Interactive effects of EDTA and oxalic acid on chromium uptake, translocation and photosynthetic attributes in Indian mustard (*Brassica juncea* L. var. Varuna)

Durga Singh, Ashish Agnihotri and Chandra Shekhar Seth*

Department of Botany, University of Delhi, Delhi 110 007, India

The accumulation and toxicity of chromium (Cr) in Indian mustard (*Brassica juncea* L.) under ethylene diamine tetraacetic acid (EDTA) and oxalic acid (OA) as chelating agents was studied. Plants were exposed to Cr(VI) and chelating agents in four experimental set-ups as Cr(VI), Cr(VI) + EDTA (1 : 1), Cr(VI) + OA (1 : 1) and Cr(VI) + EDTA + OA (1 : 1 : 1), where each set-up comprised of 0, 6.25, 12.5 and 25.0 mg of Cr(VI) and/or chelating agents in 250 g of soil. Results conferred that EDTA augmented bio-concentration factor in all the three doses of Cr(VI), OA considerably increased translocation factor in all the treatments, including control, and the combined application of the two chelates escalated both the aforesaid factors. Moreover, these chelating agents help in ameliorating Cr(VI) toxicity asserted by low degree of lipid peroxidation, insubstantial damage in root and shoot length, fresh and dry biomass, chlorophyll and leaf gas exchange parameters. Besides, plants showed a robust detoxification mechanism primarily by significant ($P < 0.05$) production of reduced glutathione and phytochelatins among other enzymatic and non-enzymatic antioxidants under these chelating agents. The present findings suggest that Indian mustard could be used as a potential phytoremediator of Cr(VI) under the combined application of EDTA and OA.

Keywords: *Brassica juncea* L., chromium uptake, chelating agents, photosynthesis.

ENVIRONMENTAL deterioration by heavy metals is a serious global concern because imbalance caused by their toxicity and harmful nature is an alarming threat to all life forms present on Earth. Among all the heavy metals, chromium (Cr) has a relatively high solubility and mobility and because of excessive anthropogenic activities, particularly tanning and electroplating industries which release large amounts of Cr into the environment, it can easily find its way into the food chain. Chromium has been reported to impart several toxic effects on photosynthetic pigments, chloroplast ultra-structure, electron transport chain and photophosphorylation in plants. It also leads to various deleterious effects on seed germination, membrane integrity, essential nutrient elements, nitrogen and protein metabolism[1] and hence demands urgent attention for its remediation.

Phytoextraction is a cost-effective and eco-friendly approach compared to physical (soil washing, stabilization/solidification, soil flushing, additives/surfactants) and chemical (adsorption using specific media, modified coagulation along with filtration, precipitation, immobilization and complexation reaction) methods, which utilizes plant-mediated clean-up of metal-polluted soil[2]. The success of phytoextraction largely depends on suitable plant species and bioavailability of metals, which can be further enhanced by appropriate use of some chelating agents. Synthetic chelating agents such as ethylene diamine tetraacetic acid (EDTA), diethylene triamine pentaacetic acid (DTPA) and ethylene glycol tetraacetic acid (EGTA) pose environmental risks due to their non-biodegradability, groundwater contamination and toxicity to plants[2]. On the contrary, organic chelating agents such as oxalic acid (OA), citric acid, malic acid, acetic acid, etc. are not reported to show any of these deleterious effects[3,4]. No doubt, synthetic chelating agents pose risks to the environment, but their wide applications cannot be ruled out and they must be used in combination with organic acids under controlled manner for efficient phytoextraction.

Relatively, few reports are available on the combined application of synthetic (EDTA) and natural (OA) chelating agents to understand their usefulness in enhancing Cr uptake, translocation and toxicity amelioration in Indian mustard. Therefore, the present study was performed to investigate the bioconcentration factor (BCF) and translocation factor (TF), whereas toxicity of Cr(VI) was assessed by several indicative parameters, viz. photosynthetic pigments, fresh and dry biomass, lipid peroxidation and leaf gas exchange parameters like net assimilation rate (A), transpiration rate (E), stomatal conductance (G_{H_2O}) and water use efficiency (WUE). Tolerance strategy against Cr(VI)-induced oxidative damage was determined

*For correspondence. (e-mail: seth_bhu@yahoo.co.in)

Declarations regarding ethical issues: The present experiment did not involve any humans or animals, and is in compliance with all ethical issues.

by estimating the enzymatic (superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), glutathione reductase (GR)) and non-enzymatic (cysteine (Cys), non-protein thiols (NP-SH), glutathione (GSH)) antioxidants along with phytochelatins.

Material and methods

Layout and treatment pattern of the experiment

Seeds of *Brassica juncea* L. (var. Varuna) were obtained from the Indian Agricultural Research Institute, New Delhi, India. The seeds were surface-sterilized with 1% sodium hypochlorite solution (20 min) and germinated on petri plates for 72 h under dark conditions. The seedlings were then transplanted into pre-prepared pots containing soil mixture spiked with different concentrations of Cr(VI) and/or chelating agents in triplicate, where 48 pots were taken and each pot contained 200 g of garden soil mixed with 50 g of peat moss. Plants were exposed to Cr(VI) alone (given as potassium dichromate – $K_2Cr_2O_7$), Cr(VI) + EDTA (1 : 1), Cr(VI) + OA (1 : 1) and Cr(VI) + EDTA + OA (1 : 1 : 1) by supplying four different concentrations of Cr(VI) and/or chelating agents: 0 (control), 6.25, 12.5 and 25 mg in total 250 g of soil. Subsequently, plants were kept in a plant growth chamber (Daihan Labtech Co. Ltd) under controlled temperature (21 ± 2°C) and illuminated (14 h daylight) by light intensity of 1500 μE m^{-2} s^{-1} for six weeks. Also, soil bioavailability test in each experimental set was performed and the respective Cr(VI) concentrations were found to be 6.19 ± 0.02, 12.42 ± 0.03 and 24.87 ± 0.05 mg against 6.25, 12.5 and 25 mg respectively.

Quantification of chromium in plant and soil

The amount of Cr(VI) in root, shoot and soil was determined by atomic absorption spectroscopy[5]. Dried and finely powdered soil and plant samples (250 mg) were digested in 10.0 ml mixture of HNO_3 : $HClO_4$ (3 : 1, v/v) at 70°C and the volume reduced up to 1 ml. Acid-digested sample solutions were filtered and diluted up to 25 ml by gradual addition of Milli-Q water and were further analysed for Cr(VI) quantification by flame atomic absorption spectrophotometer (PerkinElmer A analyst 600; detection limit: 0.01 ppb Cr). Finally, BCF and TF were derived using the following formulae: BCF = metal concentration in root/metal concentration in soil; TF = metal concentration in shoot/metal concentration in root.

