Int. J. Environ. Sci. Technol. (2015) 12:3981–3992
DOI 10.1007/s13762-015-0831-0

ORIGINAL PAPER

Exogenous application of ethylenediamminetetraacetic acid enhanced phytoremediation of cadmium by *Brassica napus* L.

M. Farid[1] · S. Ali[1] · W. Ishaque[2] · M. B. Shakoor[3] · N. K. Niazi[3,4] · I. Bibi[3,4] · M. Dawood[5] · R. A. Gill[6] · F. Abbas[1]

Received: 8 December 2014 / Revised: 19 February 2015 / Accepted: 2 June 2015 / Published online: 18 June 2015

Abstract Performance of *B. napus* in phytoextraction—an in situ environment friendly technique for the cleanup of contaminated soils—was evaluated through its response to cadmium (Cd) toxicity in combination with a chelator ethylenediamminetetraacetic acid (EDTA) while growing hydroponically in greenhouse conditions under three levels of Cd (0, 10, and 50 μM) and two levels of EDTA (0 and 2.5 mM). Cadmium presence decreased plant growth, biomass and chlorophyll concentrations, while the application of EDTA enhanced plant growth by reducing Cd-induced effects in Cd-stressed plants. Addition of EDTA improved the net photosynthetic and gas exchange capacity of plants under Cd stress. Presence of Cd at 10 and 50 μM significantly increased electrolyte leakage, the production of hydrogen peroxidase (H_2O_2) and malondialdehyde (MDA) resulting into a significant reduction in the activities of catalase, guaiacol peroxidase, ascorbate peroxidase and superoxide dismutase in Cd-stressed plants. Application of EDTA at the rate of 2.5 mM alone and with combination of Cd increased the antioxidant enzymes activities and reduced the electrolyte leakage and production of H_2O_2 and MDA. The *B. napus* actively accumulated Cd when applied with EDTA in roots, stems and leaves viz. 2817, 2207 and 1238 mg kg^{-1} DW, respectively, at higher Cd level (50 μM) followed by lower level of Cd (10 μM) viz. 1704, 1366 and 763 mg kg^{-1} DW, respectively. Results showed that this technique could be useful for the remediation of heavy metal-contaminated agricultural and industrial soils.

Keywords Antioxidant enzymes · Cadmium · Chelator · EDTA · Growth · Remediation

✉ S. Ali
shafaqataligill@yahoo.com

[1] Department of Environmental Sciences and Engineering, Government College University, Allama Iqbal Road, Faisalabad 38000, Pakistan

[2] Nuclear Institute for Agriculture and Biology (NIAB), Jhang road, P.O. Box 128, Faisalabad, Pakistan

[3] Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Faisalabad 38040, Pakistan

[4] Southern Cross GeoScience, Southern Cross University, Lismore, NSW 2480, Australia

[5] Department of Environmental Sciences, Bahauddin Zakariya University, Bosan Road, Multan, Punjab, Pakistan

[6] Department of Agronomy, Institute of Crop Science College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, People's Republic of China

Introduction

Agricultural soils worldwide are contaminated with heavy metals that hinder plants to achieve their genetic prospective and crop productivity (Gill et al. 2015: Yadav 2010). Soil pollution by heavy metals has reasonably increased since last few decades due to release of wastewater and waste from anthropogenic and geological activities to these soils (Ghosh and Singh 2005a, b; Ruttens et al. 2006). Among heavy metals, Cd is nonessential and harmful metal which is usually released in soil (Yang et al. 2005; Gill 2011a; Shakoor et al. 2014). It accumulates in different parts of plants and affects their nutrient uptake potential, inhibits the plant growth and photosynthesis, alters the chloroplast structure and induces antioxidants and lipids machinery (Wang et al. 2009; Gill 2011b).

The inherent capability of *Brassica* species to hyperaccumulate metals (such as *Brassica rapa* L., *Brassica juncea*

L., *Brassica oleracea* L. and *Brassica napus* L.) makes them able to be used for phytoextraction strategies (Szczyglowska et al. 2011; Bareen 2012). The seeds of *B. napus* grown around the globe are a vital source of cooking oil, and their potential use for biodiesel production is widely documented (Ehsan et al. 2014). Hyperaccumulator plants can easily be grown and flourish in soils where high concentration of heavy metals is present, and these plants can accumulate metal ions in their different vegetative parts. Previous studies have shown that *B. napus* can be very useful candidate for phytoextraction of many heavy metals from contaminated soils because of its faster growth, high above ground biomass and high metal uptake property (Shakoor et al. 2014; Yoon et al. 2006). A plant experiences a variety of biotic and abiotic stresses throughout its growth period which limit crop productivity in terms of yield and quality (Gill et al. 2015: Ali et al. 2013). The capability of plant genotypes to clean heavy metal can vary within and between the plant species (Metwally et al. 2005).

Cadmium toxicity increases lipid peroxidation and fats in newly developing plant cells, usually by increasing concentrations of malondialdehyde (MDA). The most important antioxidant system of plants against oxidative stress is carried out by SOD (superoxide dismutase) and CAT (catalase) (Bharwana et al. 2014: Ehsan et al. 2014). Exposure to Cd stress increases the production of reactive oxygen species (ROS) which is lethal for cell components and responsible for alterations in the antioxidant systems. An increase in lipid peroxidation can be strongly limited at both production and consumption level by increasing antioxidative systems (Ehsan et al. 2014).

Soils contaminated with heavy metals can be remediated by chemical, physical and biological methods (Ghosh and Singh 2005a, b). Phytoremediation is a solar energy-driven remediation technique for the treatment of contaminated soils, polluted groundwater and wastewaters with xenobiotics (Glass 2009; Mccutcheon and Rock 2007). In the process of phytoremediation, the contaminants are transferred from soil to root and then to shoots including plant stems and leaves (Evangelou et al. 2007). Finally, roots and shoots of plants can be harvested to eliminate the pollutants from soil (Metwally et al. 2005).

Chelate addition in combination with phytoremediation results in boosting the cleanup of heavy metals from contaminated soils and wastewater (Hernández-Allica et al. 2008). Many factors are responsible for the availability of Cd in the nutrient solution such as solution chemistry, pH and root exudation. Addition of chelator increases the solubility of heavy metals in soil or culture media for plant uptake (Ghosh and Singh 2005a, b), resulting in higher metal accumulation in different plant organs (Luo et al. 2005). If metals are valuable, then they are extracted and recycled otherwise the plants are ashed and disposed off safely (Goldate and Aldina 2010). A variety of chelators is being used around the world to successfully mobilizing bound metals. These chelators include synthetic chelators, such as EDTA which is very effective for phytoextraction of Cd, chromium, lead and nickel (Greipsson 2011). EDTA has the ability to complex Cd into Cd–EDTA in the nutrient solution which avoids Cd reaction with other species. These Cd–EDTA complexes are more available because of their mobility which makes Cd near the root surface as Cd concentration.

The objectives of this study were to evaluate the phytoextraction ability of *B. napus* against Cd in the presence of EDTA and also to observe the effects of Cd and EDTA on *B. napus* growth, biomass, gas exchange attributes and antioxidant enzymes activities.

