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BRIEF COMMUNICATION

Cadmium mitigates ultraviolet-B stress in *Anabaena doliolum*: enzymatic and non-enzymatic antioxidants

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Abstract

Impact of ultraviolet-B (UV-B) and Cd, applied individually and in combination, measured in terms of oxygen-evolution, chlorophyll (Chl) and protein contents, lipid peroxidation, and enzymatic and non-enzymatic antioxidants of *Anabaena doliolum*, revealed a greater oxidative damage induced by UV-B than by Cd. While superoxide dismutase (SOD) showed a greater stimulation by UV-B than Cd, the activities of catalase (CAT) and glutathione reductase (GR) declined after UV-B treatment. Cd treatment, however, enhanced the activities of ascorbate peroxidase (APX) and GR. CAT activity increased at low but decreased at high dose of Cd. Increase in carotenoid (Car) content in UV-B treated cells suggested a shielding effect of Car against UV-B stress. A 15- and 10-fold rise in α-tocopherol (α-TOC) content at high dose of Cd and/or UV-B offered testimony to the antioxidant role of α-TOC.

Additional key words: α-tocopherol, ascorbate, antioxidative defence system, carotenoids, chlorophyll, oxidative damage.

UV-B and Cd are two important stressors resulting from stratospheric ozone depletion and increased industrialization, which affect all types of ecosystems including aquatic biota. Both these stresses adversely affect cyanobacteria by inhibiting growth, carbon fixation, nitrogen and phosphorus metabolism, and photosynthesis (Prasad and Zeeshan 2005). Although UV-B and Cd are essentially two different stressors, they generate reactive oxygen species (ROS) stimulating the antioxidative defence system in all organisms including cyanobacteria.

Some reports on the impact of UV-B and heavy metals on the antioxidative defence system of cyanobacteria include those of Mallick and Rai (1999) using Cu on SOD, CAT, GR and APX in *Anabaena doliolum* and Nagalakshmi and Prasad (2001) on GSH mediated protection of Cd toxicity in *Scenedesmus*. Car has been reported to offer UV tolerance in cyanobacteria (Ehling-Schulz and Scherer 1999). Surprisingly not much is known about the combined effects of both stressors on the antioxidative defence system of cyanobacteria. Except the report of Jiang and Zhang (2001) on the effect of abscisic acid on enzymatic and non-enzymatic antioxidants of maize seedling, no information is available on the comparative account of two antioxidants in protecting cyanobacteria from UV-B and Cd stress. Thus the present study is first of its kind to compare the enzymatic and non-enzymatic antioxidative strategies of *Anabaena* for defence against Cd and UV-B applied individually as well as in combination.

Anabaena doliolum Bharadwaja was isolated from the rice field in Varanasi and grown axenically in a modified medium of Allen and Arnon (1955) buffered with Tris/HCl (pH 7.5) at temperature of 24 ± 2 °C and irradiance of 72 μmol(photon) m^{-2} s^{-1} PAR with a 14-h photoperiod. For UV-B treatment the culture suspensions (10 cm^3) were transferred to 75 mm glass Petri dish and exposed to artificial UV-B radiation (from UV-B lamp *CAT No. 34408, Fotodyne, Inc.*, New Berlin, WI, USA, giving its maximum output at 310 nm) in a temperature-controlled incubator. The doses selected for the study

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Abbreviations: α-TOC - α-tocopherol; APX - ascorbate peroxidase; ASA - ascorbate; Car - carotenoid; CAT - catalase; Chl - chlorophyll; GSH - glutathione reduced; GR - glutathione reductase; MDA - malondialdehyde; PAR - photosynthetically active radiation; ROS - reactive oxygen species; SOD - superoxide dismutase; UV-B - ultraviolet B radiation.

