Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (5): 90-96
Published online December 24, 2014 (http://www.cellmolbiol.com)
Received on May 13, 2014, Accepted on July 22, 2014.
doi : 10.14715/cmb/2014.60.5.15

Chromium (VI) induced oxidative stress in halotolerant alga *Dunaliella salina* and *D. tertiolecta* isolated from sambhar salt lake of Rajasthan (India)

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Abstract

Chromium (Cr) is one of the most serious pollutants in aquatic systems. This study was performed to understand the effect of Cr (VI) on halophilic algal strains of *D. salina* and *D. tertiolecta*. The results revealed good tolerance of *D. salina* towards chromium (VI) up to 8 ppm concentration, whereas tolerance level in *D. tertiolecta* was up to 2 ppm concentration. Cr (VI) not only inhibited the growth of *D. tertiolecta*, but also showed increased inhibition in the level of photosynthetic pigments, protein and carbohydrate. Results have revealed that chromium (VI) induced higher increase in lipid peroxidation and H_2O_2 production in *D. tertiolecta* than the *D. salina*, particularly at higher concentration of chromium (VI). Chromium (VI) induced increase in the rate of RNO bleaching, loss of pigments and thiol (-SH) group was relatively higher in *D. tertiolecta* than the *D. salina*, which is indicating that *D. tertiolecta* was prone to Cr (VI) induced oxidative stress. Results on RNO bleaching in the presence of radical quenchers suggested that OH° radical played an important role in the chromium (VI)-induced general oxidative stress in *D. tertiolecta*.

Key words: *Dunaliella salina*, *Dunaliella tertiolecta*, chromium, oxidative damage, SEM, FTIR.

Introduction

The sensitivity of algae to various pollutants including heavy metal stress has been thoroughly studied (1, 2). Heavy metals are known to disrupt algal metabolism either by inactivating the photosynthetic machinery, enzymatic pathways or by altering the nutrient transport and availability (3, 4). They are among the major environmental hazards due to their affinity for metal sensitive groups, such as thiol groups. Number of metals constitutes functional groups of proteins, and enzymes, if they are displaced or substitute other metals that results in to conformational changes in proteins or denaturation of enzymes and disrupt cells and organelle integrity (5).

Heavy metal stresses are also known to affect the production of active oxygen species in plants, causing oxidative stress (6, 7). The imbalance between the production of activated oxygen species and the quenching activity of antioxidant machinery setup often results in oxidative damage (6, 8). Oxygen radicals are also generated by plants during normal metabolism, and they are consumed by the intracellular defense system just for maintenance of normal growth (9). The key enzymes involved in the scavenging of oxygen radicals are the superoxide dismutase (SOD) and peroxidase which convert the superoxide radicals into non toxic radicals (10). A positive correlation has been demonstrated in the level of enzyme and oxidative stress (11).

Chromium is a highly toxic heavy metal for microorganisms and plants. Due to its widespread industrial use, chromium (Cr) has become a serious pollutant in diverse environmental settings. Chromium (VI) is one of the most common pollutants in the aquatic environment and is toxic to plants and microorganisms (12, 13). The present investigation is an attempt to study the Cr (VI) induced oxidative damage to the halophilic algal strains of *D. salina* and *D. tertiolecta*.

Materials and methods

Chemicals and reagents

All reagents used were analytical grade and used without any further purification. 1000 ppm concentrate chromium (VI) stock solution was prepared in deionized water with potassium dichromate ($K_2Cr_2O_7$). The pH of the aqueous solutions was adjusted with 1M HCl or 1M NaOH solutions.

Source of alga and growth conditions

D. salina and *D. tertiolecta* used for the present study was isolated from Sambhar salt Lake of Rajasthan, India. They were maintained in Bold Basal medium which consisted of $NaNO_3$ (2.94 mM), $MgSO_4$ (0.3 mM), $CaCl_2$ (0.17 mM), K_2HPO_4 (0.42 mM), KH_2PO_4 (1.29 mM), FeNaEDTA (0.068 mM), $NaHCO_3$ (5 mM) and NaCl (6 %), and micronutrients ; H_3BO_3 (9.7 µM), $MnSO_4$ (1.79 µM), $NaVO_3$ (0.52 µM), $ZnSO_4$ (0.15 µM), $CuSO_4$ (0.06 µM), $CoSO_4$ (0.02 µM) and $(NH_4)_6Mo_7O_{24}$ (0.003 µM). The medium was sterilized by autoclaving at 120 °C. The phosphate components were autoclaved separately and the $NaHCO_3$ solution was filter-sterilized before being added to the sterilized medium. pH of the medium was adjusted to 7.5 by adding 40 mM of Tris buffer. Cultures were kept at 25°C under white fluorescent light with an intensity of 10 Wm^{-2}.

