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Effects of Chromium VI stress on green alga *Ulva lactuca* (L.)

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Abstract: In order to contribute to the understanding of its response to metal stress, *Ulva lactuca* (L.) was treated with different concentration of chromium (VI) (0.2, 0.5, 1, and 5 mM K_2CrO_4), and alterations in Fv/Fm ratio (maximal photochemical efficiency of PSII) and cell viability were investigated. Significant alterations of the maximal photochemical efficiency of PSII (Fv/Fm) ratio were observed in response to the increase in chromium concentration. The Fv/Fm ratio decreased in *Ulva lactuca* following 2 h treatment with 1 and 5 mM Cr(VI) solution. The cell viability was decreased in cells exposed to higher Cr(VI) concentrations. Thallus cell morphology was altered in thalli exposed to chromium. A cytotoxic effect of chromium was demonstrated for the first time using acridine orange/ethidium bromide staining in macroalgae. In 1 and 5 mM of Cr(VI) treated samples, the rate of necrotic cells increased about 76.93% and 84.23%, respectively.

Key words: Chromium VI, photosynthesis, *Ulva lactuca*, acridine orange/ethidium bromide staining

Yeşil Alg *Ulva lactuca* (L.) üzerinde Krom (VI) Stresinin Etkileri

Özet: Farklı konsantrasyonlarda krom (VI) (0,2, 0,5, 1 ve 5 mM K_2CrO_4) uygulaması yapılan *Ulva lactuca* (L.)'da metal stresine yanıt olarak PSII fotosentetik kuantum verimi, hücre canlılığı, ve sitotoksik etkiler üzerindeki değişimler araştırılmıştır. Krom konsantrasyonundaki artışa bağlı olarak fotosentetik kuantum verim (Fv/Fm) oranında önemli ölçüde değişimler olduğu gözlenmiştir. U. lactuca'nın 2 saat, 1 ve 5 mM Cr(VI) uygulanmasını takiben Fv/Fm oranı düşmüştür. Evan's blue alınım sonuçları ise 1 ve 5 mM Cr(VI) uygulanmış örneklerde hücre ölümünde azalmanın olduğunu göstermiştir. Tüm deney gruplarında thallus hücre morfolojisinde değişim olduğu belirlenmiştir. Makro alglerde ilk kez acridine orange/ethidium bromide boyama tekniği kullanılarak, sitotoksik etki gösterilmiştir. 1 ve 5 mM Cr(VI) uygulanan örneklerde nekrotik hücre yüzdesinin, sırası ile % 76,93 ve % 84,23, oranında artığı da gösterilmiştir.

Anahtar sözcükler: Krom (VI), Fotosentez, *Ulva lactuca*, acridine orange/ethidium bromide boyama

Introduction

Chromium is a highly toxic non-essential metal for plants that inhibits a variety of metabolic activities in plants (1). Chromium (VI) is considered to be the most toxic form of Cr, which usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxy-anions. Forms of chromium in industrial wastewater are highly variable; one particular form is hexavalent pigments. Film and photography, galvanometry and electrical procedures,

metal cleaning, plating and electroplating, leather and mining contain undesirable amounts of Cr(VI) according to the water standards (2).

Generally, heavy metal toxicity is related to oxidative damage, which can promote both with direct increase in cellular concentration of reactive oxygen species (ROS) and decrease in cellular antioxidant capacity (3,4). ROS can be harmful to organisms at high concentrations. They can oxidize protein, lipids, and nucleic acids, thus cause alterations in cell structure and mutagenesis (5,6).

