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Effect of chromium on accumulation and antioxidants in *Cucumis utillissimus* L.: Response under enhanced bioavailability condition

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Abstract

This study compares the accumulation of Cr(VI) and biochemical changes (total chlorophyll, carotenoid, protein, malondialdehyde (MDA) and cysteine contents) and roles of antioxidant enzymes (superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX)) in tolerance to metal induced stress in *Cucumis utillissimus* L. grown in Cr contaminated soil (CS) with garden soil (GS). Furthermore, Cr bioavailability was enhanced by ethylene diamine tetra-acetic acid (EDTA) addition to the soil to forecast the plant's accumulation pattern at elevated Cr environment. Accumulation of Cr in the leaves of the plant increased with increase in substrate metals concentration. It further increased with the addition of EDTA by 1437% and 487% in GS and CS, respectively at the highest treatment level. The lipid peroxidation increased proportionately with increase in Cr accumulation in the leaves. All the activity of antioxidant enzymes (SOD, GPX and APX) and the level of cysteine increased with dose dependant manner. SOD and cysteine were observed to be higher in the GS than in CS, but APX and GPX were found to be higher in CS than in GS. The increase in GPX and APX activities with the increase in Cr concentration could be assumed that these two enzymes have a major role in the defense mechanism towards stress induced by Cr in *C. utillissimus*.

Key words: antioxidant; *Cucumis utillissimus* L.; chromium; ethylene diamine tetra-acetic acid; bioavailable

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Introduction

The main source of Cr in natural soils is weathering of their parent materials. An increase in total Cr concentration in agricultural soils originates from fall out and washout of atmospheric Cr containing particles from the chrome bearing sludge and refuse from industrial activity. Of the two stable forms of Cr, Cr(VI) is considered more toxic than Cr(III) because of its greater solubility and so available to the plant; whereas Cr(III) is assume to be less toxic because it is mainly bound to organic matter in soil rendering it less mobile and so not available to the plant.

India is one of the leading producers of leather from raw skins and hides. The use of chrome in tanning process generates huge amount of Cr contaminated sludges and wastewater. Several poor and marginal farmers use the sludge as soil conditioner and wastewater for irrigation and the rest is drained into the river. The use of Cr contaminated sludge and wastewater in agricultural practices risk the plants from being exposed to toxic metal, Cr. The impact of Cr contamination in the physiology of plants depends on its redox state, which determines its mobilization, subsequent uptake, and resultant toxicity in the plant system (Han et al., 2004; Chen et al., 2001; Mittler, 2002; Shanker et al., 2005; Sinha et al., 2005a), water relations (Pandey and Sharma, 2003). Metabolic alterations by Cr exposure have also been described in the plants by its ability to generate reactive oxygen species (ROS) (Dietz et al., 1999) and induce oxidative damages to plants (Sinha et al., 2005). The accumulation of metals by the plant is mainly governed by the availability of the metal from the total concentration present in the soil and availability is influenced by the physico-chemical properties of the soil. Chelants, mostly acidic ones, are known to bring down the pH of the soil and mobilize the elements when added in small quantity. A number of chelants have been reported in literature rendering soil trace metals more phytoavailable (Blaylock et al., 1997; Huang et al., 1997; Gupta and Sinha, 2007). Among all the chelants, the use of ethylene diamine tetra-acetic acid (EDTA) has been extensively used and studied (Cooper et al., 1999; Shen et al., 2002).

The plant of *Cucumis utilissimus* L. is grown on the river bed and near the flood plains throughout the gangetic

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plains of northern India. The species belongs to the dicotyledonous class and have bicollateral vascular bundle arrangement, which have the capability to take up large amount of water and nutrient from the soil thereby, accumulating excess metals in its biomass. Seeds of this species are used in formulations for the ailment of painful micturition and suppression of urine and are also known to posses cooling, edible and diuretic properties. The fruits of *C. utilissimus* are consumed prolifically as salad during the summer season, increasing the risk quotient from dietary intake. These sites are highly contaminated due to discharge of treated tannery wastewater containing high level of Cr in the river water (Singh et al., 2004). The plants of *C. utilissimus* collected from these sites, have shown healthy growth without any symptom of toxicity. However, higher accumulation of Cr was recorded in the plants (48.97 ± 8.68 μg/g dry weight (dw) in leaves and 17.94 ± 2.60 in μg/g dw in fruits) collected from these sites (unpublished data).