Materials and methods

Experimental design and growth sites

Mature seeds of *B. napus* genotype (variety Faisal Canola) were received from Ayub Agriculture Research Institute (AARI) Gene bank. After thorough washing with sanitized water, healthy seeds were sown in plastic trays having two inches sterilized sand and were incubated at 20–22 °C in a growth chamber. After 4 weeks of germination, uniform and healthy seedlings were transferred in hydroponically arranged setup containing modified Hoagland's nutrient solution that contain $K(NO_3)_2$ 3000 μM, $Ca(NO_3)_2$ 2000 μM, $KH_2(PO_4)$ 100 μM, $MgSO_4$ 1000 μM, H_3BO_3 50 μM, $MnCl_2.4H_2O$ 0.05 μM, $ZnSO_4.7H_2O$ 0.8 μM, $CuSO_4.5H_2O$ 0.3 μM, $H_2Mo_4.H_2O$ 0.10 μM and FeNa-EDTA 12.5 μM. All the chemicals and regents were of analytical grade and purchased from a reputed supplier company. With an air pump, aeration was supplied constantly. The solutions were renewed every 7 days. The experimental treatments were arranged in complete randomized design (CRD).

Treatments and measurements

After 2 weeks of transplanting, uniform plants were treated with $CdCl_2$ and EDTA in following combinations: T1: Control (having no EDTA and $CdCl_2$), T2: Cd (10 μM), T3: Cd (50 μM), T4: EDTA (2.5 mM), T5: Cd (10 μM) + EDTA (2.5 mM) and T6: Cd (50 μM) + EDTA (2.5 mM) with three replications, while in control, were applied. The pH of the growth medium was maintained at 6.0 ± 0.1 during the experimental period by adding 1 M NaOH or H_2SO_4 at alternate days when required.

Plant growth parameters and SPAD value

Plant height, root length and number of leaves per plant were measured after 8 weeks of treatment. Leaf area was measured by using digital leaf area meter. Data regarding fresh and dry root, stem and leaves weight were also measured. The SPAD (Soil–Plant Analyses Development) is faster way to collect chlorophyll readings. After 8 weeks of treatments, SPAD-502 (Zheijang Top Instruments Co., Ltd, China) meter was used to measure these readings.

Determination of chlorophyll contents

Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids were determined spectrophotometrically (Metzner et al. 1965).

For pigment extraction, uppermost fully expanded leaves of selected plants were reserved. The contents of photosynthetic pigment were extracted from a known fresh weight of leaves in aqueous acetone 85 % (v/v) solution. For 10 min, removal was centrifuged at 4000 rpm. Then, supernatant was collected and adulterated with aqueous acetone 85 % (v/v) solution to proper concentration for spectrophotometric measurements. At wavelengths of 452.5, 644 and 663 nm, destruction was analyzed beside the blank of a pure 85 % aqueous acetone solution. Chl a, b, total chlorophyll and carotenoids were estimated using the subsequent calculations:

$$\text{Chlorophyll a } (\mu g/L) = 10.3 * E663 - 0.98 * E644$$

$$\text{Chlorophyll b } (\mu g/L) = 19.7 * E644 - 3.87 * E663$$

$$\text{Total chlorophyll} = \text{chlorophyll a} + \text{chlorophyll b}$$

$$\text{Total carotenoids } (\mu g/L) = 4.2 * E452.5 - \{(0.0264 * \text{chl a}) + (0.426 * \text{chl b})\}$$

Lastly, these pigment fractions were calculated as mg g^{-1} fresh weight.

Gas exchange characteristics and electrolyte leakage assay

After 8 weeks of treatment, infrared gas analyzer (IRGA, CI-340, Analytical Development Company, Hoddesdon, England) was used for the measurements of stomatal conductance (Gs), photosynthetic rate (A), water use efficiency (A/E) and transpiration rate (E).

Electrolyte outflow was checked through the method defined by Dionisio-Sese and Tobita (1998). After 8 weeks of treatment applications, the uppermost completely expanded leaves were cut into small parts of 5 mm length and positioned in test tubes containing 8 ml deionized and distilled water. The tubes were processed in incubator in water bath at 32 °C for 2 h, after which the electrical conductivity of initial medium (EC1) was assessed. All of the samples were placed in autoclave at 121 °C for 20 min so that all the electrolytes were expelled out. After cooling the samples to 25 °C, final electrical conductivity (EC2) of the samples was measured and electrolyte leakage (EL) was computed with formula.

$$\text{EL} = (\text{EC1}/\text{EC2}) \times 100.$$

Antioxidant enzymes (SOD, POD, APX and CAT) and protein content assay

Antioxidant enzymes including peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and soluble protein in leaves and roots tissues were also evaluated spectrophotometrically.

Plant samples from fully stretched leaves and roots were taken for enzymatic analyses. Leaves and roots were firstly chomped with mortar and pestle under pre-chilled condition with liquid nitrogen. This pattern was standardized in 0.05 M phosphate buffer (maintaining pH at 7.8) and filtered through four layers of muslin cloth for 10 min at 4 °C centrifuged at 12,000×*g*.

For the assessment of SOD activity, the samples were homogenized in a medium having buffer of 50 mM potassium phosphate with pH 7.0 and 0.1 mM of EDTA, and 1 mM dithiothreitol (DTT) as described by Dixit et al. (2001). The SOD activity was evaluated by assessing its power to subdue the photochemical decrease in nitrobluetetrazolium (NBT) by adopting the methodology of Giannopolitis and Ries (1977). One unit of SOD activity was determined as the quantity of enzyme that induced 50 % prohibition of photochemical reduction in the NBT.

For the assessment of POD, samples were made homogenized in a potassium phosphate buffer medium which was composed of 50 mM, and its pH was 7.0, 0.1 mM EDTA and 1 mM dithiothreitol (DTT). The POD activity was calculated using method described by Chance and Maehly (1955) with few adjustments. For POD assessment, the mixture (3 ml) comprised of 50 mM phosphate buffer with pH 7.0, 20 mM guaiacol, 40 mM H2O2 and 0.1 ml of enzyme extract. The chemical reaction was started by adding up the extract of enzyme. The change in wavelength of the solution at 470 nm was read on spectrophotometer.

The activity of catalase (CAT, EC 1.11.1.6) was determined according to Aebi (1984). The assay solution (3.0 ml) was contained of 100 µl enzyme extract, 100 µl H_2O_2 (300 mM) and 2.8 ml phosphate buffer (50 mM) with 2 mM EDTA (pH 7.0). The activity of CAT was assayed by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 disappearance ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to the method of Nakano and Asada (1981). The assay mixture consisted of 100 µl enzyme aliquot, 100 µl ascorbate (7.5 mM), 100 µl H_2O_2 (300 mM) and 2.7 ml 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0). The oxidation activity of ascorbate was determined by the change in wavelength at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

The soluble protein content was analyzed according to Bradford (1976) using Coomassie Brilliant Blue G-250 as dye and albumin as a standard.