Determination of morpho-physiological and lipid peroxidation parameters

For determination of fresh and dry weight, plants were thoroughly washed (four times) with distilled water, followed by drying at 50–55°C and weighed accordingly till weight loss became constant.

Chlorophyll content was determined[6], where 100 mg of fresh leaves was homogenized in 5 ml of 100% DMSO and absorbance was taken at 452.5, 645 and 663 nm using UV-visible spectrophotometer (Beckman Coulter-DU 730).

Lipid peroxidaion was evaluated by estimating the malondialdehyde (MDA) content in 200 mg of frozen leaves homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). After centrifugation, the supernatant (1.0 ml) was mixed with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) and heated at 95°C for 30 min (ref. 7). Finally, MDA content was calculated using ΔA (A_{600}–A_{532}) and extinction coefficient (ε = 155 mM^{-1} cm^{-1}).

Determination of gas exchange parameters

Gas exchange parameters such as net assimilation rate (A), transpiration rate (E), stomatal conductance (G_{H_2O}) and WUE (derived as A/E) were analysed on fully expanded young leaves (third node from top) between 9.00 and 11.00 a.m. on a clear sunny day using portable infrared gas analyser (IRGA, GFS-3000 Portable Photosynthesis System, Walz, Effeltrich, Germany). Before taking the final readings, the analyser was adjusted for leaf surface area (3.00 cm^2), ambient CO_2 concentration (398 ppm), photosynthetic active radiation (PAR: 1000 μmol m^{-2} s^{-1}), impeller at 7 and relative humidity inside the leaf cuvette at 35%.

Estimation of antioxidant enzymes

Antioxidant enzymes were extracted by homogenizing fresh leaves (150 mg) in 0.1 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1% PVPP (w/v) and 0.5% Triton-X. Consequently, the homogenate was filtered, centrifuged and the resulting supernatant was used for the measurement of the following enzymes.

Superoxide dismutase (SOD; EC 1.15.1.1) was evaluated using 1 ml of assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 75 μM NBT, 13 mM methionine, 2 μM riboflavin and a suitable aliquot of enzyme extract[8]. For determining SOD activity, the reduction in NBT with and without enzyme was derived by taking the difference in absorbance at 560 nm.

Catalase (CAT; EC 1.11.1.6) activity was assayed using 50 mM potassium phosphate buffer (pH 7.5), 18 mM H_2O_2 and a suitable aliquot of enzyme extract taking the absorbance at 240 nm (ref. 9).

Ascorbate peroxidase (APX; EC 1.11.1.11) assay was performed in 3 ml of reaction mixture containing 0.1 M phosphate buffer (pH 7.8), 35 mM H_2O_2, 15 mM sodium ascorbate, 1.2 mM EDTA and a suitable aliquot of

Table 1. Effect of chelating agents on chromium (Cr) accumulation in soil, root and shoot, bioconcentration factor (BCF) and translocation factor (TF) of *Brassica juncea* L.

Cr concentration (mg/250 g of soil)	Treatments	Chromium concentration in soil and different parts of *B. juncea* L.				
		Soil (mg kg^{-1} soil)	Root (mg kg^{-1} DW)	Shoot (mg kg^{-1} DW)	BCF	TF
0	Control	0.21 ± 0.012^{a}	0.19 ± 0.004^{a}	0.14 ± 0.002^{a}	0.904	0.74
	Control + EDTA	0.61 ± 0.015^{b}	0.20 ± 0.009^{a}	0.15 ± 0.0007^{a}	0.328	0.75
	Control + OA	0.98 ± 0.008^{c}	0.35 ± 0.001^{a}	0.30 ± 0.002^{a}	0.347	0.85
	Control + both	$0.43 \pm 0.008^{a,b}$	0.39 ± 0.005^{a}	0.32 ± 0.004^{a}	0.929	0.82
6.25	Cr	15.0 ± 0.075^{g}	6.0 ± 0.104^{b}	2.66 ± 0.029^{c}	0.4	0.44
	Cr + EDTA	9.39 ± 0.108^{d}	6.54 ± 0.034^{c}	3.49 ± 0.014^{c}	0.695	0.54
	Cr + OA	12.2 ± 0.075^{e}	5.79 ± 0.156^{b}	4.67 ± 0.0425^{e}	0.474	0.81
	Cr + both	12.6 ± 0.092^{f}	7.61 ± 0.09^{c}	5.48 ± 0.011^{d}	0.604	0.72
12.5	Cr	28.4 ± 0.216^{j}	13.14 ± 0.179^{g}	3.81 ± 0.223^{c}	0.462	0.29
	Cr + EDTA	28.0 ± 0.242^{l}	15.93 ± 0.394^{j}	5.90 ± 0.286^{g}	0.569	0.37
	Cr + OA	30.86 ± 0.151^{k}	15.12 ± 0.54^{e}	6.16 ± 0.0607^{h}	0.490	0.66
	Cr + both	27.88 ± 0.151^{i}	14.27 ± 0.691^{g}	8.56 ± 0.256^{h}	0.511	0.60
25	Cr	40.8 ± 1.058^{h}	14.877 ± 0.229^{d}	4.17 ± 0.141^{b}	0.365	0.28
	Cr + EDTA	39.73 ± 0.252^{m}	19.627 ± 0.343^{h}	6.28 ± 0.09^{f}	0.494	0.32
	Cr + OA	42.17 ± 0.351^{n}	16.611 ± 0.804^{f}	11.13 ± 0.298^{i}	0.394	0.67
	Cr + both	42.93 ± 0.306^{o}	18.55 ± 0.264^{i}	10.39 ± 0.096^{j}	0.432	0.56

Data are means ± SD of three independent experiments and one-way ANOVA was used to test the significance level at $P < 0.05$.
Concentration of EDTA and/or OA is 6.25 mg when Cr concentration is nil.
DW, Dry weight, EDTA, ethylene diamine tetraacetic acid; OA, oxalic acid.

enzyme extract. The change in absorbance was recorded at 290 nm and activity was defined as μmoles of ascorbate oxidized per minute per gram fresh weight[10].