The present study has been carried out to assess (1) the biochemical changes (total chlorophyll, carotenoid, protein, malondialdehyde (MDA), cysteine contents) and roles of antioxidant enzymes (superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX)) in tolerance mechanism of the plant exposed to two different types of soil (garden and tannery wastewater contaminated soil) spiked with Cr and; and (2) the role of EDTA in the enhancement of Cr mobility for understanding the biochemical changes at elevated condition.

1 Materials and methods

In India, Jajmau (Kanpur) lies in the Indo-Gangetic plains between the parallels of 26′28°N and 80′24°E. It is one of the major centers for processing of raw hides. The discharge from tanning industries is treated in an up-flow anaerobic sludge blanket (UASB) treatment plant before releasing. The treated wastewater is either used by the local farmers for irrigation of edible crops/vegetables in the adjacent agricultural fields (2100 acre) or it is straightway drained into the river Ganga.

1.1 Experimental setup

Soils from two different locations were used for the pot experiment. Tannery waste contaminated soils (CS) were collected from Jajmau, Kanpur (Uttar Pradesh, India), and garden soil (GS) from National Botanical Research Institute (NBRI, Lucknow, India). The soil were air-dried, finely grounded, sieved (2 mm), and filled into each pot (8.5 kg soil + 0.5 kg manure). Seeds of *Cucumis utilissmus* L. were sterilized in 0.1% $HgCl_2$ and soaked in water overnight, and sown into pots (five each). The seedlings were grown for two weeks before it was thinned to three plants per pot; the soil were spiked with different concentrations of Cr (0, 50, 100, 150 μg/g) as potassium dichromate. Leaving the soil spiked with Cr for three weeks, a set of the same experiment was treated with EDTA (1 mmol/kg soil). All the treatments were grown in triplicates. The pots of garden soil (GS) were denoted as GS(0), GS(50), GS(100), GS(150) and GS(0)+EDTA, GS(50)+EDTA, GS(100)+EDTA, GS(150)+EDTA for without EDTA and with EDTA, respectively. Similarly, the pots of Kanpur soil (CS) were denoted as CS(0), CS(50), CS(100), CS(150) and CS(0)+EDTA, CS(50)+EDTA, CS(100)+EDTA, CS(150)+EDTA for without and with EDTA, respectively. All the pots were watered daily with 100 mL tap water to prevent from leaching from the bottom. Pots were placed in a greenhouse with an average diurnal temperature of 20–42°C in a completely randomized block design. The plants were harvested after 30 days of treatment.

1.2 Metal accumulation and quality control

Plants were uprooted from the pots, gently tapped to loosen the bound soil and subsequently washed under running tap water, and finally with double distilled water. Above ground part of the plant (leaves) was then blotted dry and oven dried at 80°C till constant weight. The oven dried samples were digested in HNO_3:$HClO_4$ in 3:1 (*V/V*) ratio and metal contents were estimated using Atomic Absorption Spectrophotometer (Avanta Σ, GBC, Australia).

The standard reference material for metals (E-Merck, Germany) was used for the calibration and quality assurance for each analytical batch. Method validation (accuracy and repeatability) was performed by analyzing the certified materials reference solution (BND 1101.02) of multi-elements (Zn, Fe, Cu) and single element reference solution, BND 102.03 (Pb); BND 402.02 (Cr) and BND 1001.02 (Ni) provided by National Physical Laboratory (NPL), New Delhi, India, and the results were found within ± 1.81% of certified values (n = 10).