Estimation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA, a product of lipid peroxidation) content by the thiobarbituric acid (TBA) reaction using the method of Heath and Packer (1968), with minor modifications as described by Dhindsa et al. (1981) and Zhang and Kirkham (1994). A 0.25 g leaf sample was homogenized in 5 ml of 0.1 % TCA. The homogenate was centrifuged at 10,000×*g* for 5 min; 4 ml of 20 % TCA containing 0.5 % TBA was added in 1 ml aliquot of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000×*g* for 10 min, the absorbance of the supernatant at 532 nm was measured and the value for the nonspecific absorption at 600 nm was subtracted. The MDA content was calculated by using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

Hydrogen peroxide (H_2O_2) was extracted by homogenizing 50 mg leaf or root tissues with 3 ml of phosphate buffer (50 mM, pH 6.5). Then, the homogenate was centrifuged at 6000×*g* for 25 min. To measure H_2O_2 content, 3 ml of extracted solution was mixed with 1 ml of 0.1 % titanium sulfate in 20 % (v/v) H_2SO_4 and the mixture was then centrifuged at 6000×*g* for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm. Hydrogen peroxide content was computed by using the extinction coefficient of 0.28 $\mu mol^{-1}\ cm^{-1}$.

Heavy metal content and statistical analysis

Plant samples (0.5 g) were taken in a 100-ml flask, and then, 15 ml of concentrated HNO_3 was added. After mixing, the flasks were put on hot plate with gradual increase in temperature up to 275 °C that resulted in appearing of dense yellow fumes from flask. When quantity of dense yellow fumes became low, then hydrogen peroxide was added until dense yellow fumes disappeared. When samples became colorless, the flasks were removed from hot plate and shifted to laboratory at room temperature where its volume was made up to 25 ml by adding distilled water. The Cd contents in root, stem and leaf tissues were determined by using flame atomic absorption spectrometry AAS (Nov Aa 400 Analytik Jena, Germany) by using the method described by Ehsan et al. (2014) with some modifications.

The concentration of Cd in plant root, stem and leaf was measured by the following method used by Shakoor et al. (2014).

$$\text{Cd concentration } \left(\text{mg kg}^{-1}\text{DW}\right) = \text{reading of AAS} \times \text{dilution factor/dry wt. of plant organ}$$

The accumulation of Cd in plant shoot and root was estimated by the following formula:

$$\text{Cd accumulation } \left(\mu\text{g plant}^{-1}\right) = \text{conc. of Cd} \times \text{dry wt. of plant organ}$$

All values described in results section are mean of three replicates ± standard deviation (SD). Analysis of variance (ANOVA) was done by using a statistical package SPSS version 17.0 (SPSS, Chicago, IL) followed by Tukey's post hoc test between the means of treatments to determine the significant difference between mean values.

Results and discussion

Effects of Cd and EDTA on plant biomass and growth parameters

The response of *B. napus* seedlings in term of biomass and growth parameters—against Cd and EDTA application, alone and in combination—is given in Table 1. The seedlings exhibited significant and noticeable symptoms of Cd toxicity by decreasing plant height, root and shoot lengths and number of leaves per $plant^{-1}$ as compared to control one. Moreover, Cd caused significant reduction in plant biomass in dose-dependent manner, whereas the Cd-induced reduction was more obvious at higher concentration (50 µM). The correlation between Cd concentration and biomass of *B. napus* is clearly described in (Table 2), where the increase in Cd concentration caused significant reduction in fresh and dry weight of leaf, stem and root.

The addition of 2.5 mM EDTA significantly decreased Cd-induced growth and biomass inhibition characteristics. The EDTA restored the growth and biomass of *B. napus* by reducing the inhibitory effects with increasing the plant tolerance toward both levels of Cd. Application of EDTA alone showed significant increase in all parameters except leaf area, while its stress-alleviating effects were more obvious at Cd 10 µM rather than 50 µM.

Table 1 Effect of Cd and EDTA on plant root length (cm), plant height (cm), leaf area (cm^2), number of leaves per $plant^{-1}$ and fresh and dry biomass of roots, stems and leaves (g $plant^{-1}$) of *Brassica napus* L.

Treatments	Root length (cm)	Plant height (cm)	Leaf area (cm^2)	No. of leaves per $plant^{-1}$	Root fresh weight (g)	Root dry weight (g)	Stem fresh weight (g)	Stem dry weight (g)	Leaf fresh weight (g)	Leaf dry weight (g)
Control	26.10 ± 0.91b	52.49 ± 1.23b	304.66 ± 6.11a	14.66 ± 0.57b	28.74 ± 1.57b	2.20 ± 0.1ab	35.41 ± 1.06ab	3.17 ± 0.30b	57.82 ± 3.14b	5.23 ± 0.15b
EDTA	30.37 ± 1.29a	59.40 ± 2.44a	309.26 ± 7.01a	16.33 ± 0.57a	32.08 ± 0.65a	2.41 ± 0.07a	37.19 ± 0.72a	3.98 ± 0.41a	65.03 ± 3.13a	5.82 ± 0.08a
Cd10	18.70 ± 0.43d	38.56 ± 0.92d	260.078 ± 4.88c	11.16 ± 0.28d	21.41 ± 1.00d	1.77 ± 0.08c	26.48 ± 1.00c	2.33 ± 0.08 cd	31.33 ± 1.50d	4.1 ± 0.10d
Cd10 + EDTA	22.63 ± 1.08c	44.91 ± 1.11c	286.12 ± 5.29b	12.63 ± 0.41c	25.41 ± 0.89c	2.09 ± 0.11b	33.17 ± 1.10b	2.85 ± 0.13bc	35.41 ± 2.10c	4.82 ± 0.08c
Cd50	13.21 ± 0.70f	23.83 ± 0.99f	194.56 ± 4.11e	8.43 ± 0.40f	14.34 ± 0.21e	1.22 ± 0.09e	18.10 ± 0.90e	1.66 ± 0.03e	22.33 ± 1.0f	2.93 ± 0.15f
Cd50 + EDTA	15.95 ± 0.47e	31.29 ± 1.49e	231.82 ± 7.62d	9.66 ± 0.12e	19.01 ± 0.42d	1.48 ± 0.03d	23.41 ± 1.38d	2.03 ± 0.10de	27.08 ± 1.10e	3.82 ± 0.08e

Values are the means of three replications ± SD. Variants possessing the different letters are statistically significant at $P > 0.05$

Abiotic stress including heavy metal stress is attracting more attention since last few decades because in trace levels some of these heavy metals are taken as micronutrients which cause morphological, physiological and biochemical effects in living organisms. The present results describe that Cd stressful condition significantly reduced the plant growth and biomass parameters (Table 1) which might be due to the entry of larger concentration of Cd into biochemical system of the plant leading toward drastic impacts, and these results are predominantly supported by the previous researchers (Shi et al. 2010; Nwugol and Huerta 2008; Zhang et al. 2008; Vaculík et al. 2009; Song et al. 2009). EDTA-assisted increase in growth and biomass has also been observed in sweet sorghum and sudangrass seedlings (Szekely et al. 2011).

Chlorophyll contents and electrolyte leakage

The effects of different levels of Cd and EDTA on chlorophyll pigments of leaves (A) and electrolyte leakage of leaves and roots (B) are illustrated in Fig. 1. A remarkable dose-dependent decrease in chlorophyll content was observed under both level of Cd (10 and 50 μM) as compared to control one having no Cd, while the contrary effects were observed in case of electrolyte leakage.