The toxic effects of Cr(VI) in plants are primarily dependent on the metal speciation, which determines its uptake, translocation, and accumulation (7) but photosynthesis is clearly an important target of its toxicity (8,9,10). Hexavalent form of Cr has been shown to generate hydroxyl radicals from hydrogen peroxide to replace Mg ion from the active site of many enzymes (11). Cr phytotoxicity can result in degradation of pigment contents, nutrient balance, enhancement of antioxidant enzymes activity, and induction of oxidative stress in algae (12,13). Different species of the genera *Ulva* are considered to be good bio-indicators of metal contamination (14). The laminar structure of *Ulva lactuca* leads to a relatively high surface area: volume ratio and the thallus cells are structurally uniform and physiologically active. In addition, the cosmopolitan nature of the alga and its capacity to grow in heavily contaminated areas increase its potential as a useful bio-indicator (15). Despite the many publications dealing with heavy metal accumulation in *Ulva* sp., there is little information about the effects of chromium toxicity on physiological parameters. In the present study, we investigated the chromium uptake, toxic effects of Cr(VI) on Photosystem II, and effects of cytotoxicity.

Materials and method

Materials and experiment design

Ulva lactuca L. samples were collected from İzmir Bay and kept in ice during transportation to the laboratory. After extensive washing with autoclaved artificial seawater (500 mM NaCl, 10 mM KCl, 30 mM $MgSO_4$, 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.8), the samples were incubated for 2 h in solutions of K_2CrO_4 (0.2 mM, 0.5 mM, 1 mM, and 5 mM in artificial seawater medium) and compared with control samples soaked in artificial seawater medium. Each treatment was comprised of 3 replicates. The photon irradiance was 150 mW/cm^{-2} at 20 °C.

Chlorophyll a fluorescence

Chlorophyll a fluorescence of samples was measured with a plant efficiency analyzer (Handy PEA, Hansatech). Before the measurements of chlorophyll fluorescence were taken, thalli were dark-adapted for 15 min. The Fv/Fm parameter was calculated by the instrument from fluorescence induction curves of 5 s durations recorded at an irradiance of 1800 μmol m^{-2}s^{-1} from light emitting diodes. The Fv/Fm parameter (maximal photochemical efficiency of PSII) was used as a stress indicator.

Chromium uptake

For the determination of Cr(VI) content, samples of 1 g of thalli were pulverized with liquid nitrogen. The powder was dried for 24 h at 105 °C. Samples of 250 mg were digested in 10 mL concentrated pure HNO_3 (Merck) in test tubes of 50 mL in a heating block for 8 h at a temperature of 120 °C. Chromium content was measured by ICP-OES.

Assesment of the integrity of cell membranes

Batches of thalli of *U. lactuca* were divided into samples of 1 g and immersed in 100 mL of double-distilled water for 60 min. The electric conductivity (EC) of the water was measured by an electric conductivity meter (WTW Cond 340i).

Analysis of cell viability

The Evan's staining method (16) was used to quantify cell death. Thalli were stained in 0.25% aqueous Evan's blue solution for 15 min at room temperature, and washed twice for 15 min with distilled water to remove the excess stain. For quantitative assessment, the dye was extracted by boiling in a solution of 50% methanol/1% SDS for 1 h at 50 °C. The Evan's blue was measured at 600 nm. Each treatment was comprised of 3 replicates.

Tissue section preparation

The thallus was cut into small pieces and then fixed with FAA (50% ethanol, 5% glacial acetic acid, 3.7 % formaldehyde, freshly prepared). The sections

were incubated in FAA solution overnight. The fixed samples were dehydrated through an ethanol series (50%, 70%, 80%, 95%, and 100% for 15 min each concentration). The dehydrated samples were immersed in ethanol-xylene (1:1) for 10 min and then in 100% xylene for 10 min. The samples were embedded in paraffin, and cut to 5 μm sections with Lecia RM 2145 microtome.

Analysis of anatomical structure

For anatomical studies, a sections (5 μm thick) stained with hematoxylin were examined for morphological changes under a light microscope (Olympus BX-51). Measurements of anatomical features were made in 25 different areas of section of *U. lactuca* thallus. Anatomical features were assessed by light microscopy (Olympus BX-51 light microscope, Olympus C-5050 digital camera) at a magnification of ×100. Abnormal cells were counted using image analysis software (Image-Pro Express 4.5, Media-Cybernetics Inc., USA).