Guaiacol peroxidase (GPX; EC 1.11.1.7) was determined using 2 ml of reaction mixture consisting of 0.1 mM phosphate buffer (pH 6.0), 45 mM guaiacol (v/v), 44 mM H_2O_2 and a suitable aliquot of enzyme extract. Oxidation of guaiacol (extinction coefficient = 26.6 mM^{-1} cm^{-1}) was calculated at 470 nm and activity was defined as μmoles of guaiacol oxidized per minute per gram fresh weight[11].

Glutathione reductase (GR; EC 1.6.4.2) activity was measured using 1 ml of reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG and a suitable aliquot of enzyme extract using extinction coefficient of NADPH (ε = 6.2 mM^{-1} cm^{-1}) at 340 nm (ref. 12).

Estimation of non-enzymatic antioxidants

Estimation of Cys, NP-SH, GSH and PCs was performed to gain an insight about major non-enzymatic antioxidants. The total amount of Cys was obtained by grinding 250 mg of leaves in 5% chilled perchloric acid (v/v) as affirmed by Gaitonde[13]. NP-SH were estimated by grinding 200 mg fresh leaves in 5% (w/v) 5-sulphosalicylic acid and Ellman's reaction mixture (5 mM EDTA and 0.6 mM 5,5′-dithiobis (2-nitrobenzoic acid) in 120 mM phosphate buffer, pH 7.5) as detailed by Ellman[14]. Reduced glutathione was analysed using 5% (w/v) sulphosalicylic acid and 0.6 mM DTNB in 100 mM phosphate buffer (pH 7.0) and taking the absorbance at 412 nm (ref. 15). After obtaining the amount of total NP-SH and GSH, quantitative estimation of PCs was derived using the formula: Total PCs = (total NP-SH) – (GSH). Finally, unused plants and soil were safely disposed-off in land fillings to avoid further contamination.

Statistical analysis

All the experimental data are means of three independent experiments and represented as means ± SD. The significant differences were tested by one-way ANOVA (SPSS software version 21 for Windows 7 Ultimate; IBM Japan Ltd., Tokyo, Japan) followed by multiple comparison Duncan's test at significance level $P < 0.05$.

Results and discussion

Uptake and translocation of chromium in Brassica juncea L.

Results showed higher Cr(VI) accumulation in the root (6.0, 13.13 and 14.89 mg/kg DW) than shoot (2.66, 3.81 and 4.67 mg/kg DW) against 6.25, 12.5 and 25.0 mg Cr(VI) exposure respectively (Table 1). Differential

Table 2. Effect of Cr and chelating agents on fresh weight, dry weight, root length and shoot length of *B. juncea* L.

Cr concentration (mg/250 g of soil)	Treatments	Growth parameters			
		Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)
0	Control	8.36 ± 0.12^{g}	34.05 ± 0.48^{i}	471.8 ± 1.25^{n}	94.36 ± 0.251^{n}
	Control + EDTA	8.85 ± 0.13^{g}	$36.27 \pm 0.65^{g,h}$	389 ± 3.60^{l}	77.8 ± 0.721^{l}
	Control + OA	8.14 ± 0.12^{g}	38.97 ± 0.46^{j}	442.4 ± 2.4^{m}	88.48 ± 0.480^{m}
	Control + both	7.72 ± 0.25^{g}	32.85 ± 0.35^{k}	372.8 ± 2.2^{n}	74.56 ± 0.451^{n}
6.25	Cr	7.10 ± 0.10^{e}	30.07 ± 0.57^{f}	332.5 ± 2.78^{i}	66.5 ± 0.557^{i}
	Cr + EDTA	7.44 ± 0.06^{e}	34.5 ± 0.3^{e}	310.5 ± 1.89^{h}	62.1 ± 0.378^{h}
	Cr + OA	7.05 ± 0.13^{e}	36.56 ± 0.40^{g}	353.5 ± 1.32^{j}	70.7 ± 0.264^{j}
	Cr + both	6.69 ± 0.20^{f}	$32.23 \pm 0.64^{h,i}$	320.5 ± 1.37^{k}	64.11 ± 0.275^{k}
12.5	Cr	5.46 ± 0.19^{c}	25.56 ± 0.60^{d}	253.7 ± 2.86^{e}	50.74 ± 0.573^{e}
	Cr + EDTA	6.04 ± 0.19^{c}	27.62 ± 0.39^{c}	241.1 ± 0.76^{d}	48.23 ± 0.153^{d}
	Cr + OA	5.67 ± 0.17^{c}	28.23 ± 0.37^{d}	267.05 ± 1.35^{f}	53.41 ± 0.271^{f}
	Cr + both	5.52 ± 0.36^{d}	25.82 ± 0.50^{e}	251.05 ± 1.41^{g}	50.21 ± 0.283^{g}
25	Cr	4.70 ± 0.18^{a}	20.45 ± 0.42^{a}	154.6 ± 2.33^{b}	30.93 ± 0.466^{b}
	Cr + EDTA	5.14 ± 0.12^{a}	26.86 ± 0.65^{a}	166.3 ± 2.10^{a}	33.27 ± 0.421^{a}
	Cr + OA	5.44 ± 0.09^{b}	25.45 ± 0.42^{a}	177.6 ± 1.27^{b}	35.52 ± 0.255^{b}
	Cr + both	4.66 ± 0.18^{c}	27.56 ± 0.26^{b}	161.7 ± 1.69^{c}	32.34 ± 0.339^{c}

accumulation in root and shoot is correlated with the proximity of root to metal-spiked soil, translocation hindrance from root to shoot, and distinctive biosynthesis of PCs[16]. After applying chelating agents, BCF rose significantly in case of EDTA (74%, 23%, 49%) compared to OA (19%, 6%, 11%) subjected to 6.25, 12.5 and 25 mg of Cr(VI), confirming EDTA augments Cr(VI) uptake more efficiently than OA. On the contrary, OA triggered Cr(VI) translocation more efficiently than EDTA, which is confirmed by a significant rise in TF (1.8-, 3.5-, three-fold) instead of EDTA (1.2-, 1.4-, 1.5-fold) against 6.25, 12.5 and 25 mg Cr(VI) exposure respectively (Table 1). This might be due to the fact that OA lowers the external pH, which increases Cr bioavailability in the rhizosphere. Also, OA (having negatively charged carboxyl group) shows high affinity towards Cr (having positive charge), thus easing its mobilization in the shoot.