1.3 Estimation of various physiological and antioxidants parameters

Leaves of the freshly harvested plants were used for the estimation of all the physiological and biochemical parameters. The chlorophyll content in the fresh leaves of the plant (100 mg) was estimated by the method of Arnon (1949) and carotenoid content by the method of Duxbury and Yantsch (1956). The protein content in the leaves of the plant was estimated by the method of Lowry et al. (1951) using BSA as the standard protein. The lipid peroxidation in the plant leaves was measured in terms of malondialdehyde content, determined by thiobarbituric acid (TBA) reaction following the method of Heath and Packer (1968). The cysteine content in the leaves of the plant was estimated following the method of Gaitonde (1967). Plant tissues (200 mg) were homogenized in 2 mL of 100 mmol/L potassium phosphate buffer, pH 7.5 containing 1 mmol/L of EDTA in presence of pinch of polyvinyl polypyrrolidone (PVP). The homogenate was centrifuged at 12,000 ×*g* for 15 min at 4°C. All steps in the preparation of enzyme extract were carried out at 0–4°C. This supernatant was used to measure the activities of SOD, APX and GPX. The activity of SOD (EC 1.15.1.1) was measured in the leaves of the plant by the method of Nishikimi and Rao (1972), using the enzyme extract.

A set devoid of enzymes served as control. APX (EC 1.11.1.11) activity was measured in the leaves of the plant by the method of Nakano and Asada (1981), estimating the role of ascorbate oxidation at 290 nm, using the extinction coefficient of 2.8/mmol/cm. The activity was calculated. GPX (EC 1.11.1.7) was measured in plant parts, following the method of Curtis (1971), modified by Kato and Shimizu (1987). Activity was calculated using the extinction coefficient of 26.6/mmol/cm at 470 nm for oxidized tetra guiacol polymer. One unit of peroxidase activity was defined as the calculated consumption of 1 μmol H_2O_2/min/g fresh weight (fw).

1.4 Statistical analysis

The experiment was performed in completely randomized block design involving four treatments of GS with CS (with and without EDTA), in triplicates. All the dataset obtained from the experiment, were subjected to two-way analysis of variance (ANOVA) using Microsoft Excel 2007 followed by Duncan's multiple range test (DMRT) calculation (Gomez and Gomez, 1984). Significance levels were compared at $p < 0.05$. The non significant groups (on par) were labeled as a, b, c and so on.

2 Results and discussion

2.1 Physico-chemical properties of the substrates

The soil (GS, CS) used as substrate to grow the plants were analyzed for their physico-chemical properties (Table 1). The results showed that pH, electrical conductivity (EC), cation exchange capacity (CEC), water holding capacity (WHC), organic carbon (OC) and organic matter (OM) of CS were higher than the level of respective parameters in GS. The level of extractable metals was significantly high in CS as compared to GS. The increase in the level of all the physico-chemical parameters in contaminated soil is due to irrigation with treated tannery wastewater (Gupta and Sinha, 2007). The level of toxic metal (Cr) in the plants collected from contaminated agricultural field of Jajmau (Kanpur) has shown high accumulation in the leaves (48.97 ± 8.68) μg/g dw and fruits (17.24 ± 2.60) μg/g dw (unpublished data).

Table 1 Physico-chemical properties of contaminated soil (CS) and garden soil (GS)

Parameter	GS	CS
pH (1:2.5)	6.7 ± 0.02	7.2 ± 0.01
EC (μS/cm)	461.0 ± 0.01	617.0 ± 0.01
CEC (cmol (p+)/kg)	32.2 ± 2.6	50.3 ± 1.2
OC (%)	0.4 ± 0.1	0.8 ± 0.01
OM (%)	0.7 ± 0.01	1.4 ± 0.01
DTPA extractable metals		
Fe (mg/kg dw)	10.0 ± 0.2	16.7 ± 0.2
Zn (mg/kg dw)	5.2 ± 0.6	13.6 ± 0.4
Mn (mg/kg dw)	20.0 ± 1.0	12.3 ± 0.2
Cu (mg/kg dw)	1.8 ± 0.2	3.5 ± 0.0
Cr (mg/kg dw)	BDL	5.6 ± 0.1
Pb (mg/kg dw)	0.7 ± 0.1	3.4 ± 0.1
Ni (mg/kg dw)	BDL	2.9 ± 0.0
Cd (mg/kg dw)	BDL	1.2 ± 0.1

All the values are mean of three replicates±SD.
BDL: below detection limits; EC: electrical conductivity; CEC: cation exchange capacity; OC: organic carbon; OM: organic matter; DTPA: diethylene triamine penta-acetic acid.