Fluorescence microscopy

Cell damage was assessed by monitoring changes in cell granularity by staining with a combination of the fluorescent DNA-binding dyes acridine orange and ethidium bromide that allowed a quantitative determination of the cells (17). Sections were stained with 0.01% (w/v) acridine orange and 0.01% (w/v) ethidium bromide in 10 mM PBS buffer (pH 7.0) for 10 min. Using this double staining, 2 types of nuclei were identified: (1) green nuclei in non-damaged cells and (2) necrotic cells with a uniformly orange to red nucleus with an organized structure. The slides were examined using an Olympus BX-51 light microscope equipped with blue fluoresce filters and photographed using an Olympus C-5050 digital camera. Necrotic formations were assessed by fluorescence microscopy at a magnification of ×100. Green or orange nuclei were counted in the algae thallus using image software (Image-Pro Express 4.5, Media-Cybernetics Inc., USA). Experiments were repeated 3 times and hundreds of tissue sections were analyzed.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, including one-way analysis of variance (ANOVA), Student's t test and Tukey.

Results

Chromium uptake

The concentration of Cr(VI) in the untreated thalli (control) was 8.91 μg/g dw for *U. lactuca.* The concentration of Cr (VI) in thallus treated with 0.2, 0.5, 1, and 5 mM thalli were 10.16, 36.44, 58.4, and 78.28 μg/g dw, respectively (Table 1).

Activity of PSII

A gradual reduction in Fv/Fm ratio with increasing Cr(VI)-concentrations was observed ($P<0.05$). The obtained values of Fv/Fm of treated with 1 mM and 5 mM of Cr(VI) samples were approx. 0.3 and 0.1, which indicated severe inhibition of photosynthetic processes by Cr (Table 1).

Table 1. Effects of different concentrations of chromium on Fv/Fm ratio and electrical conductivity of U. lactuca. Each value represents the mean of 3 replications ± SD.

Groups	Cr(VI) content (μg/gdw) X ± SD	Fv/Fm X ± SD	EC (μScm^{-1}) X ± SD
Seawater medium	8.91 ± 0.06	0.771 ± 0.01	1.8 ± 0.14
0.2 mM K_2CrO_4	10.16 ± 0.12	0.736 ± 0.04	2.06 ± 0.33
0.5 mM K_2CrO_4	36.44 ± 1.03	0.721 ± 0.06	4.78 ± 0.53
1 mM K_2CrO_4	58.4 ± 0.08	0.331 ± 0.05	10.82 ± 1.08
5 mMK_2CrO_4	78.28 ± 1.65	0.197 ± 0.07	15.03 ± 1.22
ANOVA			
F ratio	44.42	54.82	137.48
F probability	0.0002	0.0003	0.000

n = number of replicates, x = mean values, SD = standard deviation.

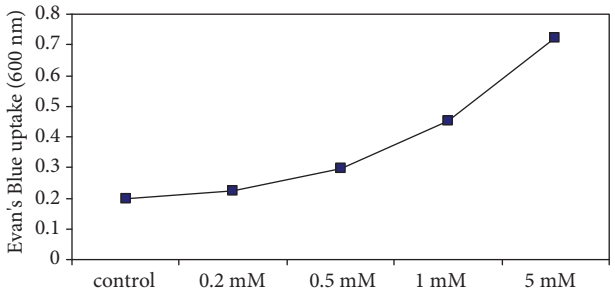

Figure 2. Acridine orange/ethidium bromide staining of the sections of U. lactuca, control group (**A**), treated with 0.2 mM Cr(VI) (**B**), treated with 0.5 mM Cr(VI) (**C**), treated with 1 mM Cr(VI) (**D**), and treated with 5 mM Cr (VI) (**E**). Acridine orange/ethidium bromide staining, original magnification ×40.

Cell viability and integrity of membrane

The cell death in thallus of *U. lactuca* exposed to 1 mM and 5 mM of Cr(VI) significantly increased to about 35.4% and 62.7% compared to the control ($P<0.05$). Similarly, high values of electric conductivity (EC) were observed in thalli treated with 1 mM and 5 mM concentration of Cr(VI) (Table 1). At lower concentrations the EC values were not significantly different from the group treated with water (EC value of control and 0.2 mM of Cr(VI)-treated samples were 1.8 and 2.06 μS cm^{-1}, respectively).