Toxicity of chromium and its alleviation by chelating agents

Plant growth indices: Alteration in root length, shoot length and plant biomass was considered for ascertaining Cr(VI) toxicity in plants. Root and shoot length declined gradually in a concentration-dependent manner from control to 25 mg Cr(VI); higher decline in root than in shoot was observed, as roots are more susceptible to Cr(VI) toxicity than the shoots[1]. This could be due to the higher accumulation of Cr(VI) in the roots, thereby enhancing its sequestration in vacuoles and inhibiting root development. The fresh and dry weight of plants followed a similar trend of toxicity and registered a significant ($P < 0.05$) fall of 30%, 46% and 67% compared to control against 6.25, 12.5 and 25 mg Cr(VI) exposure respectively (Table 2).

Photosynthetic pigments and gas exchange parameters: An exposure of 12.5 and 25.0 mg of Cr(VI) resulted in significant decline in chlorophyll *a* (31% and 61%), chlorophyll *b* (70% and 82%), total chlorophyll (20% and 61%) and carotenoids (44% and 68%) content respectively, compared to control (Figure 1 *a–d*). Toxic effects of Cr on photosynthetic pigments are primarily due to the degradation of δ-amino levulinic acid dehydratase entailed in chlorophyll biosynthesis. Other possible reasons could be replacement of Mg^{+2} ions from the porphyrin ring, changes in the thylakoid membrane ultrastructure and peroxidation of chloroplast, which ultimately causes destruction of chlorophyll[17,18].

Gas exchange parameters (A, E, G_{H_2O} and WUE) were studied for validating Cr(VI) toxicity on photosynthetic pigments as well as photosynthetic performance of plants. The least toxic Cr(VI) exposure (6.25 mg) showed the least toxicity on photosynthetic pigments and gas exchange parameters. On the contrary, higher exposures (12.5 and 25 mg) decreased the net assimilation rate by 32% (9.176 μmol CO_2 m^{-2} s^{-1}) and 59% (5.555 μmol CO_2 m^{-2} s^{-1}) respectively, compared to control (13.472 μmol CO_2 m^{-2} s^{-1}). A similar trend of variation was observed for G_{H_2O}, E and WUE (Table 3), possibly because of: (i) substitution of Mg^{+2} from Rubisco resulting in less affinity towards CO_2, (ii) stomatal conductance and transpiration by interfering with abscisic acid and thus limiting the leaf gaseous exchange.

Moreover, the impact of chelating agents on plant growth indices, photosynthetic pigments and gas exchange

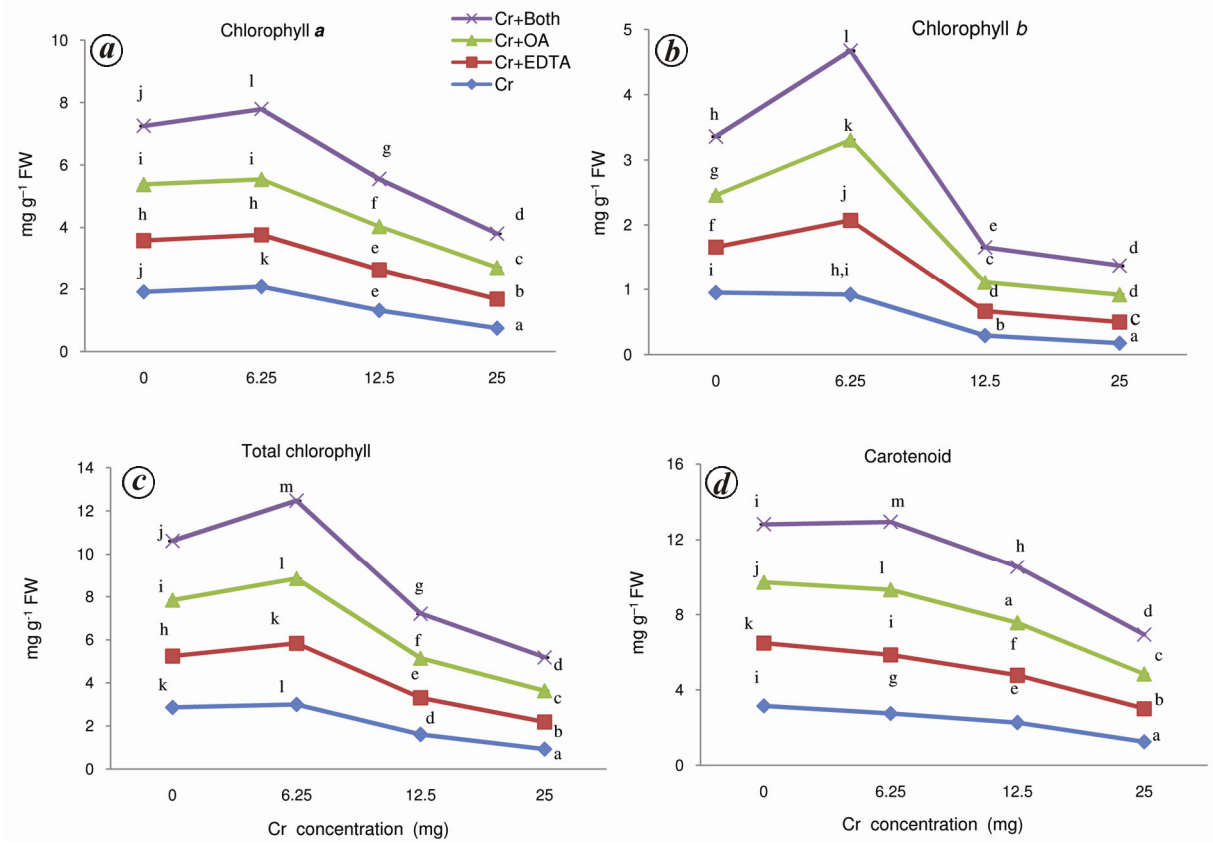

Figure 1. Effect on photosynthetic pigments: chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids of *Brassica juncea* L. treated with chromium (Cr) and chelating agents. Data are means ± SD of three independent experiments. One-way ANOVA was used to test the significance level at $P < 0.05$. Concentration of EDTA and/or OA is 6.25 mg when Cr concentration is nil.

