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Effects of chromium toxicity on leaf photosynthetic characteristics and oxidative changes in wheat (*Triticum aestivum* L.)

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Abstract

Cr(VI) significantly reduced rates of net photosynthesis and transpiration and of stomatal conductance. Cr(VI) did not affect the F_v/F_m ratio of chlorophyll fluorescence implying that the primary photochemical processes in photosystem 2 were not affected. However, the efficiency of excitation capture by open PS2 centres, *in vivo* quantum yield of PS2 photochemistry, and electron transport rate were significantly reduced by Cr(VI). The coefficient of photochemical quenching was reduced with a concomitant increase in coefficient of non-photochemical quenching, suggesting reduced demand for ATP and NADPH due to inhibition of CO_2 assimilation. Lipid peroxidation was increased by Cr(VI) and the activities of superoxide dismutase and catalase (CAT) were increased. However, the CAT activity was reduced by high Cr(VI) concentration. The activities of ascorbate peroxidase and glutathione reductase were significantly reduced by Cr(VI) treatment.

Additional key words: antioxidants; catalase; ascorbate peroxidase; chlorophyll fluorescence; glutathione reductase; lipid peroxidation; photochemical and non-photochemical quenching; photosystem 2; superoxide dismutase.

Introduction

Chromium (Cr) is fairly abundant in the earth's crust and ranks fourth among the 29 elements of biological importance. Chromium is one of the heavy metals that have nutritional importance (Kabata-Pendias and Pendias 2001). Due to industrial activities, large quantities of Cr compounds are discharged in liquid, solid, and gaseous wastes into environment and ultimately have significant adverse biological and ecological effects (Kotas and Stasicka 2000). Tanning and chrome-plating industries are prominent sources of Cr in environment. Cr(VI) is highly reactive form that influences both plants and animals. Due to Mn present in soil, Cr(III) is oxidized to Cr(VI) which remains in soil for a long time and can affect plant growth and development (Sharma *et al*. 2003). Chromium, in contrast to other toxic trace metals like cadmium, lead, mercury, and aluminium, has received little attention from plant scientists (Shankar *et al*. 2005). Toxic effects of Cr on plant growth and development include alterations in the germination process as well as in the growth of roots, stems, and leaves, which may affect total dry matter (DM) production and yield. Cr also causes deleterious effects on plant physiological processes such as photosynthesis, water relations, and mineral nutrition. Metabolic alterations by Cr exposure have also been described in plants either by a direct effect on enzymes or other metabolites or by its ability to generate reactive oxygen species which may cause oxidative stress and increased lipid peroxidation (Shankar *et al*. 2005, Sinha *et al*. 2005, Montes-Holguin *et al*. 2006). Chromium affects photosynthesis in terms of CO_2 fixation, electron transport, photophosphorylation, and enzyme activities (Clijsters and Van Assche 1985, Hörcsik *et al*. 2007). Reductions in contents of chlorophyll (Chl) and δ-aminolevulinic acid and nitrate reductase activity were reported by Vajpayee *et al*. (2000). Disorganization of chloroplast ultrastructure and inhibition of electron transport processes due to Cr and diversion of electrons from the electron-donating side of photosystem (PS) 1 to Cr(VI) is a possible explanation for Cr-induced decrease in photosynthetic rate (Shankar *et al*. 2005). The overall effect of Cr ions on photosynthesis and excitation energy transfer could also be due to Cr(VI)-induced abnormalities in the chloroplast ultrastructure like poorly developed lamellar system with widely spaced thylakoids and fewer grana (Van Assche and Clijsters 1983). I investigated the effect of excess

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Cr content on wheat leaf photosynthesis using leaf gas exchange and Chl fluorescence measurements and oxidative response of wheat.

Materials and methods

Seeds of wheat (*Triticum aestivum* cv. HD 2329) were surface sterilized with 0.1 % $HgCl_2$, washed with distilled water, sown in plastic pots containing acid washed sand, and irrigated with normal tap water. After germination only 2 seedlings per pot were allowed to grow and the remaining seedlings were removed. The plants were irrigated with full strength Hoagland's nutrient solution. At 25 d after germination, the nutrient solution was supplemented with different concentrations of Cr (0, 0.10, 0.15, 0.25 mM) supplied as $Na_2Cr_2O_7 \times 2\ H_2O$ and the plants were allowed to grow for 20 d. During the period after Cr treatment leaf photosynthetic traits and fluorescence characteristics were measured at regular time intervals. After 20 d of Cr treatment the plants were harvested, dissected into different parts, and dried in an oven at 70 °C for DM determination. Three plants from excess Cr and control treatments were separated into roots and leaves-shoot and dried in a hot-air oven set at 70 °C for 48 h. The Cr content in plant parts was determined by atomic absorption spectrophotometer after wet digestion with 4 : 1 (v/v) of HNO_3 : $HClO_4$.