Application of EDTA alone and in combination with Cd significantly increased chlorophyll (a, b), total chlorophyll and total carotenoids contents in leaves of *B. napus* as compared to their controls. The maximum chlorophyll content was observed at EDTA alone; however, this increase was not significantly different from control, while the EDTA addition decreased the electrolyte leakage in leaves and roots (Fig. 1B) when applied alone as well as in combination with Cd. Similarly, many researchers found beneficial effects of EDTA in many crops such as *R. globosa*, canola, Indian mustard and maize (Najeeb et al. 2009; Zhuang et al. 2007). Contrarily, decrease in growth, chlorophylls and SPAD value has been observed in many plants with EDTA application under metal stress (Chigbo and Batty 2013).

Effects of Cd and EDTA on gas exchange attributes and SPAD value

Figure 2 depicts the fluctuations in gas exchange characteristics of *B. napus* induced by Cd/EDTA alone or in combination in culture medium. A significant reduction was observed in gas exchange attributes such as transpiration rate, net photosynthetic rate, water use efficiency and stomatal conductance of *B. napus* leaves at both levels of Cd (10 and 50 μM) stress as compared to control ones and similar trend was followed by the SPAD value, while the reduction was dependent on dose concentration.

Table 2 Correlation between Cd concentration and biomass of *Brassica napus* L.

	Cd in leaves	Cd in stem	Cd in root	Fresh weight root	Dry weight root	Fresh weight stem	Dry weight stem	Fresh weight leaf
Cd in stem	0.997**							
Cd in root	1.000**	0.996**						
Fresh weight root	−0.860*	−0.889*	−0.854*					
Dry weight root	−0.820*	−0.852*	−0.816*	0.986**				
Fresh weight stem	−0.834*	−0.863*	−0.830*	0.985**	0.999**			
Dry weight stem	−0.855*	−0.883*	−0.852*	0.984**	0.965**	0.956**		
Fresh weight leaf	−0.869*	−0.902*	−0.865*	0.994**	0.983**	0.982**	0.981**	
Dry weight leaf	−0.781	−0.814*	−0.774	0.989**	0.979**	0.972**	0.974**	0.974**

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

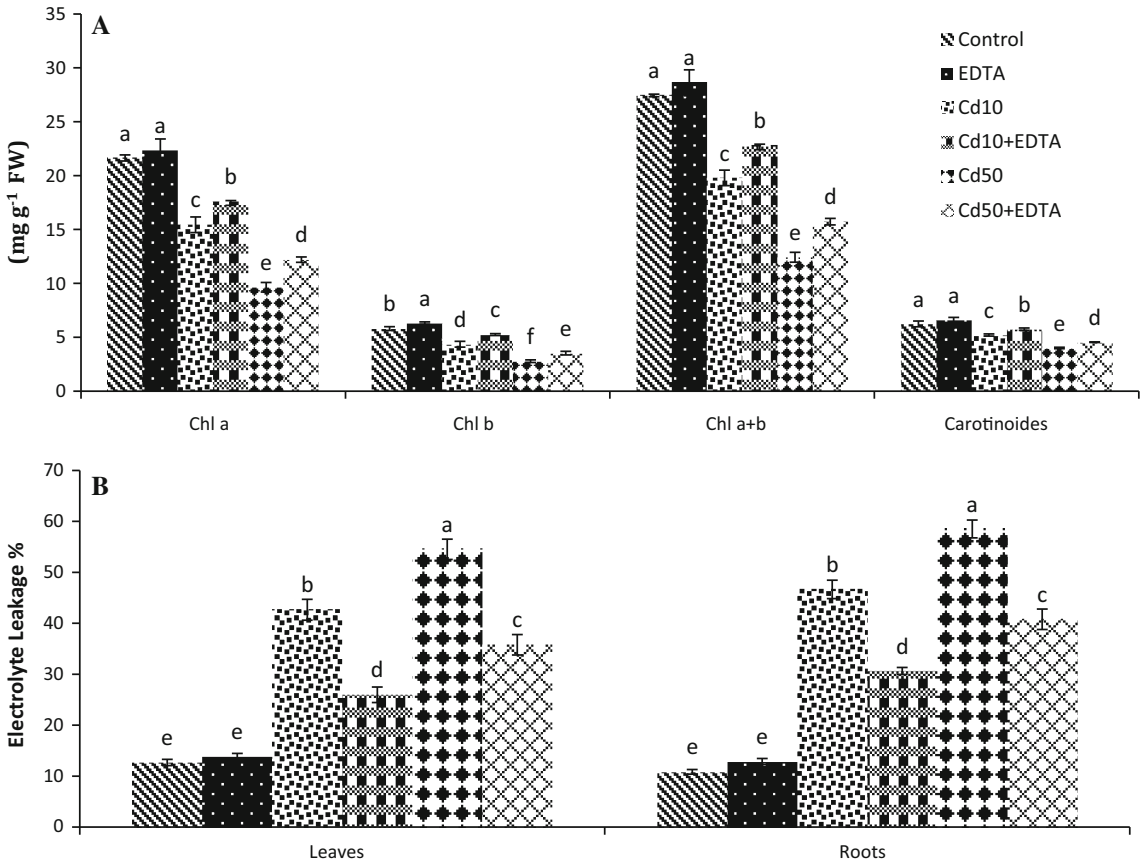

Fig. 1 Effect of Cd and EDTA on chlorophyll a, b, total chlorophyll and total carotenoids in leaves (**A**) and electrolyte leakage in roots and leaves (**B**) of *B. napus* seedlings grown in solution culture with increasing Cd concentrations (0, 10 and 50 μM) treated or not with 2.5 mM EDTA. Values are mean of three replicates ± SD. *Different small letters* indicate that values are significantly different at $P < 0.05$

Application of EDTA had a positive effect on gas exchange attributes and SPAD value of *B. napus* leaves. However, chelating behavior of EDTA seemed to be more profound and effective in decreasing the inhibitory effects of Cd stress and also improved gas exchange behavior and SPAD value. EDTA posed a distinct promoting role under