Experimental design

To study the effect of chromium (VI) on the growth and pigments synthesis in both the species of *Dunaliella* (*D. salina* and *D. tertiolecta*), experiments were

performed in 250 ml conical flask containing 100 ml of growth medium with varying chromium (VI) concentrations (0, 2, 4, 6, 8, 10 ppm). The flasks were inoculated with the 1% *Dunaliella* culture containing 10^5 cells/ml up to 21 days. All the experiments were performed in triplicates. The sample without Cr (VI) (0 ppm) was used as the control.

Growth and pigments analysis

Growth was measured in terms of optical density at 660 nm by using UV-visible spectrophotometer (Schimadzu, Japan) (14). The photosynthestic pigments were extracted and measured by the method given by McKinney (15).

Scanning electron microscopy (SEM)

Scanning electron microscopy was used to monitor the chromium (VI) induced damage in the *Dunaliella* cells. The *D. salina* and *D. tertiolecta* cells were treated with 40 ppm concentration of chromium (VI) for 24 hrs. Cells were analyzed by using scanning electron microscope (Model No. JEOL-JSM-5610LV, Japan).

Estimation of Lipid peroxidation and Hydrogen peroxide concentration

Exponentially growing cells of *D. salina* and *D. tertiolecta* were treated with the different concentration of Cr (VI) (0, 4, 10, 20, 40, 60 ppm) for 48 h. Thiobarbituric acid reactive substances production in test samples was measured by the method of Heath and Packer (16). Absorbance of thiobarbituric acid-malondialdehyde (TBA-MDA) adduct was read at 532 nm and 600 nm. Absorbance of 600 nm was subtracted from the absorbance obtained at 532 nm, in order to correct the non-specific turbidity. The amount of MDA was calculated by using extinction coefficient of 155 mM/cm (Heath & Packer, 1968). Protein was measured by the method of Lowry *et al*. (17).

The H_2O_2 concentration was determined according to Sergiev *et al*. (18). 4.0 ml of cell suspension was homogenized with 5 mL of 10 % (W/V) TCA in an ice bath. The homogenate was centrifuged at 7000 ×g for 10 min, and the supernatant (0.5 mL) was added with 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI), and the absorbance was measured at 390 nm (UV 1800, Shimadzu, Japan). H_2O_2 was used as a standard.

Measurement of RNO (N, N dimethyl p-nitrosoaniline) bleaching

Rate of RNO bleaching in the permeaplasts of *D. salina* and *D. tertiolecta* was measured as described by Joshi and Pathak (19). The reaction was started with the addition of RNO (10 µg/ml), and change in the absorbance was read at 440 nm in the UV-Vis spectrophotometer-1601 (Shimadzu, Japan). The assay mixture was incubated in the light in the presence of graded concentrations of chromium (VI) (5, 10, 20, 40, 60 ppm). The samples were withdrawn at regular intervals (20 min) and were centrifuged (5000 g, 10 min) to obtain the cell-free supernatant. The rate of RNO bleaching was expressed in terms of $O.D._{440}$ nm/mg protein/min. The radical scavengers like histidine, sodium azide, and formate (10 mM each) was added to the assay mixture about 10 minutes prior to the addition of RNO.

Measurement of sulfhydryl (-SH) groups

The *D. salina* and *D. tertiolecta* cells were incubated in the light in the presence of different concentrations of chromium (VI) (40 ppm) for 24 h. The total -SH (sulfhydryl) contents was measured by employing the Ellman's reagent (20). Absorbance of the cell-free supernatant was read at 410 nm in a UV-Vis spectrophotom-eter (Shimadzu, Japan). The total sulfhydryl -SH) content was calculated in terms of nmol -SH groups/mg protein. A standard curve was prepared by using the known concentrations of cysteine.