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were Cd_1 (0.02 µM) and Cd_2 (1.0 µM) and UV-B_1 (20 min) and UV-B_2 (60 min), which were found to be the LC_{50} (lethal doses) of Cd and UV-B as determined by the method of Rai and Raizada (1985). The experimental setup included three independent sets: *1*) cells treated with Cd for 24 h, *2*) cells exposed to UV-B, and *3*) cells pretreated with Cd for 24 h followed by UV-B exposure. All sets were then subjected to overnight incubation in dark. All the experiments were repeated thrice.

Total cell protein was estimated by the method of Bradford (1976). O_2-evolution was measured with a Clark type polarographic O_2 electrode enclosed in a 10 cm^3 airtight reaction vessel and connected to an O_2 analyzer (digital O_2 system, model 10, *Rank Brothers*, Cambridge, UK). Lipid peroxidation was measured in terms of the total thiobarbituric acid reactive substances (TBARS) and expressed as equivalent of malondialdehyde (MDA) content as per Cakmak and Horst (1991) with minor modifications.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of reduction of nitroblue tetrazolium (NBT) according to the method of Gianopolitis and Ries (1977). Catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) activities were determined by measuring the consumption of H_2O_2. The former at an coefficient of absorbance of 39.4 mM^{-1} cm^{-1} at 240 nm for 3 min (Aebi 1984) and the latter at coefficient of absorbance of 2.8 mM^{-1} cm^{-1} at 290 nm for 1 min (Nakano and Asada 1981). Glutathione reductase (GR, EC 1.6.4.2) was estimated by the method of Schaedle and Bassham (1977).

Chlorophyll (Chl) and carotenoid (Car) contents were calculated using the specific absorption coefficient (Jiang and Zhang 2001). α-TOC was extracted as per Munné-Bosch *et al.* (1999) using HPLC column (300 × 3.9 mm, C-18 column, *Waters Chromatography Division*, Millipore Corporation, Milford, MA, USA). Ascorbate was measured in terms of reduction of 2,6-dichlorophenolindophenol (DCPIP) (Keller and Schwager 1977). Total GSH was estimated by the 5,5' dithiobis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase coupled assay as described in Anderson (1985).

Results were statistically analyzed using one-way *ANOVA* followed by Pearson's correlation coefficient using *SPSS 10* software. There were three independent variables for each experiment.

The O_2 evolution showed a significant decrease following treatments with UV-B and Cd. However, a maximum decline of 85.4 % was observed with Cd_2 treatment as compared to control (Table 1). This can be explained in the light of the sensitivity of PS 2 to both Cd as well as UV-B (Rai *et al.* 1995). However, the decrease in protein and Chl contents was more pronounced for UV-B than Cd. The decline in protein content (Table 1) after UV-B and Cd exposure may be due to production of ROS, which is known to damage protein (Hernandez and Almansa 2002). However, Chl degradation by UV-B could be due to its high energy content and that by Cd may be due to inhibition of Chl biosynthesis (Larsson *et al.* 1998).

Perceptible lipid peroxidation (Table 1) was noticed following UV-B and Cd treatments; UV-B emerged as a stronger inducer of lipid peroxidation (2.0 and 3.4 fold increase after UV-B_1 and UV-B_2 treatments respectively) than Cd (46.3 and 89.8 % increase after Cd_1 and Cd_2 treatments respectively). UV-B induced production of toxic O_2 radicals may oxidize the fatty acids and upset the ratio of saturated/unsaturated fatty acid thereby disturbing the membrane fluidity (Kramer *et al.* 1991). In contrast to this Cd-induced lipid peroxidation is an indirect effect caused by H_2O_2 accumulated consequent upon inhibition of various metabolic processes including carbon fixation, nitrogen and phosphorus metabolism, and photosynthesis (Prasad and Zeeshan 2005).