Leaf gas exchange was measured using a *LI-6400* portable photosynthesis measuring system (*LICOR*, USA). Leaves were irradiated by *6400-02B* LED source providing a photosynthetic photon flux density (PPFD) of 1 200 μmol m^{-2} s^{-1} and the temperature was maintained at 25 °C, relative humidity at 70 %, and CO_2 concentration at 350 g m^{-3}.

Chl fluorescence was recorded with a portable pulse amplitude modulation fluorometer (*PAM 2000*, *Walz*, Effeltrich, Germany). The minimal (F_0) and maximal (F_m) fluorescence were recorded after pre-darkening for 10 min on attached leaf on which the gas exchange was previously measured. The plants were then exposed to natural sunlight for approximately 6 min to induce photosynthesis. The minimal fluorescence level in light-adapted state (F_0') was determined by using far-red pre-irradiation. From fluorescence parameters determined on both irradiated and dark-adapted leaves the following parameters were calculated: the maximal quantum yield of PS2 photochemistry F_v/F_m, the photochemical quenching coefficient $q_P = (F_m' - F_t)/(F_m' - F_0')$, non-photochemical quenching coefficient $q_N = 1 - (F_m' - F_0')/(F_m - F_0)$, the efficiency of excitation capture by open PS2 centres $\Phi_e = F_v'/F_m'$, *in vivo* quantum yield of PS2 photochemistry $\Phi_{PS2} = (F_v'/F_m'\ q_P)$, and apparent photosynthetic electron transport rate ETR = Y × PAR × 0.5 × 0.84. Yield Y represents the overall photochemical quantum yield [$Y = (F_m' - F_t')/F_m'$]. PAR is the flux density of incident photosynthetically active radiation [μmol(quantum) m^{-2} s^{-1}]. Transport of one electron requires absorption of two quanta, as two photosystems are involved (factor 0.5). It is assumed that 84 % of the incident quanta are absorbed by the leaf (factor 0.84).

Lipid peroxidation and activities of antioxidant enzymes were measured at 0, 8, and 14 d after the plants were subjected to Cr(VI) stress. Wheat shoots (0.5 g) were homogenized in ice cold 5 cm^3 of 50 mM phosphate buffer (pH 7.8). The homogenates were centrifuged at 14 000×*g* for 20 min. The supernatants were used for the measurement of enzyme activity and malondialdehyde (MDA) content. Superoxide dismutase (SOD), catalase (CAT), and MDA contents were estimated according to the method used by Guo *et al*. (2005). One SOD enzyme unit was defined as the amount of enzyme required for inhibition of photochemical reduction of nitroblue tetrazolium (NBT) by 50 %. One unit of CAT was defined as amount of enzyme needed for conversion of one micromole of H_2O_2 into water per minute. The concentration of MDA was calculated by its extinction coefficient of 155×10^6 cm^2 mol^{-1}, after subtracting non-specific absorbance at 600 nm (Dhindsa *et al*. 1981).

Ascorbate peroxidase (APX) activity was measured according to the method of Nakano and Asada (1987). Plant tissue was ground in 3 cm^3 of extraction solution containing 50 mM phosphate buffer (pH 7.8), 2 mM ascorbic acid, and 5 mM EDTA at 4 °C in a pre-chilled glass mortar. The homogenate was centrifuged for 15 min at 14 000×*g*. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, and 0.1 cm^3 of extract. The reaction was initiated by addition of H_2O_2. The APX activity was measured by the decrease in absorbance of 290 nm within 1 min and calculated using an extinction coefficient of 2.8×10^6 cm^2 mol^{-1} for ascorbic acid.