The reduction of Cr(VI) to Cr(III) is pH dependent and increases with decreasing pH values. The characteristic of tannery waste soil are high organic matter content and anaerobic type. Cr(VI) is known to reduced in acidic soils as acidic conditions promotes the of release of Fe(II) species from soil minerals for reaction with aqueous Cr(VI) species, and also increase the rate of Cr(VI) reduction by organic matter (Eary and Rai, 1991). The mobility of Cr in the soil is also influenced by the presence of Fe, Mn and other metals (Bartlett and Kimble, 1976; Yadav et al., 2007).

2.2 Accumulation of Cr

C. utilissimus was exposed to four concentrations of Cr (0, 50, 100 and 150 μg/g) for 30 days and observed that Cr was accumulated in all the treatments. There was no significant ($p < 0.05$) difference in Cr accumulation in the leaves in all the treatments without EDTA and in lower concentrations with EDTA. However, there was a significant ($p < 0.05$) increase at higher concentration treated with EDTA. As anticipated, the addition of EDTA in GS and CS increased the accumulation of Cr in the leaves of *C. utilissimus* as compared to without addition of EDTA in all the treatments (Fig. 1). Sinha et al. (2006) have reported Cr accumulation in the leaves of several crops grown on Cr contaminated tannery waste soil (phytoavailable Cr range 33.26 to 114.26 μg/g dw), and the accumulation were found in the range between 5.01 and 128.16 μg/g dw.

The effectiveness of EDTA in mobilizing metals in soil is well recorded (Madrid et al., 2003). Lombi et al. (2001) reported the efficiency of EDTA in overcoming the diffusion limitation of Cr to the root surface than the barrier of shoot to root translocation. It is also reported that the ability of EDTA to enhance the root to shoot translocation is metal specific. In our case, EDTA was found to be effective in transporting Cr(VI) to the leaves part of the plant. Under the enhanced (EDTA treated) Cr condition, the Cr accumulation increased by 739% and 1674% in GS and CS, respectively at the highest Cr concentration. The

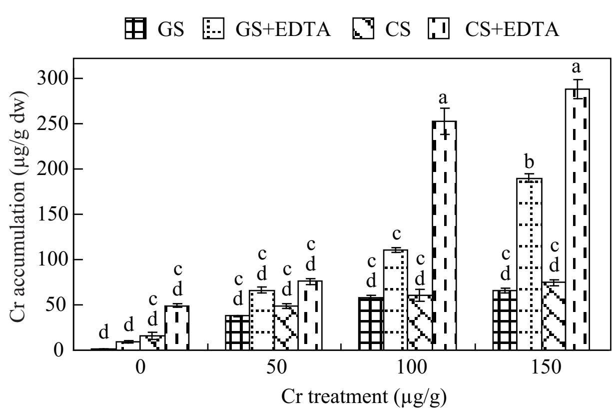

Fig. 1 Accumulation of Cr in the leaves of the plants after 30 days of exposure. All the values are means of three replicates ± SD. Values marked with the same letters are not significantly (Duncans test, $p < 0.05$) different.

higher uptake of Cr by *C. utilissimus* may be attributed to the soil water content, since it is fairly stable to reduction in water (Cary et al., 1977). This finding is also substantiated by the results of Mishra et al. (1995) where they reported that when Cr supplied to plants through irrigation water at levels ranging from 0.5 to 25 mg Cr/L, higher levels of uptake was observed in roots, shoots and even in grains.

