM)	0.267±0.003 (6.21)	0.454±0.004 (138.5)	0.741±0.004 (370.9)	0.934±0.005 (453.2)	1.24±0.006 (1089.8)	3.34±0.004 (2089.9)

Table2 (b): Effect of the different treatments on the CAT activity of *A. dololium* over a time course of 15 days.

Treatments	1st day	3rd day	6th day	8th day	12th day	15th day
Control	0.056±0.006	0.060±0.006	0.068±0.005	0.097±0.022	0.101±0.001	0.102±0.002
Cu(8.2um)	0.063±0.112 (1028.3)	0.597±0.051 (888.3)	0.522±0.006 (652.6)	0.311±0.007 (230.5)	0.203±0.010 (101.8)	0.185±0.002 (75.2)
Cu (20um)	0.979±0.017 (1681.1)	0.235±0.009 (295)	0.135±0.004 (97.5)	0.109±0.004 (15.7)	0.068±0.005 (-37.6)	0.066±0.001 (-42.2)
Cd (0.02uM)	0.486±0.027 (764.0)	0.320±0.009 (433.8)	0.161±0.008 (135.2)	0.163±0.007 (52.63)	0.122±0.003 (20.74)	0.49±0.001 (-51.8)
Cs(0.1uM)	0.440±0.102 (798.0)	0.363±0.027 (503.3)	0.275±0.018 (297.1)	0.178±0.006 (86.6)	0.151±0.004 (57.7)	0.059±0.001 (-44.7)

*All values mean+-SD of three replicates Values in parenthesis show percent increase over control as those with a negative sign indicate percent inhibition.

Table2(c): Effect of the different treatments on the APX activity of *A. dololium* over a time course of 15 days.

Treatments	1st day	3rd day	6th day	8th day	12th day	15th dqy
Control	0.926±0.005	0.568±0.008	0.749±0.004	0.695±0.00 1	0.942±0.004	1.75±0.039
Cu(8.2um)	1.30±0.003 (12.1)	2.56±0.013 (255.0)	5.35±0.002 (272.3)	27.9±0.010 (3928.7)	47.5±0.014 (4970.4)	14.9±0.016 (8437.2)
Cu (20um)	1.22±0.013 (33.2)	2.59±0.005 (272.3)	3.81±0.002 (408.5)	50.59±0..02 4(7147.6)	52.66±0.035 (5526.0)	254.3±0.019 (14184.0)
Cd (0.02uM)	2.02±0.007 (82.47)	15.7±0.047 ((1303.1)	18.9±0.097 (2478.6)	27.2± 0.038 (377.3)	38.4±0.016 (4007.6)	115.4±0.043 (6489.5)
Cs(0.1uM)	1.89±0.008 (105.2)	11.75±0.027 (1513.3)	23.04 ±0.007 (2963.0)	29.9±0.025 (4165.4)	54.4±0,125 (5627.0)	184.0±0.125 (10428.4)

Table2(d) Effect of the different treatments on the GR activity of *A. dololium* over a time course of 15 days.

Treatments	1st day	3rd day	6th day	8th day	12th day	15th day
Control	0.204±0.007	0.251±0.001	0.239±0.003	0.257±0.005	0.252±0.005	0.283±0.005
Cu(8.2 uM)	0.302±0.007 (53.8)	0.776±0.002 (202.3)	0.884±0.012 (240.8)	0.948±0.003 (264.8)	1.802±0.009 (621.0)	7.51±0.003 (1779.8)
Cu (20 uM)	0.187±0.002 (-26.6)	0.509±0.005 (108.1)	0.699±0.012 (177.4)	0.724±0..01 2 (195.4)	0.902±0.004 (259.8)	10.9±0.015 (13715.5)
Cd (0.02 uM)	0.172±0.001 (-13.5)	0.740±0.008 (191.8)	0.772±0.011 (215.4)	0.878± 0.002 (261.4)	2.465±0.019(8 76.1)	5.208±0.014 (1822.6)
Cd(0.1 uM)	0.149±0.002 (-3.3)	0.533±0.004 (131.2)	0.656 ±0.002 (163.4)	1.418±0.001 (364.6)	1.418±0.001 (465.2)	10.54±0.009 (3666.6)

*All values mean+-SD of three replicates Values in parenthesis show percent increase over control as those with a negative sign indicate percent inhibition.