Glutathione reductase (GR) activity was measured as described by Gamble and Burke (1984). Leaf (0.3 g) was extracted in 3 cm^3 of 0.1 M Tricine-NaOH (pH 7.8). The homogenate was centrifuged for 15 min at 14 000×*g*. The 1 cm^3 of reaction mixture contained 50 mM Tricine-NaOH (pH 7.8), 0.5 mM glutathione (GSSG), 0.1 mM NADPH, and 0.2 cm^3 of extract. GSSG was replaced by water in the blank. The oxidation of NADPH (extinction coefficient 6.22×10^6 cm^2 mol^{-1}) was monitored at 340 nm for 2 min. The data was subjected to statistical analysis as per completely randomized design using in-house developed software with 4 replications.

Results

Toxic concentrations of Cr significantly reduced DM production in different plant parts of wheat plants at 20 d after treatment. The reduction in DM was more pronounced in shoots than roots (Table 1). The reduction in Chl *a* content amounted 16.5, 22.3, and 40.5 % and that of Chl *b* 19.4, 46.1, and 55.6 % over control for plants grown at 0.10, 0.15, and 0.25 mM Cr(VI), respectively (Table 1). Roots contained much more Cr than leaves and stem (Table 1).

Table 1. Effect of Cr(VI) on dry matter (DM) [mg per plant] and Cr accumulation [mg kg^{-1}(DM)], and chlorophyll (Chl) content [g kg^{-1}(FM)] in wheat plants determined after 20 d of growth in presence of different Cr(VI) concentrations. Means of 4 replications ± SD.

		Cr concentration [mM] Control	0.10	0.15	0.25	LSD (*p=0.05*)
DM	leaves	610±27	402±61	311±48	196±54	9.41
	stem	412±21	310±32	139±51	91±19	3.35
	roots	189±11	136±21	96±14	61±16	2.88
	total	1211±19	848±38	546±32	348±28	–
Cr	leaves	0.0	89.1±11	131.3±9	168.6±16.0	4.98
	stem	0.0	56.6±8.0	69.8±11.0	88.9±12.0	5.77
	root	0.0	597.4±24.0	789.8±31.0	931.3±28.0	8.60
Chl	Chl *a*	1.21±0.60	1.01±0.30	0.94±0.08	0.72±0.07	0.06
	Chl *b*	0.36±0.04	0.29±0.05	0.21±0.02	0.16±0.02	0.03

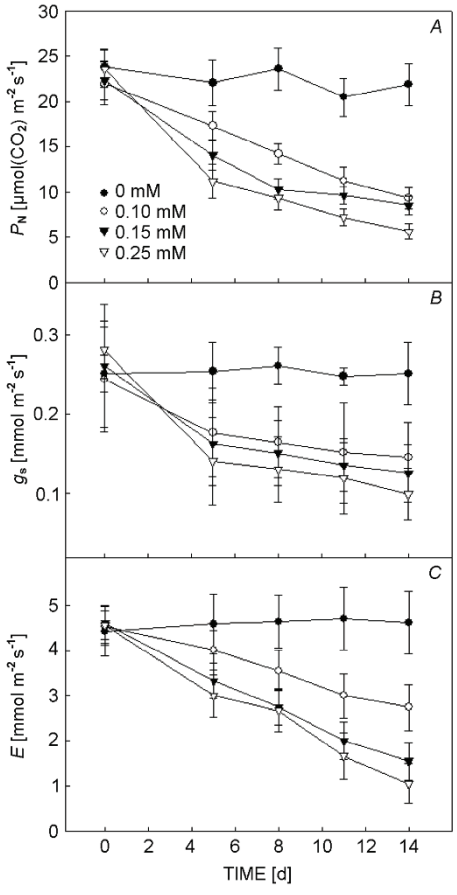

Fig. 1. Effect of chromium on (*A*) leaf net photosynthetic rate, P_N, (*B*) stomatal conductance, g_s, and (*C*) rate of transpiration, *E*. Means of four replications ± SE.

Net photosynthetic rate (P_N) was significantly reduced by Cr(VI) and the reduction graduated with increasing time of Cr treatment (Fig. 1*A*). Maximum inhibition in leaf photosynthesis was observed at 14 d after emergence by using 0.25 mM Cr. Chromium significantly reduced the stomatal conductance (g_s) and transpiration rate (*E*) of wheat leaves. The reduction was more pronounced at higher Cr concentrations. A gradual reduction in both the parameters was observed with increasing time (Fig. 1*B,C*).