Table 3. Effect of Cr and chelating agents on *A* (μmol CO_2 m^{-2} s^{-1}); *E* (mmol H_2O m^{-2} s^{-1}); G_{H_2O} (mmol H_2O m^{-2} s^{-1}) and water use efficiency (WUE) in leaves of *B. juncea* L.

Cr concentration (mg/250 g of soil)	Treatments	Gas exchange parameters			
		A	*E*	G_{H_2O}	WUE
0	Control	13.47 ± 0.0006^{k}	11.31 ± 0.002^{o}	$601.3 \pm 0.01^{g,h}$	$1.19 \pm 0^{b,c}$
	Control + EDTA	12.26 ± 0.002^{i}	10.62 ± 0.001^{l}	603.5 ± 0.0044^{h}	$1.15 \pm 0.0006^{a,b}$
	Control + OA	$12.42 \pm 0.001^{i,j}$	10.81 ± 0.001^{m}	632.8 ± 0.003^{i}	$1.15 \pm 0^{a,b}$
	Control + both	12.63 ± 0.016^{j}	10.97 ± 0.0006^{n}	$593.4 \pm 0.001^{f,g,h}$	$1.15 \pm 0.0015^{a,b}$
6.25	Cr	13.85 ± 0.003^{l}	9.71 ± 0.001^{i}	$577.4 \pm 0.01^{e,f,g,h}$	1.43 ± 0.0006^{h}
	Cr + EDTA	$12.47 \pm 0.001^{i,j}$	9.46 ± 0.001^{h}	$575.6 \pm 0.002^{e,f,g}$	$1.32 \pm 0^{f,g}$
	Cr + OA	13.43 ± 0.002^{k}	9.92 ± 0.002^{j}	$567.8 \pm 0^{e,f}$	1.35 ± 0.0006^{g}
	Cr + both	14.55 ± 0.0006^{m}	10.13 ± 0.001^{k}	561.3 ± 0.01^{e}	1.44 ± 0^{h}
12.5	Cr	9.17 ± 0.003^{e}	7.10 ± 0.0006^{d}	462.3 ± 57.7^{d}	$1.15 \pm 0.0006^{a,b}$
	Cr + EDTA	10.54 ± 0.001^{f}	8.345 ± 0.0006^{e}	430.5 ± 0.001^{c}	$1.26 \pm 0.0006^{d,e}$
	Cr + OA	10.94 ± 0.0006^{g}	8.57 ± 0.002^{f}	460.1 ± 0.0006^{d}	$1.28 \pm 0.0006^{d,e,f}$
	Cr + both	11.78 ± 0.003^{h}	8.89 ± 0.001^{g}	474.3 ± 0.010^{d}	$1.326 \pm 0^{f,g}$
25	Cr	5.55 ± 0.003^{a}	4.97 ± 0.001^{a}	255.6 ± 0.0006^{a}	1.12 ± 0.0006^{a}
	Cr + EDTA	6.61 ± 0.0006^{b}	4.96 ± 0.001^{a}	318.4 ± 0.001^{b}	$1.33 \pm 0.0006^{e,f}$
	Cr + OA	6.54 ± 0.0006^{c}	5.49 ± 0.0006^{b}	335.1 ± 0.001^{b}	$1.191 \pm 0^{c,d}$
	Cr + both	7.56 ± 0.001^{d}	5.74 ± 0.0006^{c}	346.4 ± 0.001^{b}	$1.32 \pm 0.0006^{f,g}$

A, Net assimilation rate; *E*, transpiration rate; G_{H_2O}, stomatal conductance.

parameters followed a common pattern of toxicity: EDTA + OA < OA < EDTA < Cr(VI) indicates that a combined application of EDTA and OA showed less toxicity than their individual counterparts. This is because chelates rapidly form metal–chelate complexes in the apoplast and/or symplast, rendering the metals non-toxic[19]. Also, these chelates enhance the solubility and uptake of essential macro- and micronutrients by facilitating soil cation exchange, which promotes chlorophyll biosynthesis leading to greater biomass and productivity.

Lipid peroxidation: The amount of malondialdehyde (an indicator of degree of lipid peroxidation) escalated significantly ($P < 0.05$) in a concentration-dependent manner by 3.5- and 5.1-fold compared to control against 12.5 and 25 mg of Cr(VI) respectively. As the accumulation of Cr(VI) increased, it led to the production of reactive oxygen species (ROS) and/or free radicals following lipid peroxidation. Further, chelates improved the extent of damage revealed by lowering in the MDA content: 13% (EDTA), 35% (OA), 41% (EDTA + OA), than Cr(VI) alone (Figure 2). This might be due to the fact that chelating agents bind with Cr(VI) inside plant cells and thereafter make it unavailable for ROS production, thus preventing ion leakage and membrane damage.

Plant's mechanism(s) for tolerance and detoxification against Cr(VI) toxicity: The tolerance strategy is mainly achieved by concerted action of enzymatic and non-enzymatic antioxidants. Among enzymatic antioxidants, SOD plays a pivotal role in the first line of defence by quenching superoxide radicals, which act as a precursor to other ROS produced in different compartments of the cells. The activity of SOD increased by 2.1- and 6.8-fold against 6.25 and 12.5 mg Cr(VI) respectively, compared to control (Figure 3 *a*). Higher SOD activity can efficiently detoxify the superoxide radicals either by *de novo* synthesis of enzymatic proteins, or by the induction of SOD genes-mediated signal transduction[17]. To a large extent, H_2O_2 produced either by superoxide radicals or directly inside a cell is scavenged by CAT present in peroxisomes and mitochondria, where it is responsible for decomposing H_2O_2 into H_2O and O_2. An exposure of 12.5 mg Cr(VI) resulted in CAT activity as 0.085 μmol mg^{-1} protein, which was 2.4-fold higher than control (0.035 μmol mg^{-1} protein), emphasizing higher scavenging of H_2O_2 (Figure 3 *b*). Ascorbate peroxidase, an important enzyme of the ascorbate–glutathione cycle, is localized mainly in the chloroplast as well as the cytoplasm, where it plays a central role in combating oxidative stress[20]. Upon 12.5 mg of Cr(VI) exposure, APX activity was found to be 0.397 μmol mg^{-1} protein, which was significantly ($P < 0.05$) 4.8-fold higher than control (0.083 μmol mg^{-1} protein). Similarly, GPX is located in the cytosol, cell wall, vacuole and extracellular space and is considered as a stress-marker enzyme showing higher affinity towards H_2O_2-scavenging than CAT. The activity of GPX escalated significantly ($P < 0.05$) by 1.4- and 2-fold upon 6.25 and 12.5 mg Cr(VI) exposure respectively, compared to control (0.405 μmol mg^{-1} protein), suggesting an integrative contribution of APX and GPX towards ROS and/or free radicals scavenging for attenuation of oxidative stress (Figure 3 *c* and *d*). Furthermore, GR (Figure 3 *e*) is the most important component maintaining high GSH/GSSG ratio for uninterrupted running of the ascorbate–glutathione cycle along with the functioning of the glyoxalase defence system and PC synthesis in plants. Its activity increased by 1.3- and 1.9-fold against 6.25 and 12.5 mg of Cr(VI) respectively, compared to control which shows similar trends as in *Cuscuta reflexa* upon Cd exposure[21].