The SOD activity was enhanced by 2-fold after UV-B_2 exposure. A greater increase in SOD activity by UV-B than Cd (Fig. 1*A*) can be explained in the light of the work of Mackerness *et al.* (2001) where $O_2^{\bullet -}$ was the first ROS generated in response to UV-B stress. Since SOD is stimulated both by UV-B and Cd, the chances of O_2-induced damage of cell should be minimum. Nonetheless, even the transient presence of the $O_2^{\bullet -}$ in the

Table 1. Effect of different doses of Cd and UV-B on oxygen evolution, chlorophyll, protein and MDA contents of *Anabaena doliolum.* Mean ± SD, $n = 3$, * - values significantly different at $P < 0.05$ from corresponding controls.

Treatments	Oxygen evolution [mM (O_2) mg^{-1}(protein) s^{-1}]	Chlorophyll [mg dm^{-3}]	Protein [mg dm^{-3}]	MDA [mmol g^{-1}(protein)]
Control	0.344 ± 0.02	6.63 ± 0.10	66.6 ± 1.00	0.227 ± 0.03
UV-B_1	0.125 ± 0.01*	3.58 ± 0.08*	51.6 ± 1.11*	0.462 ± 0.01*
UV-B_2	0.100 ± 0.01*	2.82 ± 0.06*	42.7 ± 2.02*	0.780 ± 0.01*
Cd_1	0.147 ± 0.02*	4.33 ± 0.05*	57.1 ± 1.52*	0.332 ± 0.03*
Cd_2	0.050 ± 0.00*	4.31 ± 0.11*	52.0 ± 2.00*	0.430 ± 0.04*
Cd_1+ UV-B_1	0.153 ± 0.01*	2.67 ± 0.06*	59.6 ± 1.00*	0.470 ± 0.03*
Cd_1+ UV-B_2	0.144 ± 0.03*	2.44 ± 0.10*	41.4 ± 1.50*	0.630 ± 0.01*
Cd_2+ UV-B_1	0.175 ± 0.02*	4.21 ± 0.08*	64.1 ± 2.47	0.560 ± 0.05
Cd_2+ UV-B_2	0.139 ± 0.01*	1.85 ± 0.04*	53.2 ± 1.56*	0.640 ± 0.05*