F_v/F_m is a measure of the intrinsic efficiency of PS2 photochemistry in the dark-adapted state and this parameter was not significantly affected by the Cr concentration or by duration of treatment (Fig. 2*A*). However, the Φ_e (F_v'/F_m') which represents the photochemical efficiency of PS2 under steady state irradiation and *in vivo* quantum yield of PS2 photochemistry Φ_{PS2} ($F_v'/F_m' \times q_P$) was significantly reduced by the Cr(VI) concentration and by duration of treatment (Fig. 2*B,C*). The effect was most pronounced in plants treated with 0.25 mM Cr(VI). The reduction was discernable only after 8 d of Cr(VI) treatment under 0.10 and 0.15 mM Cr. The coefficient of photochemical quenching (q_P) was significantly reduced by Cr(VI) concentrations and by duration of treatment; highest reduction was found after 8-d Cr treatment (Fig. 2*D*). The coefficient of non-photochemical quenching (q_N) increased significantly by Cr(VI) treatment and with treatment duration (Fig. 2*E*). The increase was most pronounced in plants treated with 0.25 mM Cr(VI). However, the increase was discernable only after 8 d of Cr(VI) treatment with 0.10 and 0.15 mM Cr. Cr(VI) concentrations and duration of treatment significantly

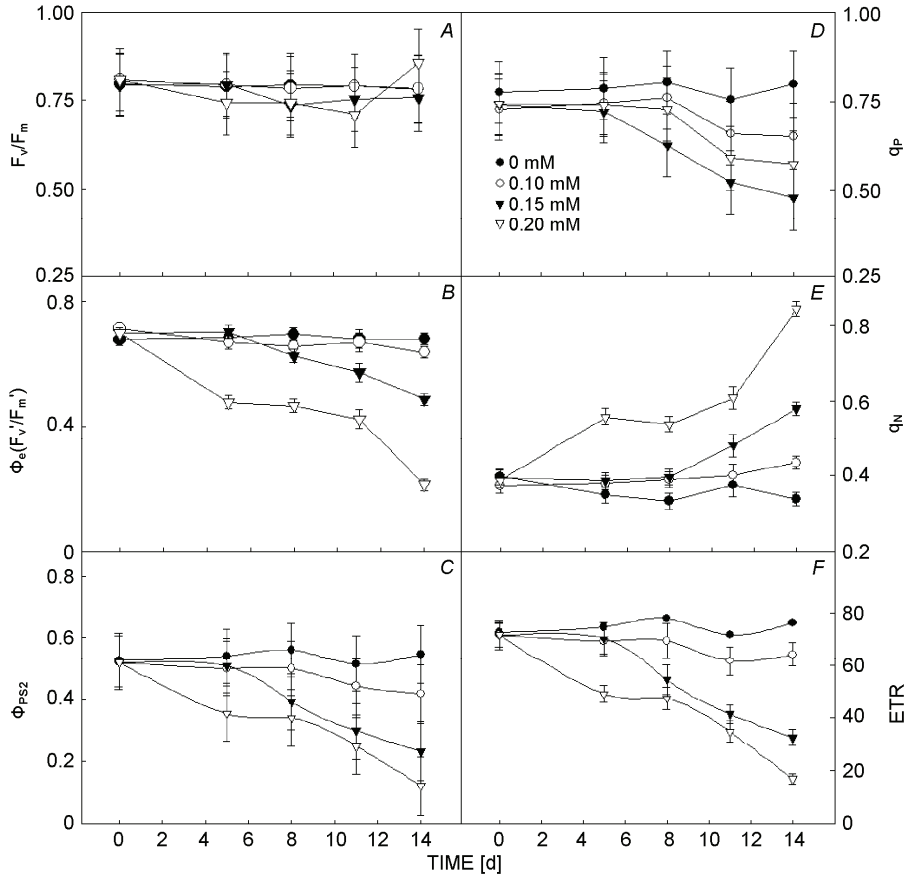

Fig. 2. Effect of chromium on (*A*) the maximal quantum yield of PS2 photochemistry, F_v/F_m, (*B*) the efficiency of excitation capture by open PS2 centres, Φ_e (F_v'/F_m'), (*C*) *in vivo* quantum yield of PS2 photochemistry, Φ_{PS2} ($F_v'/F_m' \times q_P$), (*D*) coefficient of photochemical quenching, q_P, (*E*) coefficient of non-photochemical quenching, q_N, and (*F*) apparent electron transport rate, ETR. Means of 4 replications ± SE.

reduced the apparent electron transport rate (ETR) of wheat leaves (Fig. 2*F*).