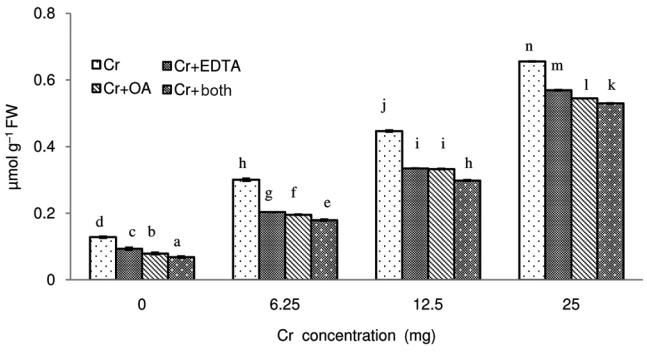

Figure 2. Effect of malonidaldehyde content of *B. juncea* L. treated with Cr and chelating agents.

On the contrary, the highest toxic exposure of Cr(VI) (25 mg) significantly declined SOD activity either by replacing Cu^{+2} and/or Zn^{+2} with Cr(VI) cations from the active site(s) of enzyme, or inactivation by H_2O_2 (ref. 22). Simultaneously, CAT activity deteriorated by 47% due to the damage suffered in its structure and function[20]. Moreover, significant ($P < 0.05$) fall of 64% and 25% in APX and GPX activities respectively, was due to the accumulation of excess H_2O_2 resulting in either inactivation and inhibition of their biosynthesis, or change in the assemblage of their subunits[1]. Finally, GR activity accounted for 30% decline, but still remained higher than control, thus indicating: (i) direct reaction of Cr(VI) with sulphydryl groups of enzyme resulting in decreased activity, which in turn affects the ascorbate–glutathione cycle, and (ii) its central role in all defence systems by maintaining the GSH pool[22].

In case of non-enzymatic antioxidants (Figure 4 *a–c*), results revealed that Cys content rose significantly ($P < 0.05$) by 2.3- and 3.2-fold upon 6.25 and 12.5 mg Cr(VI) respectively, compared to control (0.112 μmol g^{-1} FW), while NP-SH increased by 3.3-fold than control (1.825 μmol g^{-1} FW). This could be due to the augmented sulphate uptake and transport, which is catalysed by sulphate reduction enzymes like ATP-sulphurylase, adenosine-5′-phosphosulphate (APS) reductase, sulphite

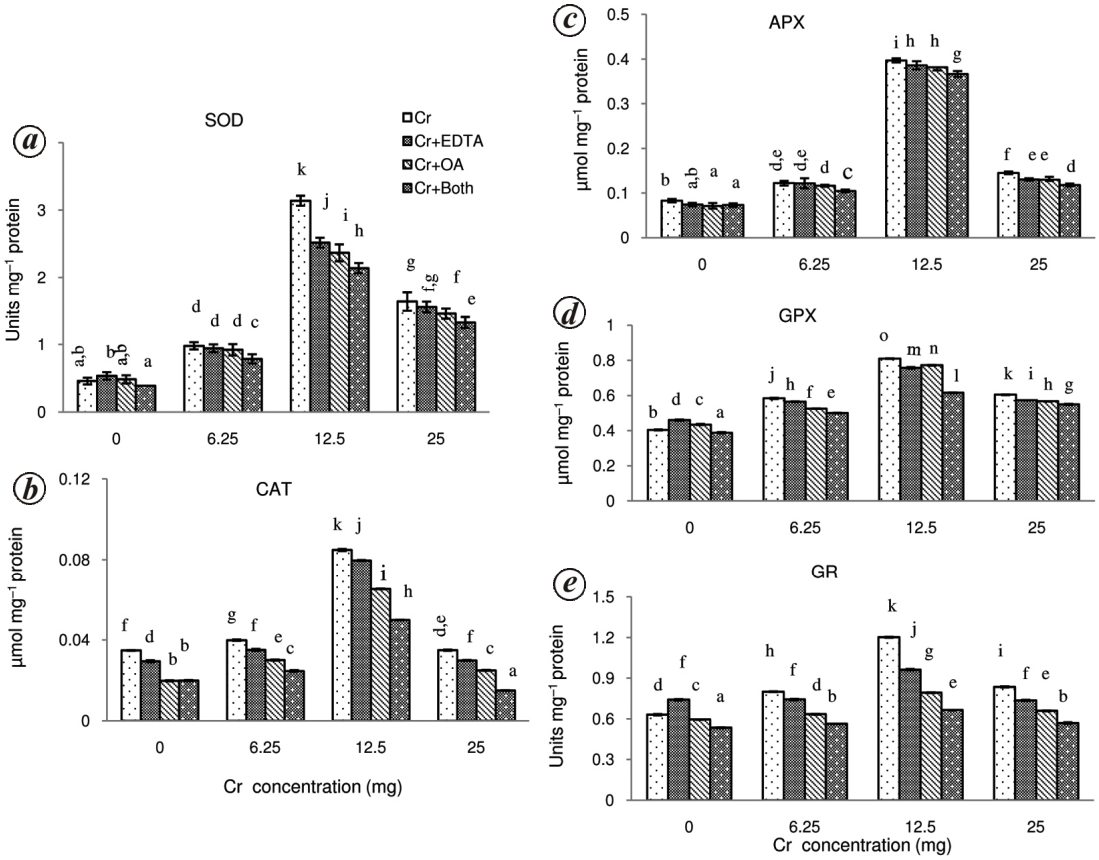

Figure 3. Activities of antioxidant enzymes SOD, CAT, APX, GPX and GR in *B. juncea* L. treated with Cr and chelating agents.