Measurement of superoxide dismutase (SOD) activity

D. salina and *D. tertiolecta* cells were treated with chromium (VI) (40 ppm) for 24 h before the measurement of superoxide dismutase (EC 1.15.1.1) activity. Activity of SOD enzyme was measured by the method described by Misra and Fridovich (21). The assay mixture containing the permeaplasts of *Dunaliella* was supplemented with xanthine-oxidase (0.4 units), xanthine (0.4 mM) prepared in Tris-HCl buffer (50 mM, pH 8.0), and epinephrine (0.1mM). The mixture was light incubated. The superoxide radicals (O_2^-) generated by xanthine and the xanthine-oxidase system react with the epinephrine at pH 8.0 (10 mM, Tris-HCl buffer) to yield the adrenochrome with absorption maxima at 480 nm. Absorbance of the supernatant was read at 480 nm by using an appropriate blank. The rate of epinephrine oxidation catalyzed by xanthine and the xanthine-oxidase system in the absence of enzyme (permeaplasts) was taken as the control (100%). The enzyme causing 50% inhibition of epinephrine oxidation was taken as one unit enzyme activity.

Results

Growth analysis of algae at different concentrations of chromium (VI)

To monitor the effect of Cr (VI) on the growth of isolated *Dunaliella* strains (*D. salina* and *D. tertiolecta*), the cells were grown in presence of varying concentrations of Cr (VI) (from 0 ppm to 10 ppm) upto 21 days. Growth was monitored in terms of optical density (665 nm). Growth of *D. tertiolecta* was significantly inhibited in presence of Cr (VI), whereas no growth was observed beyond 2 ppm of Cr (VI). On the other hand, *D. salina* showed growth up to 8 ppm. *D. salina* showed the concentration dependent growth pattern, while maximum growth was observed at 0 ppm concentration.

Results showed a decline in the percent survival of *D. salina* and *D. tertiolecta* with increasing concentration of the metal. The LD-50 concentrations (50% growth inhibitory concentrations) of Cr (VI) for *D. salina* and *D. tertiolecta* were found to be 6.75 ppm and 0.96 ppm, respectively. These results indicated that cells of *D. salina* were more tolerant to Cr (VI) in comparison to *D. tertiolecta* (Figure 1).

Effect of chromium (VI) on the pigments
Effect on the chlorophyll 'a'

The effects of chromium (VI) concentrations (0-10 ppm) on photosynthetic pigments have been observed in

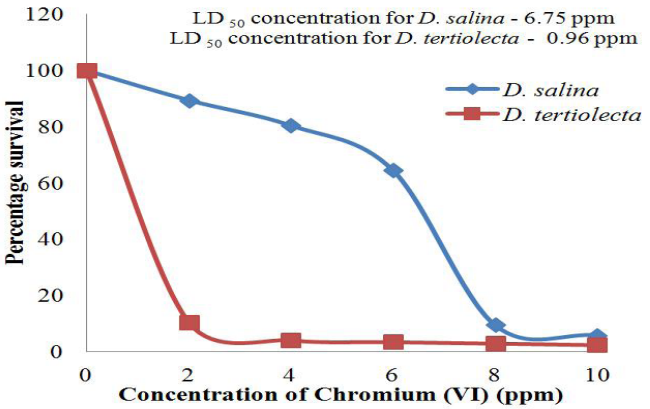

Figure 1. Effect of Chromium (VI) (0-10 ppm) on the growth of *D. salina* and *D. tertiolecta*. All the values are mean of three replicates.

both the strains of *Dunaliella*, *D. salina* and *D. tertiolecta*. Both the alga was treated with the different concentration of Cr (VI). The chlorophyll a and carotenoids were extracted during exponential phase (15th day). The results obtained with methanolic extract of cells revealed that the chlorophyll 'a' content of *D. salina* and *D. tertiolecta* gradually decreased with increasing concentration of Cr(VI) concentration when compared with the control (without metal). There was a marginal decline in chlorophyll 'a' content upto 6 ppm in *D. salina* while a steep decline in the level of Chlorophyll 'a' was observed in *D. tertiolecta* at higher concentration of Cr (VI). It has been observed that *D. salina* showed greater tolerance against Cr (VI), while *D. tertiolecta* was more susceptible (Figure 2).