The MDA content as a measure of lipid peroxidation increased significantly with Cr(VI) concentration and duration of treatment. Maximum increase (45 %) over control was observed after 14 d of treatment with 0.25 mM Cr(VI) (Fig. 3*A*). The SOD activity was slightly increased by the Cr(VI) treatments. Maximum increase (6 %) was observed with 0.25 mM Cr(VI) after 14 d of Cr treatment (Fig. 3*B*). A marginal increase in CAT activity was observed under 0.1 M Cr(VI) both after 8 and 14 d of treatment (Fig. 3*C*). However, the activity was reduced by higher Cr(VI) concentrations (0.15 and 0.25 mM). The reduction was 7 and 28 % after 8 and 14 d of Cr(VI) treatment, respectively. A gradual reduction in the activity of APX activity was observed in plants treated with Cr(VI): the reduction depended on duration of treatment (Fig. 3*D*), the highest reduction of 22.3 % was observed in plants subjected to 14 d of 0.25 mM Cr(VI). Similar significant reductions were found for GR activity: they depended both on Cr concentration and treatment duration (Fig. 3*E*). Maximum reduction over control plants at 0 time (27 %) was observed in plants treated with 0.25 mM Cr for 14 d.

Discussion

Chromium significantly reduces the biomass production in wheat. The reduction in DM production by Cr is documented in different plants (Sharma and Sharma 1993). Reduction in DM of *Valisneria spiralis* by Cr(IV) concentration of above 25 g m^{-3} was reported by Vajpayee *et al*. (2001). Our results confirm these reports. Roots contain more Cr(VI) than leaves or stem. The reasons for greater accumulation of Cr in roots is due to accumulation of Cr in vacuoles of root cells, thus rendering it less toxic, which may be natural toxicity response of plant (Shankar *et al*. 2004). The Chl content of plants grown in presence of Cr(VI) was lower than in control plants. The decrease was more pronounced in Chl *b* than Chl *a*. Reduction in Chl content as a marked effect of Cr in different plants like wheat, cauliflower, and *Salvinia* has already been reported (McGrath 1982,

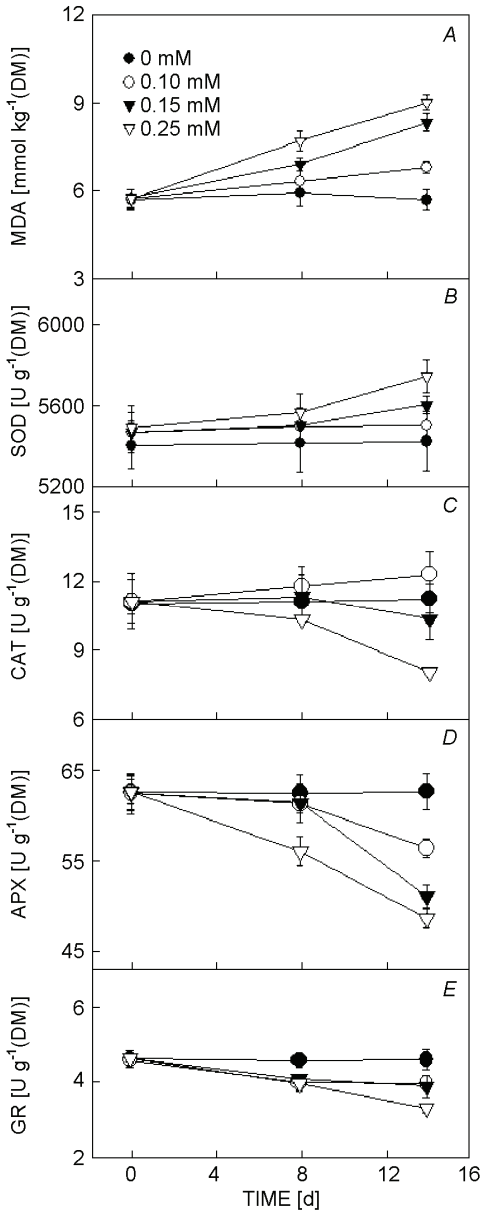

Fig. 3. Effect of chromium on (*A*) malondialdehyde (MDA) content and activities of (*B*) superoxide dismutase (SOD), (*C*) catalase (CAT), (*D*) ascorbate peroxidase (APX), and (*E*) glutathione reductase (GR) of wheat plants. Means of 4 replications ± SE.