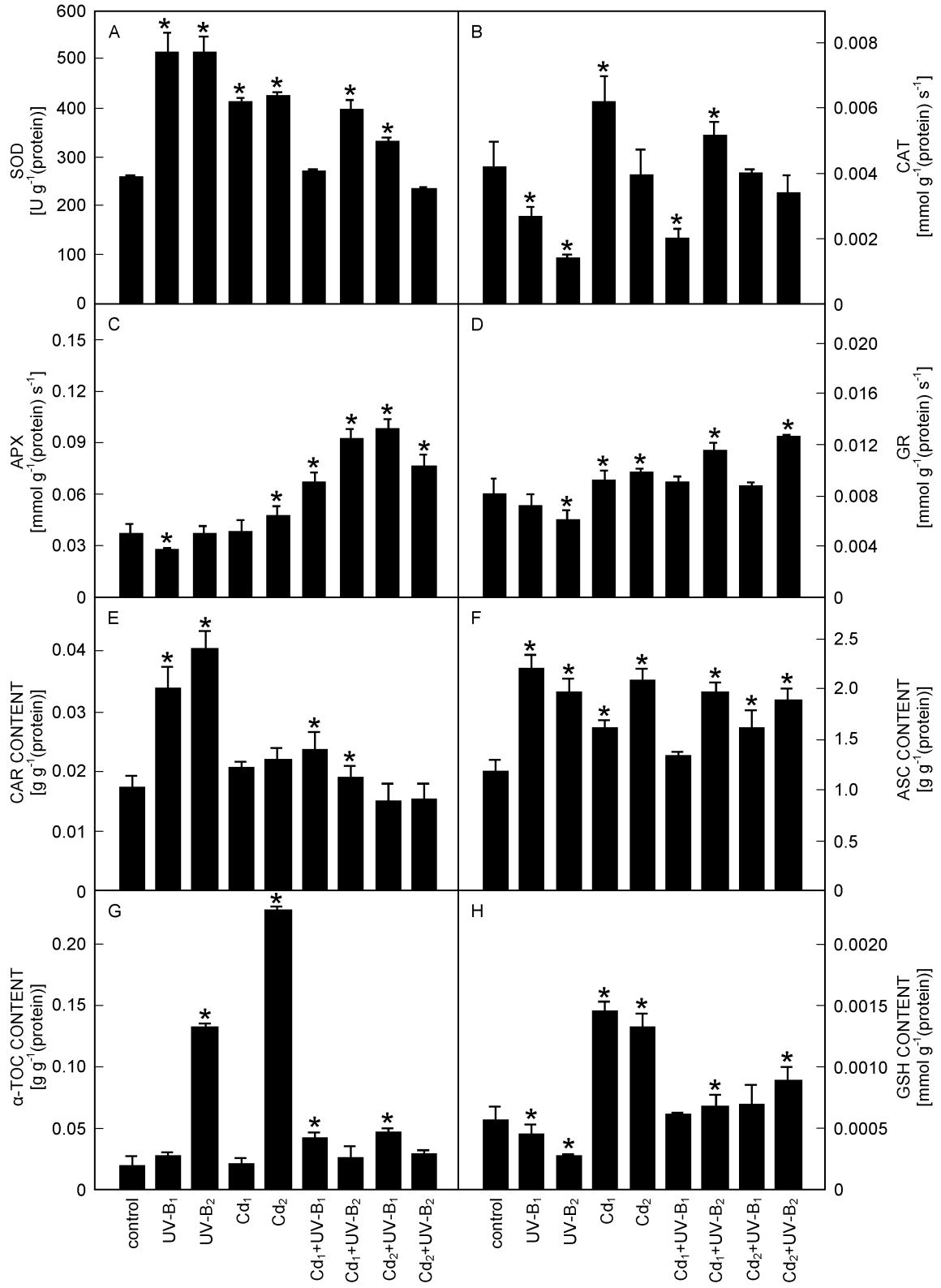

Fig. 1. Effect of different treatments of Cd and UV-B on superoxide dismutase, SOD (*A*), catalase, CAT (*B*), ascorbate peroxidase, APX (*C*), glutathione reductase, GR (*D*) activities and carotenoid, CAR (*E*), ascorbate, ASA (*F*), α-tocopherol, α-TOC (*G*) and glutathione reduced, GSH (*H*) contents of *Anabaena doliolum*. Means ± SD, $n = 3$, * - values significantly different at $P < 0.05$ from corresponding controls.

cell have the potential to oxidize membrane fatty acids and initiate lipid peroxidation (Kramer *et al.* 1991). Under such circumstances it is essential to scavenge H_2O_2 either by CAT or APX and GR.

Catalase was found to be sensitive to UV-B and higher dose of Cd (Cd_2) (Fig. 1*B*). UV-B-induced inactivation of CAT could also be due to its photoinactivation and degradation (Streb *et al.* 1993) due to sensitivity of its haem group. The inhibition of Cd can be explained on the basis of the following: Cd increases the production of peroxide which combines with CAT forming CAT-H_2O_2 complex before its detoxification. This complex under low concentration of peroxide forms H_2O and O_2 and at high concentration gives rise to $OH^{\cdot}$ (Wirstam *et al*. 1999). Thus Cd-induced inhibition could be a part of avoidance of the formation of $OH^{\cdot}$ and hence survival strategy of the cell.