reductase resulting in greater synthesis and assimilation of cysteine and NP-SH[22]. The key non-enzymatic antioxidant, i.e. glutathione exhibited significant ($P < 0.05$) enhancement by 1.9- and 2.7-fold against 6.25 and 12.5 mg Cr(VI) respectively, compared to control (1.791 mol g^{-1} FW). This elevation could be either by induction of GSH transcription genes: γ-glutamylcysteine synthetase (*gsh1*) and glutathione synthetase (*gsh2*), or by H_2O_2-driven derepression of existing mRNA-encoding enzymes of GSH biosynthetic pathway reported in *Arabidopsis*[20]. On the contrary, the extreme toxic exposure of 25 mg Cr(VI) exhibited significant ($P < 0.05$) decline in Cys and NP-SH by 57% and 55% respectively, compared to previous treatments, indicating failure in their biosynthesis either by decrease in activities of the aforesaid sulphate reduction enzymes, or by higher consumption for synthesizing GSH and PCs[23]. Similarly, GSH slumped by 57% attributed to its utilization in many metabolic pathways like PC synthesis, glyoxalase defence system, ascorbate–glutathione cycle and/or as a reductant in antioxidant system to combat oxidative stress[17].

The detoxification of Cr(VI) is primarily done by its chelation with PCs and the result exhibited significant ($P < 0.05$) synthesis of PCs (0.807 and 1.129 μmol g^{-1} FW) against 6.25 and 12.5 mg Cr(VI) respectively, but a sharp decline (45%) at the highest toxic exposure of 25 mg Cr(VI) compared to control (0.034 μmol g^{-1} FW) (Figure 4 *d*). The significant amount of PCs in 6.25 and 12.5 mg Cr(VI) could be due to the upregulation of its biosynthetic pathway, while the declining amount in 25 mg Cr(VI) could be due to either degradation of PCs or inhibition in the activity of phytochelatin synthase (PCS) during excessive Cr accumulation[17,23].

Alleviating effects of chelates on enzymatic, non-enzymatic antioxidants, and phytochelatins

All the studied enzymatic antioxidants, non-enzymatic antioxidants and PCs exhibited their response during chelates exposure in the order EDTA + OA < OA < EDTA < Cr(VI). This clearly indicates that a combined application of chelates ameliorates Cr(VI) toxicity; thus the aforesaid parameters showed their activity to a smaller extent. Results demonstrated that EDTA + OA accounted for lesser SOD activity by 20%, 32%, 19% and GR by 30%, 45% and 32% against 6.25, 12.5 and 25 mg of individual Cr(VI) treatment respectively. Also, CAT, APX and GPX followed a similar pattern (Figure 3 *a–e*). Similarly, in case of non-enzymatic antioxidants, EDTA + OA

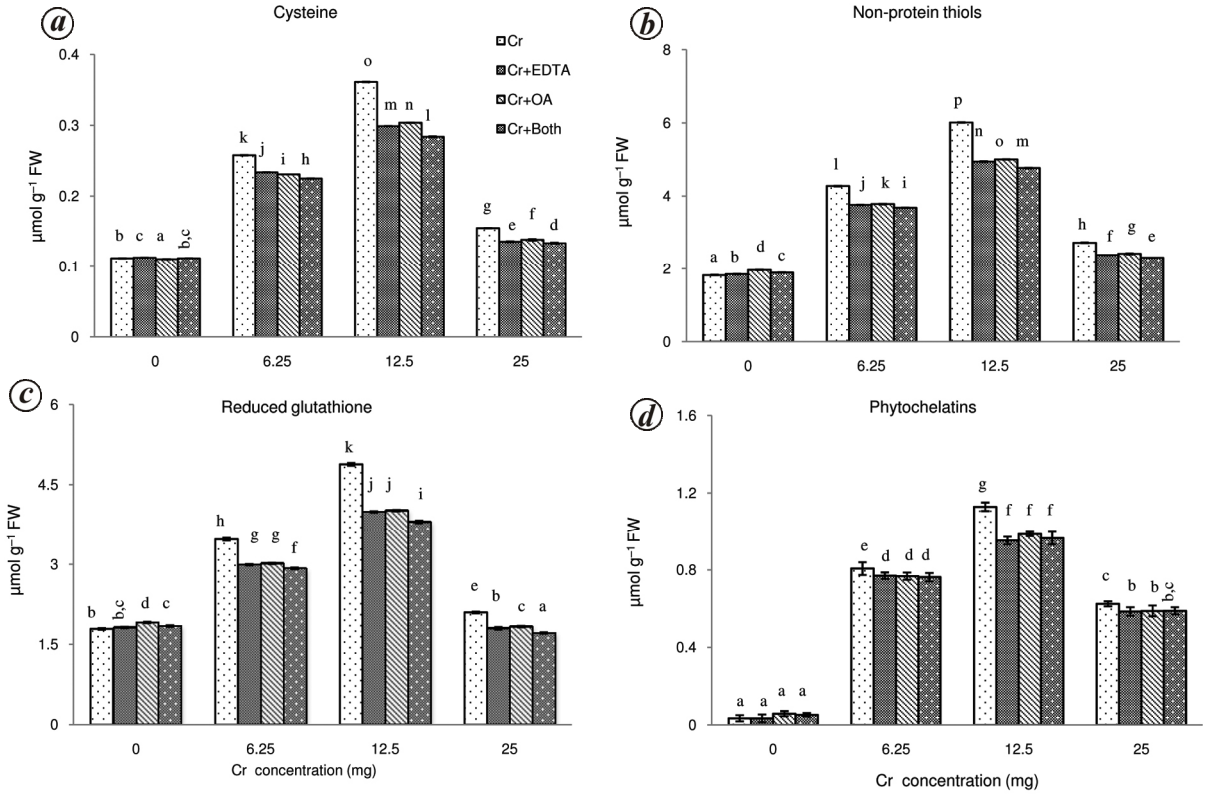

Figure 4. Effect of Cr and chelating agents on non-enzymatic antioxidants Cys, NP-SH, GSH and PCs in *B. juncea* L.

registered lesser synthesis of NP-SH by 14%, 21% and 15%, and GSH by 16%, 22% and 18% against 6.25, 12.5 and 25 mg of Cr(VI) treatment respectively, while Cys and PCs also followed a similar pattern (Figure 4 *a–d*). This could be due to low phenylalanine ammonia-lyase (PAL) activity resulting in lesser synthesis of phenolic compounds, which has a major role in the production of many antioxidants[24] as well as PCs. Thus, the protective role of chelating agents against metal toxicity cannot be attributed to enhanced synthesis of antioxidants, but by diminishing oxidative stress probably by decreasing free radicals production in plant cells.