SOD activity of the cell was much elevated in case of Cu 20 (90 fold) as compared to Cd (36.8 fold). Similarly the increase of APX, was found to more in 20 um Cu (141.8 fold). Than .1um Cd (104.2 fold). Contradictory to this GR activity was enhanced much in .1um Cd (36.6 fold) as compared to Cu (37.1 fold) whereas the activity of CAT was found to be inhibited much by Cd 1 (-44.2% then by Cu 20 (-42.2%).The logarithmic phase of cells of *A. dololium* were taken and exposed to 20uM Cd and for 20 (UV1) and 50 (UV2) min exposure of UV B. These treatments were found to increase the extent of oxidative damage measured in terms of lipid peroxidation by 71.7, 89.2 and 108.7% respectively with respect to control. However, the BSA pretreated cells on subjecting to the above stress condition exhibited increase in lipid concentration by 78.6, 80.4 and 89.17 % respectively over the control. Further, both Cd and UV–B were found to enhance the activity of SOD which the first line of defense against ROS, catalysis the desiccation of O_2^- to O_2 and H_2O_2. SOD activity is also considered to be an indirect measurement of O_2 production and hence the extent of oxidative damage. However, increase in SOD after Cd in the cell without BS0 was 87.1% whereas UV1 and UV2 produced a rise. The results pertaining to CAT. Although similar trend was observed in BSO treated cells. UV1 and UV2 exhibited a much greater increase in SOD activity of 205 and 475% respectively with respect to control. The results pertaining to CAT in table 3 a, showed that Cd induced the CA activity by 62.7%. However, UV1 and UV2 inhibited its activity by 64.4 and 74.2% respectively as compared to control. This demonstrates that UV-B has an

inhibitory effect on CAT activity. This is also supported by Streb (1993) who reported Photo-inhibition of CAT activity. On the other hand BSO pretreatment inhibited CAT in all the cases, probably due to toxicity of CAT. Our results also revealed that these two stresses significantly increased the APX activity (Duncan's multiple range test)by 5.6,2.4 and 4.9 fold in case of non BSO treated cells whereas 5.7,4.5 and 8.5 fold in BSO pretreated cells after the exposure of Cd, UV1 and UV2 respectively as compared to control. GR was increased by 2.84 and 1.09 fold after Cd and UV1 treatment respectively but decreased by 17.7% in the care of Uv1 as compared to control. On the other hand the GR activity in BSO pretreated cells was found to be enhanced by 3.17, 1.88 and 1.59 fold after Cd, Uv1 and Uv2 stress respectively. A low molecular weight protein PC was found to be induced after Cu and Cd treatment.

Table 3(a). Effect of different concentrations of Cd and UV-B on melondialdehyde content, activities of the antioxidant enzymes (SOD,CAT, APX and GR) and phytochelatin concentration in cells of Anabaena dololium. All values are mean+- SD of three replicates. Values having different letters are significantly different (P<0.05). Different analysis was done for each column (Duncan's new multiple range test). Values in parenthesis show %increase over control and those with negative sign indicate percent inhibition.

Treatment	MDA content (uM mol ng^{-1} protein)	SOD (uM SOD mg^{-1} $protin^{-1}$.	CAT(uM min^{-1}mg $protein^{-1}$	APX (uM min^{-1}mg $protien^{-1}$	GR(uM min^{-1}mg $protein^{-1}$	PC concentration (uM mg $protein^{-1}$
Control	0.015 ±0.011	0.0131±0.011	0.045±0.002	0.953±0.020	0.056±0.002	0.054±0.000
Cd	0.023±0.002 (53.7)	0.246±0.002 (87.1)	0.074±0.003 (62.7)	5.373±0.030 (463.7)	0.160±0.003 (185.7)	0.074±0.001 (34.6)
UV1	0.029±0.002 (89.2)	0.288±0.005 (119.1)	0.016±0.002 (-64.9)	2.256±0.035 (136.7)	0.061±0.002 (8.9)	0.056±0.000 (2.6)
UV2	0.032±0.001 (108.7)	0.311±0.005 (137.2)	0.013±0.002 (-72.2)	1.676±0.025 (390.6)	0.046±0.001 (-17.8)	0.056±0.002 (23.6)
Cd+UV1	0.022 ±0.002 (41.3)	0.365±0.004 (178.5)	0.080±0.003 (75.5)	3.456±0.034 (262.6)	0.124±0.003 (121.4)	0.067±0.002 (23.6)
Cd+UV2	0.016±0.001 (9.3)	0.256±0.002 (119.1)	0.062±0.004 (35.7)	1.543±0.032 (61.9)	0.072±0.003 (28.5)	0.078±0.002 (42.9)

Table 3b. Effect of different concentrations of Cd and UVB on malondialdehyde content, activities of the antioxidant enzymes (SOD, CAT, APX and GR) and phytochelation concentration in BSO pretreated cells of Anabaena dololium. All values are mean+- SD of three replicates. Values having different letters are significantly different (P<0.05). Different analysis was done for each column (Duncan's new multiple range test) Values in parenthesis show %increase over control and those with negative sign indicate percent inhibition.