Sharma and Sharma 1993, Chatterjee and Chatterjee 2000, Nichols *et al*. 2000). The pronounced decrease in Chl *b* is due to the destabilization and degradation of the proteins of the peripheral part of antenna complex (Shankar 2003).

P_N, E, and g_s were significantly reduced by Cr(VI) stress. It is not well understood to what extent the Cr-induced inhibition of photosynthesis is due to disorganization of chloroplast membranes (Vazques *et al*. 1987), inhibition of electron transport, or to the influence of Cr on the enzymes of the Calvin cycle. Zeid (2001) reported dramatic inhibition of photosynthesis by Cr(VI). Significant reductions in E and g_s were also observed. Little information is available on the effect of Cr on water relations of plants: Barcelo *et al*. (1986) observed a decrease in leaf water potential. Reduction in E and g_s was reported in cauliflower (Chatterjee and Chatterjee 2000). My results support these observations.

Cr(VI) did not affect the F_v/F_m ratio, which measures the maximal PS2 efficiency in primary photochemical reactions. Negligible effect of heavy metals such as Cd and Mn was observed on F_v/F_m indicating that the primary photochemical reactions were not affected (Krupa *et al*. 1992, Subrahmanyam and Rathore 2000). However, the light-adapted fluorescence parameters like Φ_e and Φ_{PS2} were significantly reduced by Cr(VI) indicating establishment of non-photochemical quenching in the leaves (Krupa *et al*. 1993). The values of q_P and q_N under steady-state irradiation were significantly affected by Cr(VI). A significant reduction in q_P with a concomitant increase in q_N was observed. This implies that the utilization of NADPH and ATP was inhibited under reduced CO_2 assimilation caused by Cr(VI) stress. The increase in q_N is due to an increased rate constant of thermal dissipation of excitation energy and this increase represents a mechanism to down regulate photosynthetic electron transport (Lu and Zhang 1999, Subrahmanyam *et al*. 2006). The increase in q_N induced by irradiation is predominantly due to an increase in the energy-dependent quenching which is associated with an increased transthylakoid pH gradient (Rees and Horten 1990). The q_N related to photoinhibition can be excluded here since the plants were grown at moderate irradiation. The reductions in both F_v'/F_m' and q_P are responsible for considerable decrease in Φ_{PS2}.

Cr(VI) increased the MDA content in wheat leaves which reflects the level of lipid peroxidation resulting from oxidative stress induced damage to membranes. Lipid peroxidation as indication of oxidative stress in plants can be induced *via* free radicals of reactive oxygen species that are generated as a result of heavy metal toxicity in plants (Panda and Chaudhury 2005). Induction and activation of SOD and of CAT are some of the major metal detoxification mechanism in plants (Shankar 2003). Gwozdz *et al*. (1997) reported that at lower heavy metal concentrations the activity of antioxidant enzymes increased, whereas at higher concentrations the SOD activity did not increase further and CAT activity decreased. The reduction in CAT activity observed in my study supports this observation. Cr-exposure of wheat seedlings resulted in decreased CAT activity (Panda and Patra 2000). The reduction in peroxidase and CAT in wheat treated with Cr was reported by Sharma and Sharma (1996). In my study the activities of APX and GR were reduced by Cr treatment. Rai *et al*. (2004) reported significant reduction in the activity of APX in *Ocimum tenuiflorum*. Reduction in GR activity under Ni stress has been reported in *Scenedesmus acutus*

f. *alternans* (Ranadhawa *et al*. 2001). My results suggest that both APX and GR are sensitive to high Cr(VI).

In conclusion, I found that the PS2 primary photochemical reactions were not affected by Cr(VI). The reduction in P_N shows that Cr(VI) had significantly affected the utilization of ATP and NADPH generated in light reactions. The CO_2 assimilation by PCR-cycle acts as a sink for the products of photosynthetic electron transport. Any process which interferes or reduces the utilization of products of electron transport and affects non-photochemical quenching will modify the rate of electron transport through PS2 and reduce quantum yield of linear electron transport. Cr(VI) especially at higher concentration increased lipid peroxidation and increased the activity of SOD and reduced the activities of CAT, APX, and GR indicating that these enzymes are susceptible to Cr(VI) toxicity.

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