Conclusion

The results of the present study suggested that a combined application of EDTA and OA improved phytoextraction potential of *B. juncea* L. as EDTA promoted Cr(VI) uptake while OA supported efficient translocation (prime requirement for effective phytoextraction). Furthermore, it alleviates Cr(VI) toxicity asserted by low degree of lipid peroxidation and insubstantial damage to chlorophyll and, fresh and dry biomass compared to Cr(VI) alone. Gas exchange attributes (A, E, G_{H_2O} and WUE) also confirmed similar trends. The plant has developed a good tolerance and robust detoxification mechanism(s) against Cr(VI) toxicity as evidenced by significant ($P < 0.05$) synthesis of enzymatic and nonenzymatic antioxidants and PCs. Based on the present study, it can be deduced that Indian mustard could be used as an appropriate plant species for effective Cr(VI) remediation under the combined application of EDTA and OA by augmenting bio-concentration factor, translocation factor and defence system.

1. Singh, H. P., Mahajan, P., Kaur, S., Batish, D. R. and Kohli, R. K., Chromium toxicity and tolerance in plants. *Environ. Chem. Lett.*, 2013, **11**, 229–254.
2. Seth, C. S. *et al.*, Phytoextraction of toxic metals: a central role for glutathione. *Plant Cell Environ*., 2012, **35**, 334–346.
3. Ogunleyel, J. H., Dagari, M. S., Balogun, O. L. and Maigari, A., Effect of oxalic acid on Cr^{+3} ion uptake, accumulation and oxidative stress by sorrel (*Hibiscus sabdariffa* L.) seedlings in hydroponic solution. *Int. J. Chem. Mater. Environ. Res*., 2016, **3**, 49–55.
4. Li, H. *et al.*, Enhanced efficiency of cadmium removal by *Boehmeria nivea* (L.) Gaud. in the presence of exogenous citric and oxalic acids. *J. Environ. Sci*., 2014, **26**, 2508–2516.
5. APHA, AWWA and WEF, *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, Washington (DC), 2005, 21st edn.
6. Hiscox, J. D. and Israelstam, G. F., A method for extraction of chlorophyll from leaf tissue without maceration. *Can. J. Bot.*, 1979, **57**, 1332–1334.
7. Heath, R. L. and Packer, L., Photo-peroxidation in isolated chloroplasts: kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys*., 1968, **125**, 189–198.

8. Beyer, W. F. and Fridovich, I., Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Chem*., 1987, **161**, 559–566.
9. Aebi, H., Catalase. In *Methods of Enzymatic Analysis* (ed. Bergmeyer, H. U.), Verlag Chemie, Weinheim, 1974, p. 680.
10. Nakano, Y. and Asada, K., Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.*, 1981, **22**, 867–880.
11. Hemeda, H. M. and Klein, B. P., Effects of naturally occurring antioxidants on peroxidase activity of vegetable extracts. *Egypt. J. Food Sci.*, 1990, **55**, 184–185.
12. Foyer, C. H. and Halliwell, B., The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*, 1976, **133**, 21–25.
13. Gaitonde, M. K., Spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem. J.*, 1967, **104**, 627–633.
14. Ellman, G. L., Tissue sulfhydryl groups. *Arch. Biochem. Biophys*., 1959, **82**, 70–77.
15. Anderson, M. E., Determination of glutathione and glutathione disulphide in biological samples. *Methods Enzymol.*, 1985, **113**, 548–555.
16. Hsiao, K. H., Kao, P. H. and Hseu, Z. Y., Effects of chelators on chromium and nickel uptake by *Brassica juncea* on serpentine-mine tailings for phytoextraction. *J. Hazard. Mater.*, 2007, **148**, 366–376.
17. Foyer, C. H. and Shigeoka, S., Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol*., 2011, **155**, 93–100.
18. Zhang, Y. Xu, S., Yang, S. and Chen, Y., Salicylic acid alleviates cadmium-induced inhibition of growth and photosynthesis through upregulating antioxidant defense system in two melon cultivars (*Cucumis melo* L.). *Protoplasma*, 2015, **252**, 911–924.
19. Jean, L., Bordas, F., Moussard, C. G., Vernay, P., Hitmi, A. and Bollinger, J. C., Effect of citric acid and EDTA on chromium and nickel uptake and translocation by *Datura innoxia*. *Environ. Pollut*., 2008, **153**, 555–563.
20. Lim, B., Meyer, A. J. and Cobbett, C. S., Development of glutathione-deficient embryos in Arabidopsis is influenced by the maternal level of glutathione. *Plant Biol*., 2011, **13**, 693–697.
21. Srivastava, S., Tripathi, R. D. and Dwivedi, U. N., Synthesis of phytochelatins and modulation of antioxidants in response to cadmium stress in *Cuscuta reflexa* – an angiospermic parasite. *J. Plant Physiol.*, 2004, **161**, 665–674.
22. Foyer, C. H. and Noctor, G., Stress-triggered redox signalling: what's in pROSpect? *Plant Cell Environ*., 2016, **39**, 951–964.
23. Hernández, L. E., Plata, J. S., Palmero, M. B. M., Gil, S. C., Cáceres, M. L. F., Villasante, C. O. and Escobar, C., Contribution of glutathione to the control of cellular redox homeostasis under toxic metal and metalloid stress. *J. Exp. Bot*., 2015, **66**, 2901–2911.
24. Nazi, N., Ali, A., Javad, H. and Shahram, P., Phenylalanin ammonia-lyase activity, total phenolic and flavonoid content in flowers, leaves, hulls and kernels of three pistachio (*Pistacia vera* L.) cultivars. *Am.-Eur. J. Agric. Environ. Sci*., 2012, **12**, 807–814.

ACKNOWLEDGEMENTS. C.S.S. thanks the University of Delhi for providing R&D grant vide letter no. DRCH/R&D/2013-14/4155 for the present work. D.S. thanks the Central Instrumentation Facility, Department of Botany, University of Delhi for providing the instrumentation facilities required for this experiment.

Received 1 March 2016; revised accepted 22 December 2016

doi: 10.18520/cs/v112/i10/2034-2042