Treatment	MDA content (uM mol ng^{-1} protien	SOD (uM mgl^{-1} $protin^{-1}$	CAT(uM $min^{-1}mg$ $protein^{-1}$	APX (uM $min^{-1}mg$ $protien^{-1}$	GR(uM $min^{-1}mg$ protein^{-1}	PC concentration (uM mg $prtien^{-1}$)
BSO	0.018 ±0.000	0.260±0.005	0.019±0.001	1.730±0.051	0.056±0.002	0.054±0.000
Cd	0.027±0.002	0.355±0.001	0.026±0.004	5.480±0.020	0.160±0.003	0.074±0.001
	(46.6)	(87.1)	(43.0)	(463.7)	(185.7)	(34.6)
UV1	0.026±0.001	0.795±0.008	0.029±0.003	4.360±0.052	0.061±0.002	0.056±0.000
	(80.4)	(119.1)	(-36.9)	(136.7)	(8.9)	(2.6)
UV2	0.029±0.003	1.500±0.010	0.020±0.003	8.123±0.015	0.046±0.001	0.056±0.002
	(89.1)	(137.2)	(-55.5)	(390.6)	(-17.8)	(23.6)
Cd+UV1	0.028 ±0.002	0.981±0.004	0.015±0.005	5.663±0.047	0.124±0.003	0.067±0.002
	(82.6)	(178.5)	(-67.1)	(262.6)	(121.4)	(23.6)
Cd+UV2	0.023±0.002	1.791±0.004	0.027±0.002	6.383±0.076	0.072±0.003	0.078±0.002
	(51.9)	(119.1)	(-40.8)	(61.9)	(28.5)	(42.9)

Discussions

Heavy metal toxicity to algae is one of the most debated environmental problem. Algae, cyanobacteria and other aquatic plants show sensitivity to metal toxicity displaying metabolic disturbances and growth inhibitions by heavy metal content only slightly higher than the normal level. Our study reveals a reduction in the survival and growth of *Anabaena dololium* with increasing concentration of Cu and Cd thereby confirming the toxicity of these metals. With the increasing concentration of Cu and Cd there was a remarkable reduction of 124 and 32.6% at 50uM Cu and 0.1 uM Cd respectively by the 15th day. These results are in agreement with the findings of Fillips and Pallaghy (7). Rachin *et al* (19) who reported inhibition of growth and metabolism of algae and cyanobacteria by Cu and Cd. The toxicity of these two heavy metals may be either due to the disruption of the permeability of the cell membrane or inhibition of photosynthetic pigment and enzymatic activities. These results offer support to the contention that growth reduction is reasonable determent of metal toxicity and that the degree of response of algae is deep dependent on the amount of metal that traverse for reduction of growth of cyanobacteria after exposure to heavy metal. This reduction could be due to the binding of the test metals to sulphydryl group, which is responsible for regulation of cell division in plants including cyanobacteria. A concentration dependent decrease in content of all pigments was also observed in *Anabaena dololium* . The trend of pigment inhibition in this organism was Chl followed by

Caretenoid. Likewise protein content also depicted a gradual decrease in the metal supplemented cultures. The inhibition of Chl could be due to the bleaching of the pigment. Further the loss of CAR indicates that this pigment has little role in regulating metal toxicity.

Further decrease in the lipid content (data not given) with time could be due to the increased peroxidation of the membrane lipid by the heavy metals. All the studied parameters e.g. carbohydrate, lipid, DNA, RNA and protein. Except carbohydrate registered decrease in their content with the increase in the concentration of both the metals (Cu and Cd). The increase in carbohydrate content might be due to the thickening of the mucilaginous sheath. The reason for the reduction in DNA content may be the reduction of growth. As the growth rate is inhibited the number of cells will decrease and proportionally the DNA content will also decrease. This decrease could in turn cause a loss in the transcription rate hereby reducing the RNA content. Further, the reduction in RNA content could also bring about a reduction in protein content could also be due to the oxidative damage caused by the metals as observed in table 2.a, b, c, d.