2.3 Effect of Cr on photosynthetic pigments

The total chlorophyll content (Fig. 2A) in the leaves of the plants grown on GS and CS (with and without EDTA) has shown significant decrease as compared to their respective controls. Maximum decrease of 83.05%, 66.58%, 87.75% and 78.01% was observed in the plants grown on GS, GS+EDTA, CS and CS+EDTA treated with 150 μg/g Cr, respectively, compared to their respective controls. Although contradictory to the speculation, the plants grown on Cr spiked substrates along with EDTA have shown lesser decrease in their chlorophyll content in both the GS and CS as compared to the respective substrates without EDTA. Similarly, the carotenoid content in all the treatments (Fig. 2B) was found to decrease with increase in metal concentrations as compared to their respective controls, with exception to CS(50)+EDTA. Maximum decrease of 90.99%, 87.58%, 85.48% and 81.20% was observed in all treatments of 150 μg/g Cr, in GS, GS+EDTA, CS and CS+EDTA, respectively, as compared to their respective controls. Carotenoids are known to quench the photodynamics reactions leading to loss of chlorophylls, replace peroxidation and collapse of membrane in chloroplasts (Knox and Dodge, 1985), and an increase in its content have been reported in many studies (Sinha et al., 2005b; Gupta and Sinha, 2009) to increase with increase in metal concentration. However, in this case, the decreasing pattern clearly indicates that the carotenoid is not playing a role in its tolerance mechanism.

2.4 Effect on protein and lipid peroxidation

The protein content (Fig. 3A) also exhibited a gradual decrease with increase in Cr concentration in both GS and CS (with and without EDTA) as compared to their respective controls. The maximum decrease at the highest metal concentration was 83% and 73% in GS and CS (with or without EDTA) respectively, with respect to their respective controls. The levels of protein were slightly lower in the EDTA treated than the soil without EDTA, the change being a non-significant one. Reactive oxygen species induced by Cr are known to damage protein (Sinha et al., 2005b) and this may be attributed to the decrease in the protein level in the leaves of *C. utilissimus*. Although the change may be small, but this small change in chlorophyll and protein can be very serious to plant system.

The effect of free radical on the lipid membrane is best assessed by estimating the byproduct of lipid peroxidation – the malondialdehyde (MDA). An increase in MDA content was recorded with increase in metal concentrations in all the treatments as compared to their respective controls. The absolute level of MDA content (Fig. 3B) was recorded more in CS than GS. Both in GS and CS, the addition of EDTA increased the level of MDA content than non-spiked GS and CS. Level of MDA is an indirect method of quantification of oxidative stress in biological system. The gradual increase in MDA levels with increase in Cr concentration corroborates the oxidative stress due to Cr (Sinha et al., 2005b). Maximum increase of 304.93%, 390.85%, 206.61% and 197.63% was recorded in the plants grown on GS, GS+EDTA, CS and CS+EDTA, respectively treated with 150 μg/g Cr, as compared to their respective controls.

Heavy metal promotes lipid peroxidation via generation of free radicals (Mittler, 2002). In this study, an increase in MDA content under enhanced Cr environment in the leaves could be considered as an apparent reflection of oxidative damage caused due to metal stress. Several authors have also reported an increase in MDA content under Cr stress (Shanker et al., 2005; Sinha et al., 2005b).

2.5 Antioxidant and antioxidant enzymes

Cysteine is an important constituent of phytochelatins and plays an important role in metal detoxification. The