Effect on the caotenoids

D. salina and *D. tertiolecta* cells were taken for the measurement of total carotenoid level in the presence of different concentration of Cr (VI) (0-10 ppm). The level of total carotenoid showed a concentration dependent decease under Cr (VI) supplemented condition. A steep decline in total carotenoid level of *D. tertiolecta* was recorded under Cr (VI) supplemented condition, throughout the concentration range. Again the carotenoid content pattern registered higher susceptibility of *D. tertiolecta* than *D. salina* to Cr (VI).

Scanning electron microscopy analysis

The selected strains of *D. salina* and *D. tertiolecta* were treated with 40 ppm concentration of chromium

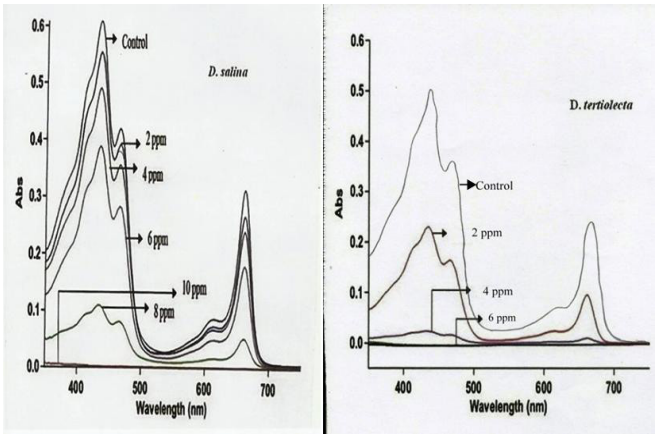

Figure 2. Effect of Chromium (VI) on the pigment production of *D. salina* and *D. tertiolecta* (after 15 days of growth) (all data presented are the mean of three replicate).

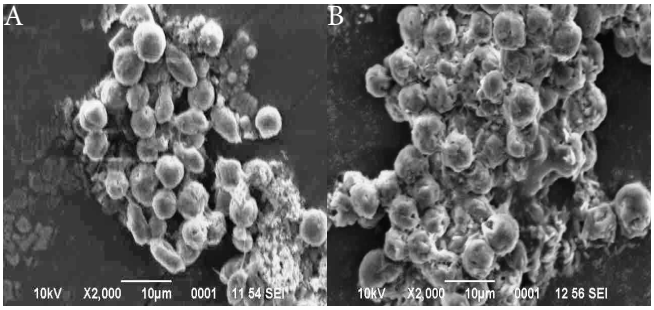

Figure 3. Scanning electron microscope analysis showing Chromium (VI) induced changes in *Dunaliella* cells. **(A)** *D. salina* control, **(B)** *D. salina* treated (40 ppm).

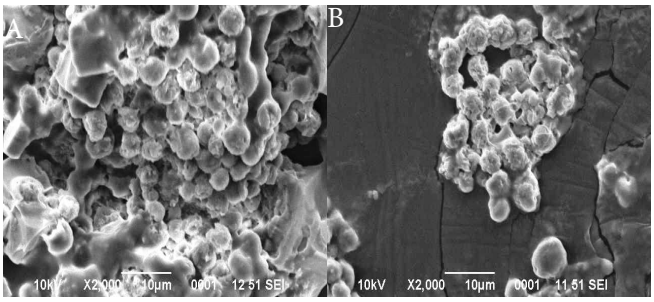

Figure 4. Scanning electron microscope analysis showing Chromium (VI) induced changes in *Dunaliella* cells. **(A)** *D. tertiolecta* control **(B)** *D. tertiolecta* treated (40 ppm).

(VI) and processed cells were analyzed by scanning electron microscopy, results obtained were compared with untreated cells (control). Results showed that both the strains (*D. salina* and *D. tertiolecta*) were damaged by selected concentration (40 ppm) of chromium (VI) when compared with untreated control cells. The cells of *D. tertiolecta* were apparently more damaged than the *D. salina*. Results also showed that *D. salina* has better tolerance for chromium (VI) than the *D. tertiolecta* (Figure 3 (a, b) and Figure 4 (a, b)).