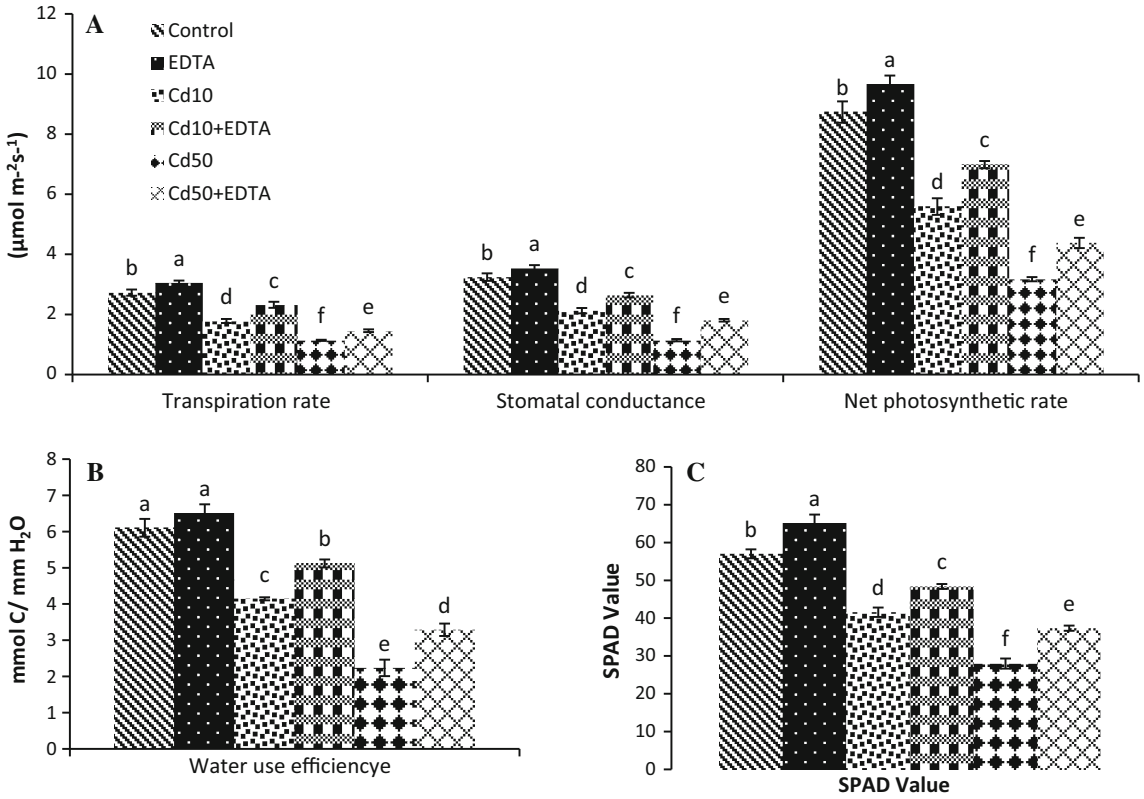

Fig. 2 Effect of Cd and EDTA on transpiration rate, stomatal conductance and net photosynthetic rate (**A**), water use efficiency (**B**) and SPAD value (**C**) of *B. napus* seedlings grown in solution culture with different Cd treatments (0, 10 and 50 µM) treated or not with 2.5 mM EDTA. Values are mean of three replicates ± SD. *Different small letters* indicate that values are significantly different at $P < 0.05$

Cd stressful conditions, and its positive effects were more pronounced when applied with Cd.

A noticeable inhibition was recorded in gas exchange attributes such as [net photosynthetic rate (Pn), water use efficiency (Pn/E), stomatal conductance (Gs), transpiration rate (E)], chlorophyll a, chlorophyll b, total chlorophyll, total carotenoids and SPAD value at higher level of Cd stress (50 µM) (Azevedo et al. 2005; Nwugol and Huerta 2008; Wahid et al. 2008; Ali et al. 2013). The plant growth, biomass, chlorophyll content and gas exchange attributes were enhanced when EDTA added which proved the positive and beneficial effects of EDTA on *B. napus* (Fig. 1A). EDTA is considered as one of the successful and admired chemical reagents because it is a powerful, recoverable and comparatively bio-stable chelator which has ability to remediate soil (Meers et al. 2005). The process of EDTA addition is considered as an important aspect controlling the leaching of metals (Luo et al. 2005).

The correlation among photosynthetic pigments, gas exchange attributes and Cd concentration in *B. napus* as shown in Table 3 clearly indicates that the Cd concentration in leaf stem and root tissues had a significant effect on these parameters.

Activities of antioxidant enzymes and protein content under Cd and EDTA

The activities of APX, SOD, POD, CAT, total soluble protein contents, H_2O_2 and MDA in roots and leaves of *B. napus* exposed to Cd and EDTA are described in Fig. 3.

Cadmium significantly affected the activities of antioxidant enzymes (Fig. 3A–C) and protein content in roots and leaves of *B. napus* at both stress levels, while the contrary trend was found in case of H_2O_2 and MDA contents (Fig. 3D, E). The application of Cd 10 µM significantly enhanced the activities of APX, SOD, POD, CAT and protein content as compared to Cd 50 µM and control one. These enhanced effects are clearly presented in Table 4 showing the correlation between Cd concentration and antioxidant enzymes activities, while in case of MDA and H_2O_2 contents, Cd-induced enhancement was more pronounced at Cd (50 µM). Addition of EDTA significantly enhanced the antioxidant enzymes activities and protein content showing a synergetic effect, while it decreased MDA and H_2O_2 activities by scavenging reactive oxygen species (ROS). The addition of Cd and EDTA alone has no significant effect on the activities of APX and POD in roots

Table 3 Correlation among Cd concentration, gas exchange attributes and photosynthetic pigments in *Brassica napus* L.

	Cd in leaves	Cd in stems	Cd in roots	Transpiration rate	Stomatal conductance	Net photosynthetic rate	Water use efficiency	Chl a	Chl b	Chl a + b
Cd in stems	0.997**									
Cd in roots	1.000**	0.996**								
Transpiration rate	−0.882*	−0.911*	−0.879*							
Stomatal conductance	−0.870*	−0.899*	−0.865*	0.994**						
Net photo synthetic rate	−0.899*	−0.925**	−0.895*	0.998**	0.996**					
Water use efficiency	−0.878*	−0.903*	−0.872*	0.993**	0.997**	0.995**				
Chl a	−0.912*	−0.934**	−0.907*	0.990**	0.993**	0.995**	0.997**			
Chl b	−0.871*	−0.895*	−0.866*	0.994**	0.993**	0.993**	0.998**	0.992**		
Chl a + b	−0.904*	−0.927**	−0.899*	0.992**	0.995**	0.996**	0.998**	1.000**	0.995**	
Carotenoids	−0.883*	−0.906*	−0.879*	0.992**	0.993**	0.994**	0.998**	0.995**	0.999**	0.997**

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

and leaves; however, significant decline was observed in SOD activity.

The incorporation of EDTA into Cd treatments enhanced the antioxidant enzymes activities and protein content and lowered the activities of MDA and H_2O_2 as compared to Cd alone. This indicates that the Cd-induced stress was alleviated by EDTA application, and an increase was also observed in EDTA-treated plants alone as compared to control.

The present results also showed that Cd decreased soluble protein in both roots and leaves of *B. napus.* It may be due to oxidative damage that inhibited the protein contents (Gupta et al. 2009). Increased reactive oxygen species (ROS) was found under metal stress as indicated by electrolyte leakage. Plants generally face the oxidative damage when exposed to Cd and other metals (Ehsan et al. 2014). Oxidative stress is indicated by decrease in antioxidant levels and increase in ROS (O_2^-, H_2O_2) accumulation under Cd stress. Similar to our observations, lipid peroxidation is reported to be induced under Cd^{2+} toxicity in *B.napus* and rice (Ehsan et al. 2014), Pb toxicity in *B.napus* (Shakoor et al. 2014), Zn^{2+} toxicity in cotton (Anwaar et al. 2014), severe water stress in wheat (Gong et al. 2005) and high temperature in tobacco (Wang et al. 2006). It is parallel to the fact that peroxidases are necessary components of plant defense system against Cd by H_2O_2 scavenging (Singh et al. 2006) as was observed in *Phaseolus vulgaris* (Smeets et al. 2005).