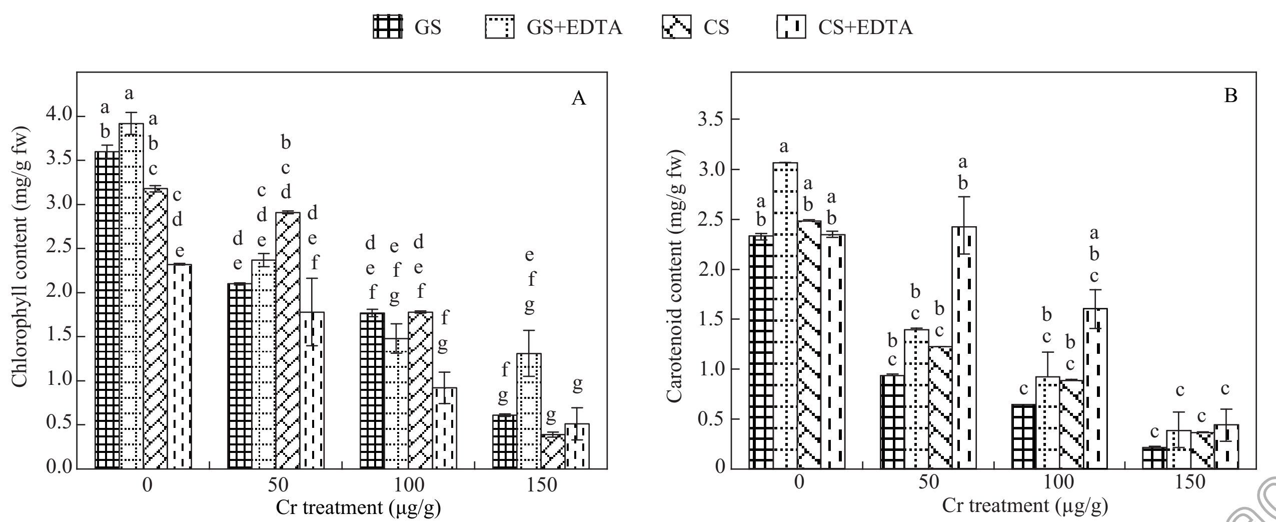

Fig. 2 Effect on chlorophyll (A) and carotenoid (B) contents in *C. utillissimus* after 30 days. All the values are means of three replicates ± SD. Values marked with the same letters are not significantly (Duncans test, $p < 0.05$) different.

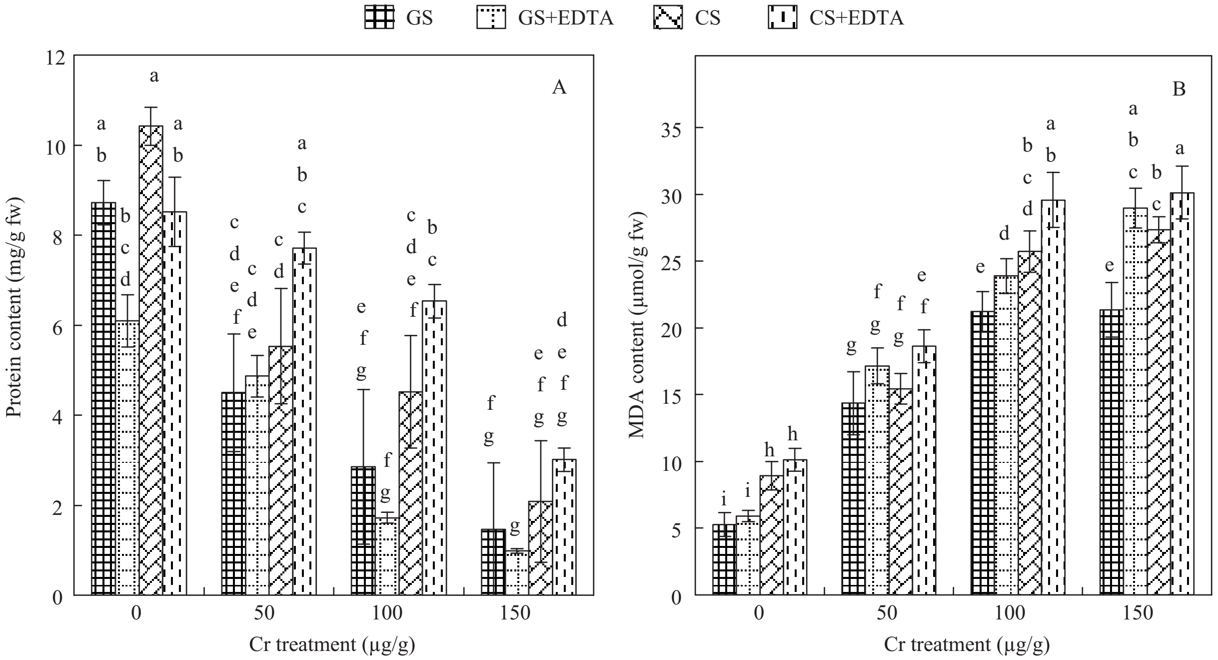

Fig. 3 Effect on protein (A) and MDA (B) contents in *C. utillissimus* after 30 days. All the values are means of three replicates ± SD. Values marked with similar alphabets are not significantly (Duncans test, $p < 0.05$) different.

values increased with increasing metal concentrations in both with and without EDTA conditions. Maximum increase of 209% and 132% in GS(150) and CS(150) was recorded respectively, with respect to their controls. The levels of cysteine in the EDTA treated soil were relatively less than the non-treated soil within the same treatment (Fig. 4A).