Effect of Chromium (VI) on Lipid peroxidation and H_2O_2 production in D. salina and D. tertiolecta

Rate of lipid peroxidation (µmol MDA formed/mg protein/h) in *D. salina* and *D. tertiolecta* cells were measured in the presence of different concentration of Cr (VI). Results (Fig.5) showed that lipid peroxidation in *D. salina* and *D. tertiolecta* increased with addition of Cr (VI), which indicated a metal induced free radical generation under stressed condition. In *D. tertiolecta* an abrupt increase in the MDA formation was observed at 20 ppm concentration. A further increase in the Cr (VI) concentration resulted into reduced rate of MDA formation. On the contrary, rate of lipid peroxidation in *D. salina* gradually increased with increasing Cr concentrations (0-60 ppm). The results suggested that the higher concentration of Cr (VI) (20-60 ppm) induce the oxidative stress as evident from the results of lipid peroxidation. Therefore a study on production of H_2O_2 was necessitated. Enhanced level of H_2O_2 was noticed in the Cr (VI) treated cells of *D. salina* and *D. tertiolecta*. The rate of H_2O_2 production was higher in *D. tertiolecta* cells (Figure 5).

A sudden increase in Cr (VI) induced H_2O_2 production in *D. tertiolecta* at 20 ppm could be because of weak antioxidative defense mechanism of cells, leading to high rate of peroxidative damage. On the contrary, *D. salina* showed a steady response to Cr (VI) toxicity

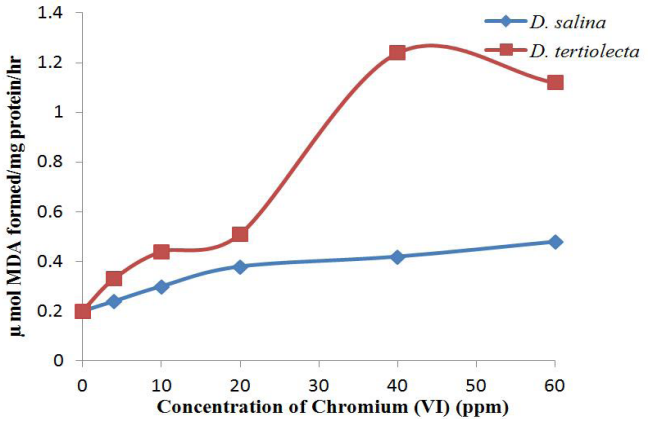

Figure 5. Effect of Chromium (VI) (0-60 ppm) on lipid peroxidation (µmol MDA formed/mg protein/hour) of *D. salina* and *D. tertiolecta*. All the values are mean of three replicates.

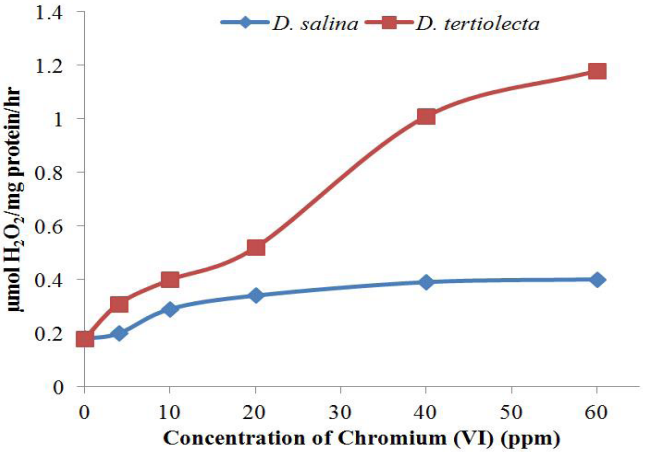

Figure 6. Effect of Chromium (VI) (0-60 ppm) on H_2O_2 production (µmol H_2O_2 formed/mg protein/hour) of *D. salina* and *D. tertiolecta*. All the values are mean of three replicates.

throughout the concentration range of Cr (VI) (Figure 6).

Effect of chromium (VI) on RNO bleaching

Rate of N,N-dimethyl 4-nitrosoaniline (RNO) bleaching (ΔOD 440 nm/mg protein/min) was measured in the presence of varying concentrations of chromium (VI) (0-60 ppm) in *D. salina* and *D. tertiolecta*. Results showed chromium (VI) induced, concentration-dependent steep rise in the rate of RNO bleaching up to 40 ppm concentration of chromium (VI) in case of *D. tertiolecta*, and thereafter it was followed by a slight decline in rate of bleaching above it. Same pattern was followed by *D. salina* also but the rate of RNO bleaching was stimulated in *D. tertiolecta* in comparison with *D. salina* (Figure 7).