To meet and check the oxidative damage induced by biotic and abiotic stresses, plants have developed complex antioxidant system. Our results advocated that ROS might be produced in *B. napus* seedlings under Cd stress and cause lipid peroxidation, as shown by the increased levels of MDA and H_2O_2. There is need to tightly regulate the ROS scavenging mechanism in plants exposed to Cd stress. Plants have developed protective and repairing mechanisms for the mitigation ROS-induced damages which are the activation of antioxidant enzymes (Sharma et al. 2012). The ROS are not directly produced by Cd stress but can bind and interfere with targets. By competing for binding sites, the Cd ultimately altered the target protein functions which involve the production of ROS (Zhang et al. 2009). The present results demonstrated that the activities of antioxidant enzymes such as CAT, SOD, APX and POD both in leaves and roots were firstly increased up to 10 μM but again decreased at higher Cd level (50 μM) (Fig. 3A, B). By contrast, Cd plus EDTA addition significantly prevented accumulation of lipid peroxidation compared with the Cd treatment alone, proposing a protective role of EDTA in reducing oxidative stress in *B. napus*.

Cadmium concentration and accumulation by plant tissues

Cadmium concentration and accumulation in roots and shoots (leaves and stems) of *B. napus* are shown in Fig. 4A, B, respectively. Increase in Cd concentration in all plant parts viz. leaves, stems and root was dose dependent. At higher concentration, the Cd was significantly higher in roots irrespective of Cd levels tailed by stem and leaves. In case of Cd accumulation, the shoot showed higher cumulative Cd content rather than roots (Fig. 4B). EDTA addition significantly boosted Cd concentrations and

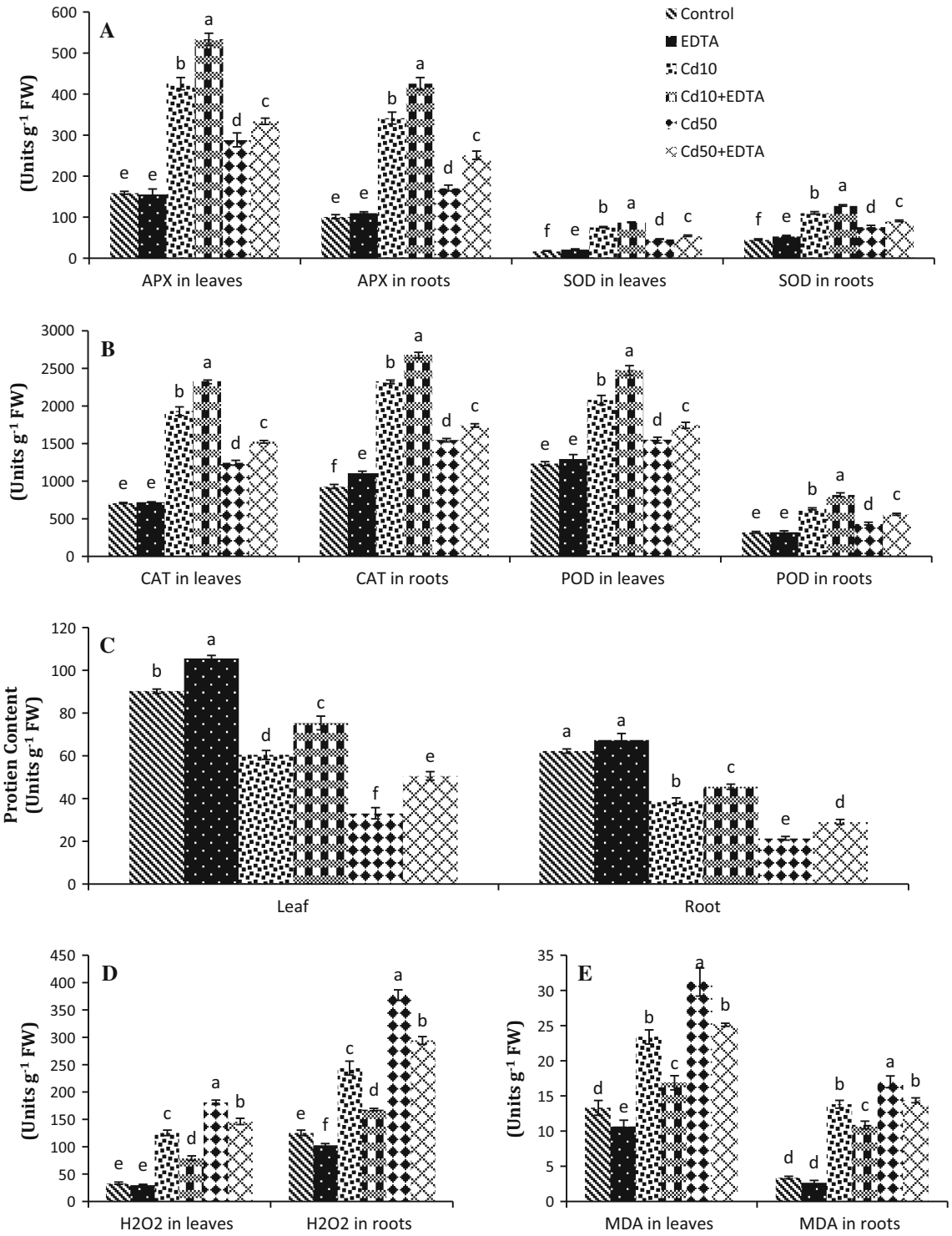

Fig. 3 Antioxidative enzyme activities (APX) and SOD (**A**), CAT and POD (**B**), protein contents (**C**), H2O2 (**D**) and MDA (**E**) in leaves and roots of *B. napus* seedlings grown in solution culture with increasing Cd concentrations (0, 10 and 50 μM) treated or not with 2.5 mM EDTA. Values are mean of three replicates ± SD. *Different small letters* indicate that values are significantly different at $P < 0.05$

accumulation in leaves, stems and roots of plants at both Cd levels. Moreover, the use of EDTA also enhanced the translocation of Cd from roots to aboveground parts of the plant (Fig. 4A, B).

Exogenous application of EDTA increased heavy metal availability because of its chelating capacity (Chigbo and Batty 2013). The main reason of greater Cd uptake in existence of chelates is possibly due to the fact that roots of plants

Table 4 Correlation among Cd concentration and antioxidant enzymes of *Brassica napus* L.

	Cd in leaf	Cd in stem	Cd in root	CAT in leaf	CAT in root	POD in leaf	POD in root	APX in leaf	APX in root	SOD in leaf
Cd in stem	0.997**									
Cd in root	1.000**	0.996**								
CAT in leaf	0.571	0.589	0.57							
CAT in root	0.516	0.538	0.515	0.994**						
POD in leaf	0.479	0.494	0.479	0.991**	0.992**					
POD in root	0.537	0.544	0.538	0.990**	0.980**	0.994**				
APX in leaf	0.57	0.587	0.569	0.998**	0.992**	0.992**	0.990**			
APX in root	0.485	0.5	0.486	0.993**	0.991**	0.996**	0.993**	0.989**		
SOD in leaf	0.588	0.611	0.587	0.996**	0.995**	0.981**	0.974**	0.993**	0.983**	
SOD in root	0.582	0.6	0.582	0.997**	0.996**	0.988**	0.985**	0.993**	0.989**	0.997**