Change in anatomical structure

In the samples treated with 0.2 and 0.5 mM Cr(VI), abnormal cells constitute 19% and 34.64% of the total thallus cells. When treated with 1 and 5 mM of Cr(VI), the percentage of abnormal cells was 64.73% and 93.52%, respectively.

Fluorescence microscopy

The results obtained with acridine orange/ethidium bromide (AO/EB) double staining are presented in Figure 2. It showed that the percentage of necrotic cells of *U. lactuca* thalli was significantly modified by the Cr(VI) treatments. Thallus of *U. lactuca* was stained as brilliant green/orange in control and 0.2 and 0.5 mM of Cr(VI)-treated samples. Concering the groups that received Cr(VI) treatment, in the samples that receivied 0.2 and 0.5 mM treatment, necrotic cell number in thallus was 18.56% and 38.9%, respectively, whereas necrotic cell number was determined to be 76.93% and 84.23% in the groups that received 1 and 5 mM treatment, respectively (Table 2).

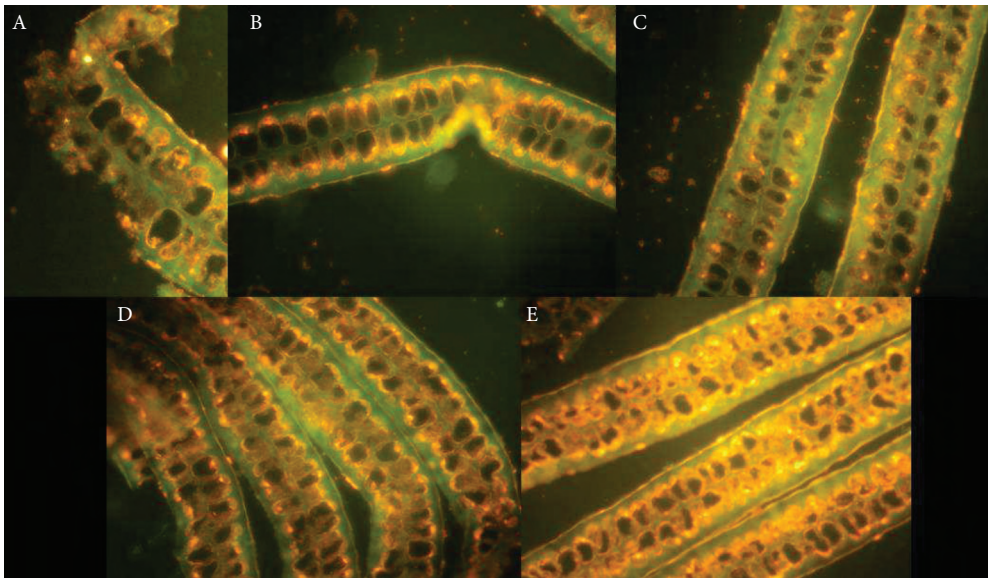

Figure 1. Influence of chromium treatments on cell viability assessed by staining with Evan's blue.

Table 2. Phytotoxic effects of high chromium concentrations on *U. lactuca* L. thalli cells. Each value represents the mean of 3 replications ± SD.

Groups	deformed cell % X ± SD	necrotic cell % X ± SD
Seawater medium	3.6 ± 0.08	5.5 ±1.23
0.2 mM K_2CrO_4	19.17 ± 7.13	18.55 ± 4.22
0.5 mM K_2CrO_4	34.64 ± 0.55	38.88 ± 3.42
1 mM K_2CrO_4	64.73 ± 6.29	76.93 ± 2.31
5 mMK_2CrO_4	93.52 ± 1.84	84.23 ± 3.72
ANOVA		
F ratio	71.33	146.29
F probability	0.0006	0.000

x = mean values, SD = standard deviation.