The results presented in table 2.a,b,c and d clearly demonstrate oxidative damage caused by Cu and Cd as expressed in terms of enzymatic oxidant activity. These stresses were found to produce ROS, which are highly deleterious to the cell. To scavenge the cellular defense system is stimulated inducing either production of antioxidants and stress proteins. The activity of the enzymatic antioxidant SOD was stimulated by Cd as well as Cu but more so by Cu. The increase in SOD activity after metal treatment could be due to the production of 0_2 anions whose detoxification is necessary for the growth of organism, thus it converted into peroxide by the activity of SOD. This could be due to the redox nature of Cu, which facilitates the production of superoxide anion.Cd being non redox metal stimulates the production of peroxide. The data obtained clearly show that the enzyme activity increased consistently with time. It is worth stating that SOD is the first enzyme of the superoxide of the antioxidant pathway responsible for the scavenging and conversion of the superoxide anion into H_2O_2. H_2O_2 is known to generate OH radicals by interacting with reductants such as transition metal ions (Fe^{2+}) generated as a result of the reduction of(Fe^{3+})by the superoxide radical. Fe^{2+} complex + H_2O_2..... Fe^{3+}(complex) + OH^{+}+OH^{-} . Both these species (H_2O_2 and OH^{-}) have the potential to oxidize membrane fatty acids, thereby initiating lipid peroxidation OH^{-} is more reactive and is more reactive and damaging than H_2O_2 because no specific enzyme is available for its scavenging. Morever, its reaction with cellular components proceeds at diffusion controlled rates (10^{8}-10^{9} M^{-1} S^{-1}). Thus even a trivial and transient expression of OH^{-} can cause damages to the membrane lipid (Asada 1999). Under these circumstances, it is essential that H_2O_2 produced scavenged either by CAT or with the help of well known enzymes of the Halliwell Asada pathway (APX and GR). The activity of SOD and GR was remarkably enhanced. Contrary to this CAT activity was suppressed with the passage of time. Table 2-b reveals that by -51.8 and 44.7% Cu (8.2 uM) on the other hand inhibited its activity by -42.2% by the 15^{th} day. The inactivation of CAT can be explained in the light of the fact that Cd and Cu can replace the metal present in CAT, which is responsible for its proper functioning (Hall,2002). Thus the insignificant effect of Cu and Cd on CAT leaves the problem of H_2O_2 scavenging unresolved. Under the circumstances other peroxide scavenging enzyme

have to take up the job. Our results show a 84, 14, 64 and 104 fold increase after the treatment of Cu1, Cu2, Cd1 and Cd2 respectively provide a testimony to the view that APX has greater affinity for H_2O_2 than CAT and is a major H2O2 scavenging enzyme under stress conditions (Streb *et al.*and Foyer *et al*, 1994). Further, the GR was also found to be increased by 17, 37, 18 and 36 fold after the treatment of Cu (8.2uM and 20 uM) .Our results are also supported by Nagalakshmi and Prasahad (17) and Mallick and Rai (15) who reported increase in APX and GR activities at increasing doses of Cu in *Scendesmus bijugatus* and *Anabaena doliolum* respectively. Cd and Cu induced increase in GR activity can be explained on the basis of the transcriptional activation of gr1 and gr2 genes. Further APX activity was also induced due to increase in H2o2 concentration, which not acts as signal for apx opron but also as direct inducer of apx gene. Nevertheless, the decrease in the oxidative damage and suppression of antioxidant enzyme activity as well as lipid peroxidation in the Cd pretreated cells exposed to UV-B clearly supports the hypothesis that PC probably has a role in UV-B tolerance.

Conclusion

All the studied parameters viz. growth, Chl/CAR content, biochemical fractionation as well as antioxidative defense system clearly demonstrated that Cd and Cu induce the oxidative damage in the cells of *A. doliolum* .The SOD activity in control cultures did not increase with time, but the other enzymes were found to increase continuously. Further in the metal treated cells the activity of SOD does not increase but metal stress tremendously increased its activity. This observation can be of significant practical importance in that the SOD activity can be used as a bio-marker of metal pollution. Further it is also found that under stress conditions the activities of the enzymes increased continuously with time until the 15^{th} day. This shows that adaptation of the cell to metal stress is very much dependent on the antioxidative defense system.

An off shoot of this work was to study the involvement of PC in UV stress. The results revealed -very significant and first hand information that the induction PC after metal stress co-tolerance in the cyanobacterium to UV-B. However, the actual reason for this could not be established and this becomes an area of future research.

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