SOD is considered to be the first line of defense for plant's anti-oxidative defense system which plays an important role in dismutation of free hydroxyl radicals by the formation of hydrogen peroxide. SOD levels of the plants grown on GS had higher than that of the CS and the same pattern has been exhibited with EDTA (Fig. 4B). However, a reverse trend was observed for APX (Fig. 4C) and GPX (Fig. 4D) where the values were higher in EDTA than without it. A gradual increase in all the antioxidant levels with increase in substrate Cr concentration was observed, with maximum levels in 150 μg/g Cr. This pattern, therefore, indicates that the antioxidative defense mechanism can vary with different substrates having different contamination levels or physico-chemical properties. Dixit et al. (2002) observed that the SOD activity in *Pisum sativum*, increased by 29% when grown on 20 μmol/L for 7 days, thereafter with increase in Cr concentration (200 μmol/L), significant decline was observed. Quite contrary to these observations, plants of *C. utilissimus* have shown increase in SOD activity grown on 150 μg/g dw (2800 μmol/L). Shankar et al. (2005) also observed increase in SOD activity of *Vigna radiata* cv. CO 4 after 12 hr of application 50 μmol/L of Cr(VI).

Comparison of percentage increase in the activity of the antioxidant enzymes, however, revealed that the increase of GPX (142.5% and 113.9%) and APX (355.11% and 326.03%) were higher than that of SOD (36.51% and 27.29%) in the plants grown on GS without and with EDTA, respectively, whereas, on CS the activity of SOD (129.43% and 53.04%) and APX (418.01% and 481.56%) were higher than GPX (43.42% and 22.77%), respectively.

This phenomenon of varied levels of antioxidant enzymes can be explained as redundancy in ROI-scavenging mechanisms (Mittler, 2002). Plants with suppressed APX production induce SOD, CAT and GR to compensate for the loss of APX, and perhaps vice-versa. The lower level of SOD in the plants grown on GS than on the CS could not be attributed to Cr level alone, as the Cr levels in the leaves of the plant grown on both the GS and CS did not have significant difference, however, addition of EDTA and subsequent increase in bioavailability of Cr had certainly enhanced the SOD synthesis. The saturation of cysteine synthesis when exposed to elevated levels of Cr is very well exhibited in Fig. 4A. There is a consistent increase in the cysteine level both in the GS and CS without EDTA, however, upon addition of EDTA and subsequent enhancing the availability of Cr, the rate of synthesis of cysteine declined. Thus, when exposed to higher levels (above 100 μg/g) of Cr, cysteine contributes lesser antioxidant defense. GPX and APX on the contrary, are the major contributor towards the defense mechanism of the plant as their levels have consistently increased with increase in Cr concentration.

3 Conclusions

C. utilissimus (whose fruit is consumed) has not shown any significant accumulation under natural condition but has shown significantly high accumulation of Cr(VI) by the addition of EDTA. The accumulation in CS was higher than that of GS as the level of contamination was four times higher in the substrate which has also been reflected in the level of lipid peroxidation. The oxidative damage in terms of peroxidation of lipids increased proportionately with