APX showed a significant decline after UV-B_1 and GR after UV-B_2 treatment (Fig. 1*C,D*). However, Cd_2 enhanced the activity of APX (27.5 %) as well as GR (13.4 %). Although lipid peroxidation showed negative correlation with CAT activity, it was significantly correlated with SOD, APX and GR ($P < 0.05$). A decline in APX *vis a vis* GR activity following UV exposure is in accordance with the reports of Strid *et al.* (1994). This decrease could be due to decrease in reduced glutathione (GSH) pool (Fig. 1*H*), responsible for the inhibition of NADPH requiring enzymes including GR (Mallick and Rai 1999). Further, Cd_2-induced increase of APX finds support from the work of Nagalakshmi and Prasad (2001) in *Scenedesmus bijugatus* and can be explained on the basis of the report that H_2O_2 is a systemic signal for *apx* gene (Karpinsky *et al.* 1999). GR stimulation by Cd can be explained in the light of its essentiality for GSH biosynthesis. Above results depict a differential stimulation of antioxidative enzymes by UV-B and Cd and revealed that at high dose of UV-B and Cd the enzymatic machinery could not provide adequate protection as reflected by a fall in chl a content and decrease in the rate of oxygen evolution (Table 1).

UV-B significantly increased the Car content by 80 and 133.3 % ($P < 0.05$) in response to UV-B_1 and UV-B_2 treatments respectively (Fig. 1*E*). However, Cd failed to significantly affect the Car content. UV-B induced increase in Car content can be explained on the basis of the report of Campos *et al.* (1991) who observed UV-B-induced increase in 3-hydroxy-3 methyl glutaryl CoA reductase RNA responsible for the induction of Car. However, induction of Car by UV-B but not by Cd suggests a possible involvement of UV-B photoreceptor as known for *Nostoc commune* (Ehling-Schulz and Scherer 1999). α-TOC showed a 15 and 10-fold increase in its content after Cd_2 and UV-B_2 treatments, respectively (Fig. 1*G*). Increase in α-TOC appears justified and essential for the cell in view of its known involvement in the prevention of lipid peroxidation, Chl photooxidation, thymine dimerization and formation of singlet oxygen (McVean and Leiber 1999).

Ascorbate was found to be increased after all the treatments registering maximum increase by 87.5 % after UV-B_1 treatment (Fig. 1*F)*. Increase in ASA content of the test cyanobacterium following exposure to UV-B and Cd could be due to its requirement for scavenging superoxide anion, singlet oxygen, and peroxide. In aqueous phase of cell ASA can efficiently carry out regeneration of α-TOC from α-tocopheroxyl radical bound to the cell membrane (Beyer 1994). A significant ($P < 0.05$) positive correlation between ASA and α-TOC provides testimony to the above view. GSH on the other hand appeared sensitive to UV-B and showed a decline of 18.5 and 51.9 % in its content after UV-B_1 and UV-B_2 treatments (Fig. 1*H*). However, a rise of 2.6 and 2.3 fold in GSH content was observed after Cd_1 and Cd_2 treatments, respectively. Further, stimulation of GSH by Cd may be due to transcriptional activation of *gsh*1 and *gsh*2 genes coding for GSH (Xiang and Oliver 1998), which is required for phytochelatin synthesis and Cd sequestration. In addition to this, due to the presence of thiol groups, GSH can also act as a defence compound against oxidative stress. The sensitivity of GSH to UV-B could be because it absorbs in the range of 200 - 300 nm (Tyagi *et al.* 2003), leading to its oxidation.

When *A. doliolum* was exposed to the two stresses in combination, Cd-pretreated cells showed comparatively less lipid peroxidation and lesser inhibition of O_2-evolution and protein content than by UV-B alone. Further, mild stimulation of SOD, decrease in the level of inhibition of CAT, greater stimulation of APX and GR by Cd + UV-B than UV-B alone was also observed. All the non-enzymatic antioxidants showed an increase in their content; this being less than that by UV-B alone.

Above results show that both enzymatic and non-enzymatic antioxidants were stimulated under Cd stress. Contrary to this, UV-B inhibited all the studied enzymatic parameters except SOD and APX, and stimulated the non-enzymatic compounds, *e.g*., ASA, Car and α-TOC. This observation lead us to conclude that the antioxidative defence system was differentially induced by UV-B and Cd. Comparatively less lipid peroxidation, recovery of O_2-evolution, Chl and protein contents by the combination of UV-B + Cd than UV-B alone suggested Cd mediated protection of UV-B in the cyanobacterium.

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