** Correlation is significant at the 0.01 level (2-tailed)

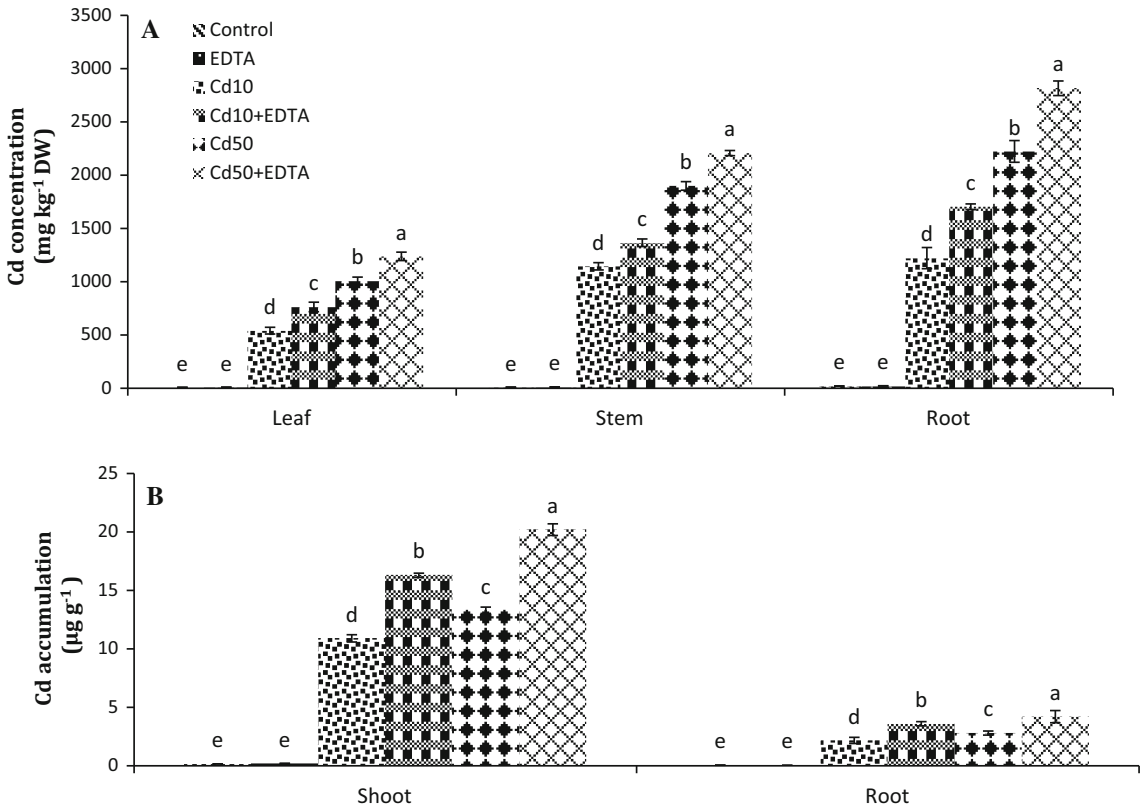

Fig. 4 Cadmium concentration (**A**) and accumulation (**B**) in leaf, stem and roots of *B. napus* seedlings grown in solution culture with different Cd treatments (0, 10 and 50 μM) treated or not with 2.5 mM EDTA. Values are mean of three replicates ± SD. *Different small letters* indicate that values are significantly different at $P < 0.05$

are able to liberate trace metals from dissolved organometallic complexes (Meers et al. 2005), and these lower stability constants of organometallic complexes agreed with greater metal uptake (Han et al. 2005). Once these complexes reach at the root surface, this surface will promote their dissociation into free Cd^{2+} particles which are more easily absorbed or taken up by roots (Wan et al. 2007). Increase in biomass of plant may be due to higher nutrient uptake (Najeeb et al. 2011) or due to synthesis of phytochelation (PCs) in plants (Muhammad et al. 2009) or due to the ability of plant species to detoxify Cd (Ghani 2011) or due to higher nutrient uptake by plants (Najeeb et al. 2011).

The Cd contents in all three parts of *B. napus* plants in present results increased as Cd concentration increased in solution culture. Luo et al. (2005) also reported that the concentrations of Cd, Cu, Pb and Zn in shoots of high biomass plants were significantly increased after EDTA application. It was observed that Cd was greatly translocated from roots to shoots in *B. napus* plants. Generally, the plants with highest resistance take up less proportion of the total solution metal and have the lowest upper ground metal contents (Ali et al. 2013.). But the application of EDTA increased the uptake of Cd in all three parts of *B. napus* such as roots, stem and leaves which indicated the greater ability of *B. napus* to uptake and accumulate Cd. The success of phytoextraction is mainly determined by the extent of biomass, heavy metals concentration in plant tissues/organs and bioavailable fraction of heavy metals in rooting medium (Wei et al. 2008).

Conclusion

In present study, it was found that Cd significantly decreased the plant growth and biomass, photosynthetic characteristics, protein content and antioxidant enzymes activities and increased electrolyte leakage, MDA and H_2O_2 content. In the meanwhile, the application of EDTA increased the morphology, photosynthetic characteristics and Cd uptake by alleviating the toxic effects of Cd stress. It can be concluded that application of EDTA at 2.5 mM is effective in reducing the toxic effect of Cd due to its chelating property. The EDTA application would be beneficial in accelerating the phytoextraction of Cd in soil and water through hyperaccumulating *B. napus* plant. Ultimately, this strategy can be used for management of soil contaminated with wastewater carrying Cd and other heavy metals sourced from industrial and anthropogenic activities.

Acknowledgments This study is the part of M.Phil. Environmental Sciences Thesis of Mujahid Farid. We are highly thankful to the Higher Education Commission (HEC), Pakistan, and Government College University, Faisalabad, Pakistan, for their financial support.

References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Ali B, Wang B, Ali S, Ghani MA, Hayat MT, Yang C, Xu L, Zhou WJ (2013) 5-Aminolevulinic acid ameliorates the growth, photosynthetic gas exchange capacity, and ultrastructural changes under cadmium stress in *Brassica napus* L. J Plant Growth Regul 32:604–614

Anwaar SA, Ali S, Ali S, Ishaque W, Farid M, Farooq MA, Najeeb U, Abbas F, Sharif M (2014) Silicon (Si) alleviates cotton (*Gossypium hirsutum* L.) from zinc (Zn) toxicity stress by limiting Zn uptake and oxidative damage. Environ Sci Pollut Res. doi:10.1007/s11356-014-3938-9

Azevedo H, Gomes C, Pinto G, Fernandes J, Loureiro S, Santos C (2005) Cadmium effects on Sunflower growth and photosynthesis, Dept of Biology, University of Aveiro, Portugal. J Plant Nutr 28:2211–2220

Bareen FE (2012) Chelate assisted phytoextraction using oilseed brassicas. Environ Pollut 21:289–311

Bharwana SA, Ali S, Farooq MA, Iqbal N, Hameed A, Abbas F, Ahmad MSA (2014) Glycine betaine-induced lead toxicity tolerance related to elevated photosynthesis, antioxidant enzymes suppressed lead uptake and oxidative stress in cotton. Turk J Bot 38:192–281

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Chem 72:248–254

Chance B, Maehly AC (1955) Assay of catalases and peroxidases. Methods Biochem Anal 136:764–775

Chigbo C, Batty L (2013) Effect of EDTA and citric acid on phytoremediation of Cr-B [a] P-co-contaminated soil. Environ Sci Pollut Res 20:8955–8963

Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. Plant Sci 135:1–9

Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Exp Bot 52:1101–1109

Ehsan S, Ali S, Noureen S, Mahmood K, Farid M, Ishaque W, Shakoor MB, Rizwan M (2014) Citric acid assisted phytoremediation of cadmium by *Brassica napus* L. Ecotoxicol Environ Safe 106:164–172