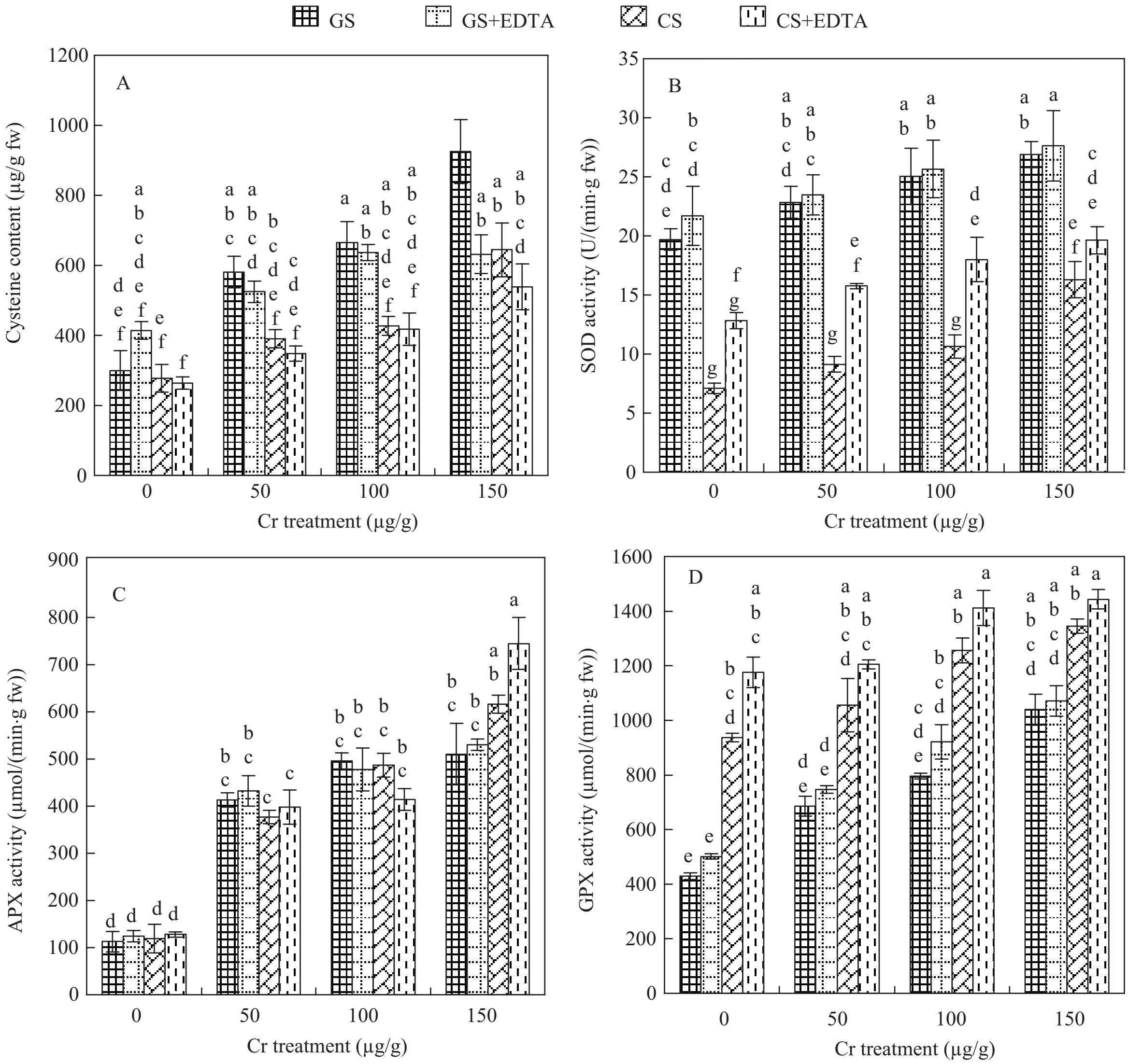

Fig. 4 Effect on the cysteine content (A) and on the activities of SOD (B), APX (C) and GPX (D) in the leaves of *C. utillissimus* after 30 days. All the values are means of three replicates ± SD. Values marked with the same letters are not significantly (Duncans test, $p < 0.05$) different.

increase in plant Cr accumulation. The antioxidant defense system exhibited redundancy as evident from the cysteine and SOD levels of the plants grown on GS was higher than that of the CS and the same pattern was exhibited with EDTA, whereas, a reverse trend was observed for APX and GPX. All the values were higher in EDTA than without it. In higher Cr levels, the APX and GPX played major roles towards the overall defense mechanism of the plant. It is evidently clear from this experiment that further increase in Cr load to the environment would face risk of hampering the growth of the plant and passing on the toxic Cr to those consuming this fruit. But, further studies needs to be undertaken to ascertain the percent of translocation to its fruit.

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