Discussion

Maximal photosystem II efficiency (Fv/Fm ratio) is frequently used to monitor stress in photosynthetic organisms. Under non-stressed conditions, higher plants, lichen, and algae typically possess Fv/Fm in the range of 0.78-0.87, 0.45-0.65, and around 0.66-0.8, respectively (18,19,20). Exposure of the thalli to 0.2 and 0.5 mM of Cr(VI) did not result in significant reduction in Fv/Fm. However, exposure to high Cr(VI) (1 and 5 mM) resulted in significant decrease in Fv/Fm ratio. Similarly, Hörcsik et al (21) showed that in algae *Chlorella pyrenoidosa*, quantum yield of PSII reaction centre reduced by Cr(VI) and this inhibitory effect increased with increasing concentration of Cr (VI). Hörcsik et al. (21) also found that inhibition of PSII activity can be accounted for by the enhanced photodestruction of the reaction centers in the cells cultivated in the presence 20 g m^{-3} of Cr(VI) using chlorophyll fluorescence transients, thermoluminescence, oxygen polarography, and Western blot analysis for D1 protein. The concentration of Cr(VI) in the untreated thalli (control) was 8.9 µg/g dw for *U. lactuca*. An increase in exposure to 0.2 and 0.5 mM of Cr(VI) content up to 10.2 and 36.4 µg/g dw had no effect on the Fv/Fm ratio of *U. lactuca* thalli, respectively (Table 1). However when the Cr(VI) concentration exceeded 58.4 µg/g dw a marginal reduction in Fv/Fm ratio was observed and the reduction in Fv/Fm ratio was much higher when the thallus Cr(VI) concentrations was 78.28 µg/g dw (Table 1).

The effects of excess Cr(VI) on the viability of *U. lactuca* thalli were examined in order to assess the degree of Cr toxicity. The Evan's blue was excluded from viable cells that retain intact plasma membranes, whereas those with damaged membranes incorporated the dye (16). Exposure of thalli to 0.2 and 0.5 mM of Cr (VI) had no significant effect on cell viability as compared to the control (Figure 2). However, accumulation of high concentration of Cr(VI) in thallus significantly reduced the cell viability. EC is considered to indicate injury to cell membranes and has been previously used for assessment of electrolyte leakage (22). Significantly high values EC were observed in thalli treated with the highest concentration of the Cr(VI) (Table 2). At lower Cr(VI) concentrations, the differences in EC values were not significant.

Our results showed that morphology of *U. lactuca* cells treated with 0.5, 1, and 5 mM Cr(VI) was significantly affected. Rodriquez et al. (4) showed that morphology of *Chlamydomonas* sp. cells affected by increasing concentrations of Cr. In *Chlamydomonas* sp., the exposure to 10 µM Cr caused an acute effect and damage to cellular compounds (4). Our results obtained with acridine orange/ethidium bromide (AO/EB) double staining showed that the number of necrotic cells significantly increased by the Cr(VI) treatments. Thalli of *U. lactuca* was stained as brilliant green in control and 0.2 and 0.5 of Cr(VI)- treated samples (Figure 2). These results showed that thalli of *U. lactuca* showed tolerance against Chromium stress. However 1 and 5 mM of Cr(VI) concentration caused cytotoxicity. In the present study, the number of necrotic cells increased in 1 mM and 5 mM application samples (Figure 2). These results showed that DNA damage occurred high concentrations of Cr(VI).

Although cell and DNA damage occurred at a very low rate, photosynthetic yield was not affected by Cr(VI) stress. These data indicate that *U. lactuca* has tolerance to Cr(VI) at low concentrations (0.2 and 0.5 mM of Cr). In addition, 0.2 and 0.5 mM of Cr concentration is very high compared to other studies on microalgae. For example *Chlorella* sp. (23),

Dunaliella sp. (24), and *Euglena gracilis* (25) can tolerate a much higher value, but these organisms are sensitive to our application concentration. Our results also indicated for the first time that 58.40 and 78.28 μg/g dw concentration of Cr(VI) resulted in formation of abnormal and necrotic cells. In the future, our aim is to explore the extent of DNA damage caused by Cr(VI) stress and find a repair mechanism.

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