Effect of oxygen radical quenchers on RNO bleaching

RNO bleaching was measured in the *D. salina* and *D. tertiolecta* cells under chromium (VI) (40 ppm) treated and untreated conditions. The radical quenchers like sodium azide, formate, histidine (10 mM each) were used in the present study. Results obtained with radical quenchers showed a more pronounced quenching effect of formate- a hydroxyl (OH°) radical quencher on RNO bleaching. The effect of histadine (O_2^- radical) and sodium azide a singlet oxygen ($^1O_2$) quencher on the rate of RNO bleaching were less pronounced in both the strains of *Dunaliella*, D. *salina* and *D. tertiolecta*. It

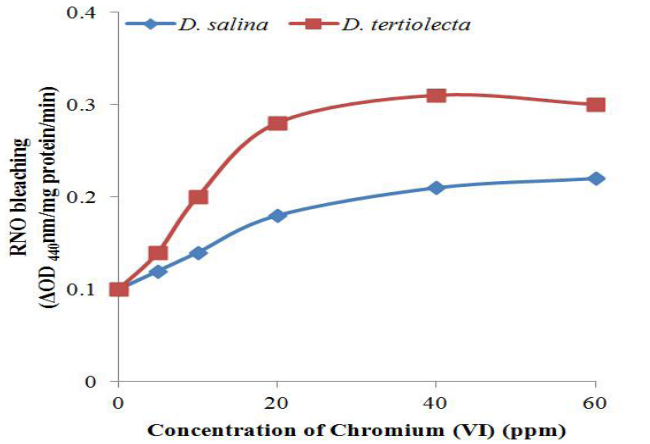

Figure 7. Effect of Chromium (VI) (0-60 ppm) on the rate of RNO bleaching ($\Delta O.D._{440}$ nm/mg protein/min) in *D. salina* and *D. tertiolecta.*

was observed that formate showed highest quenching effect in *D. tertiolecta* (66.66%) than in the *D. salina* (76.19%), while histadine and sodium azide showed slight more quenching effect in *D. tertiolecta* than in the *D. salina* (Table. 1).

Effect of chromium (VI) on superoxide dismutase (SOD) activity, sulfhydryl (-SH) content and carotenoids content

D. salina and *D. tertiolecta* cells treated with (40 ppm) concentrations of chromium (VI) for 24 h were used for measurement of the total -SH content. The results indicated a decrease in the total -SH content of the cells of *D. salina* and *D. tertiolecta*, but decrease in the total -SH content was more in case of *D. tertiolecta* than the *D. salina*. This suggested that oxidative damage to *D. tertiolecta* is an important mode of chromium (VI) injury.

D. salina and *D. tertiolecta* were treated with (40 ppm) concentration of chromium (VI) for 24 h before the cells were used for measurement of SOD activity. Rate of superoxide dismutase (SOD) activity in *Dunaliella* cells measured in terms of epinephrine oxidation (ΔOD_{480} nm/mg protein/min) revealed that SOD activity declined in the presence of chromium (VI) in both the strains of *Dunaliella*, *D. salina* and *D. tertiolecta*. However, the chromium (VI) induced decline was found to be higher in case of *D. tertiolecta* (Table. 2).

However the same results were obtained with the carotenoids synthesis, the decrease in the carotenoids synthesis (in the Cr (VI) treated cells) was much higher in *D. tertiolecta* than the *D. salina*.

Discussion

Photosynthetic organisms are highly sensitive to metal compounds. The effect of metal ions on higher plants includes the disruption of many physiological functions such as water uptake, respiration, mineral nutrient uptake and photosynthesis (22).

The results revealed good tolerance of *D. salina* towards chromium (VI) up to 8 ppm concentration, whereas tolerance level in *D. tertiolecta* was up to 2 ppm concentration. Growth of *D. tertiolecta* was significantly reduced in presence of Cr (VI). These results are in agreement with Thapar *et al.,* (23), who showed re-

Table 1. Effect of radical quenchers on RNO bleaching ($\Delta O.D._{440}$ nm/mg protein/min). The values are the mean of three independent observations.

Radical quenchers	RNO bleaching ($\Delta O.D._{440}$ nm/mg protein/min)	
	D. salina	***D. tertiolecta***
Control (without Cr (VI)	0.41	0.52
With 40 ppm Cr (VI)	0.63(100%)	0.78(100%)
Chromium (VI) (40 ppm) + Formate	0.48 (76.19%)	0.52 (66.66%)
Chromium (VI) (40 ppm) + Histadine	0.54 (85.71%)	0.69 (88.46%)
Chromium (VI) (40 ppm) + Sodium azide	0.58 (92.06%)	0.71 (91.02%)

The values given in the brackets indicate the percentage activity in relation to the control.

Table 2. Table showing the effect of Chromium (VI) on parameters of oxidative stress in *D. salina* and *D. tertiolecta*.

Parameters	**Without Cr (VI)**		**With Cr (VI) (40 ppm)**	
	D. salina	***D. tertiolecta***	***D. salina***	***D. tertiolecta***
Lipid per oxidation (μmol MDA formed/mg protein/hr)	0.21	0.20	0.38	1.08
RNO bleaching	0.41	0.52	0.63	0.78
Superoxide dismutase (SOD) (ΔOD/mg protein/min)	4.9	3.7	3.8	1.1
Total –SH group content (nmol –SH group/mg protein/min)	13.6	12.8	13.1	11.1

duced growth of A*nabaena doliolum* in the presence of Cd. The *Synechocystis aquatilis* also showed a decrease in growth with increase in the copper ion concentration (24).

A Cr (VI) concentration dependent decrease in both chlorophyll 'a' and carotenoids was observed in *D. salina* and *D. tertiolecta* cells. The results on the effect of exposure time showed that chlorophyll 'a' content of *D. salina* and *D. tertiolecta* gradually decreased with increase in the exposure time as well as metal concentration when compared with the control (without metal). The Cr (VI) induced damage in the level of carotenoid content indicated higher susceptibility of this pigment in *D. tertiolecta* than *D. salina*. The Cr (VI) induced reduction in the chlorophyll 'a' content of both the strains may be due to inhibition of chlorophyll biosynthesis brought about by inhibition of d-aminolevulinic acid dehydrogenase and protochlorophyllide reductase (25).

Scanning electron microscopic (SEM) analysis also showed Cr (VI) induced greater damage to the *D. tertiolecta* cells than *D. salina* cells when treated with 40 ppm concentration of chromium (VI). Thus the foregoing results indicated that the species *D. salina* was more tolerant to chromium as compared to *D. tertiolecta.*

A concentration dependent steady decline in the total carbohydrate and protein content of *D. salina* was found throughout the concentration range, while a sharp decline was observed in *D. tertiolecta* in presence of Cr (VI). Thus, it was concluded that carbohydrate and protein synthesis in *D. tertiolecta* was more susceptible to Cr (VI) than *D. salina*. Metal stress induced inhibition in growth and biomolecule synthesis of *D. tertiolecta* might be due to damage of the cellular constituents or inactivation of vital processes such as nutrient uptake, enzyme activities and photosynthesis as observed by Altamirano *et al.,* (26) in *Ulva rigida* and Thapar *et al.,* (23) in *Anabaena doliolum.* Lamai *et al.,* (27) also observed significant decrease in the relative growth and total chlorophyll content with increasing exposure time and concentration.

The heavy metal induced enhanced photo-susceptibility of the *D. tertiolecta* than *D. salina* cells under the metal stress may be due to involvement of the toxic oxygen radicals. Results have also revealed chromium (VI) induced increase in the rate of lipid peroxidation and H_2O_2 production, particularly at higher concentration of chromium (VI). Similar results on metal stress indicated MDA production was obtained in case of higher plants, indicative of oxidative stress (28, 29). Further, chromium (VI) induced high increase in the rate of RNO bleaching, loss of pigment and thiol (-SH) groups in *D. tertiolecta*, were indicative of relatively high intensity oxidative stress than that in *D. salina*. Results obtained with radical quencher demonstrated that formate- a known OH radical quencher (30) has more pronounced inhibitory effect on RNO bleaching than histadine and sodium azide as observed in *D. tertiolecta*. These results suggested that OH radical plays a more predominant role in the chromium (VI) induced general oxidative stress (30).

In view of the foregoing results, it was deduced that Cr (VI) at high concentration is capable of inducing a general oxidative stress, perhaps mediated by OH° radicals. The Cr (VI) induced stress was more pronounced in the *D. tertiolecta* than *D. salina*, causing overall damage to the cells.

Other articles in this theme issue include references 31-46).

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