Evangelou MW, Ebel M, Schaeffer A (2007) Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity and fate of chelating agents. Chemosphere 68:989–1003

Ghani A (2011) Varietal differences in canola (*Brassica napus* L.) for the growth, yield and yield components exposed to cadmium stress. J Anim Plant Sci 21:57–59

Ghosh M, Singh SP (2005a) A review on phytoremediation of heavy metals and utilization of it's by products. Asian J Energy Environ 6:214–231

Ghosh M, Singh SP (2005b) A comparative study of cadmium phytoremediation by accumulator and weed species. Environ Pollut 1:365–371

Giannopolitis CN, Ries SK (1977) Superoxide dismutases: occurrence in higher plants. Plant Physiol 59:309–314

Gill SS, Khan NA, Tuteja N (2011a) Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. Plant Stress 5:1–23

Gill SS, Khan NA, Tuteja N (2011b) Differential cadmium stress tolerance in five Indian mustard (*Brassica juncea* L.) cultivars an evaluation of the role of antioxidant machinery. Plant Signal Behav 6:293–300

Gill RA, Zang L, Ali B, Farooq MA, Cui P, Yang S, Zhou W (2015) Chromium-induced physio-chemical and ultrastructural changes in four cultivars of *Brassica napus* L. Chemosphere 120:154–164

Glass DJ (2009) US and international markets for phytoremediation, 1999–2000. D. Glass Associates, lnc, Needham

Goldate WC, Aldina G (2010) Mixing a portion of the bottom ash with all the fly ash to obtain a stabilized material of sufficient alkalinity to prevent the leaching of toxic metals in landfills. U.S. Patent No. 7,682,446. US Patent and Trademark Office, Washington

Gong H, Zhu X, Chen K, Wang S, Zhang C (2005) Silicon alleviates oxidative damage of wheat plants in pots under drought. Plant Sci 169:313–321

Greipsson S (2011) Phytoremediation. Nat Edu Knowl 3:1–5

Gupta DK, Nicolosoa FT, Schetingerb MRC, Rossatoa LV, Pereirab LB, Castroa GY, Srivastavac S, Tripathi RD (2009) Antioxidant defense mechanism in hydroponically grown Zea mays seedlings under moderate lead stress. J Hazard Mater 172:479–484

Han F, Shan XQ, Zhang J, Xie YN, Pei ZJ, Zhang SZ, Zhu YG, Wen B (2005) Organic acids promote the uptake of lanthanum by barley roots. New Phytol 165:481–492

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hernández-Allica J, Becerril JM, Garbisu C (2008) Assessment of the phytoextraction potential of high biomass crop plants. Environ Pollut 152:32–40

Luo CL, Shen ZG, Li XD (2005) Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. Chemosphere 59:1–11

Mccutcheon SC, Rock SA (2007) Phytoremediation: state of the science conference and other developments. Int J Phytorem 3:1–11

Meers E, Ruttens A, Hopgood MJ, Samson D, Tack FMG (2005) Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. Chemosphere 58:1011–1022

Metwally A, Safronova VI, Bellimov AA, Dietz KJ (2005) Genotypic variation of the response to cadmium toxicity in *Pisumsativum* L. J Exp Bot 56:167–178

Metzner H, Rau H, Senger H (1965) Untersuchungen zur synchron isierbakeitein zelnerpi gmentmangel-mutation von chlorella. Planta 65:186–194

Muhammad D, Chen F, Zhao J, Zhang G, Wu F (2009) Comparison of EDTA- and citric acid-enhanced phytoextraction of heavy metals in artificially metal contaminated soil by Typha angustifolia. Int J Phytorem 11:558–574

Najeeb U, Xua L, Ali S, Jilani G, Gong HJ, Shen WQ, Zhou WJ (2009) Citric acid enhances the phytoextraction of manganese and plant growth by alleviating the ultrastructural damages in *Juncuseffusus*L. J Hazard Mater 170:1156–1163

Najeeb U, Jilani G, Ali S, Sarwar M, Xu L, Zhou W (2011) Insights into cadmium induced physiological and ultra-structural disorders in *Juncus effusus* L. and its remediation through exogenous citric acid. J Hazard Mater 186:565–574

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:679–690

Nwugol CC, Huerta AJ (2008) Silicon-induced cadmium resistance in rice (*Oryza sativa*). Soil Sci 171:841–848

Ruttens A, Mench M, Colpaert JV, Boisson J, Carleer R, Vangronsveld J (2006) Phytostabilization of a metal contaminated sandy soil. I: influence of compost and/or inorganic metal immobilizing soil amendments on phytotoxicity and plant availability of metals. Environ Pollut 144:524–532

Shakoor MB, Ali S, Hameed A, Farid M, Hussain S, Yasmeen T, Najeeb U, Bharwana SA, Abbasi GH (2014) Citric acid improves lead (Pb) phytoextraction in *Brassica napus* L. by mitigating pb-induced morphological and biochemical damages. Ecotoxicol Environ Saf 109:38–47

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot. doi:10.1155/2012/217037

Shi GR, Cai QS, Liu CF, Wu L (2010) Silicon alleviates cadmium toxicity in peanut plants in relation to cadmium distribution and stimulation of antioxidative enzymes. Plant Growth Regul 245:261–288

Singh S, Eapen S, D'Souza SF (2006) Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in an aquatic plant, *Bacopamonnieri* L. Chemosphere 62:233–246

Smeets K, Cuypers A, Lambrechts A, Semane B, Hoet P, Van AL, Vangronsveld J (2005) Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. Plant Physiol Biochem 43:437–444

Song S, Li Z, Zhang J, Xue G, Fan F, Liang Y (2009) Silicon-enhanced resistance to cadmium toxicity in *Brassica chinensis* L. is attributed to Si-suppressed cadmium uptake and transport and Si-enhanced antioxidant defense capacity. J Hazard Mater 172:74–83

Szczygłowska M, Piekarska A, Konieczka P, Namiesnik J (2011) Use of brassica plants in the phytoremediation and biofumigation processes. Int J Mol Sci 12:7760–7771

Szekely A, Poor P, Bagi I, Csiszar J, Gemes K, Horvath F, Tari I (2011) Effect of EDTA on the growth and copper accumulation of sweet sorghum and sudangrass seedlings. Acta Biol Szeged 55:159–164

VaculíK M, Lux A, Luxovac M, Tanimoto E, Lichtscheidl I (2009) Silicon mitigates cadmium inhibitory effects in young maize plants. Ecotoxicol Environ Saf 67:52–58

Wahid A, Ghani A, Javed F (2008) Effect of cadmium on photosynthesis, nutrition and growth of mungbean. Agron Sustain Dev 28:273–280

Wan H, Shan XQ, Liu T, Xie Y, Wen B, Zhang S, Han F (2007) Genuchten, organic acids enhance the uptake of Pb by wheat roots. Planta 225:1483–1494

Wang P, Duan W, Takabayashi A, Endo T, Shikanai T, Ye JY, Mi H (2006) Chloroplastic NAD (P) H dehydrogenase in tobacco leaves functions in alleviation of oxidative damage caused by temperature stress. Plant Physiol 141:465–474

Wang C, Sun Q, Wang L (2009) Cadmium toxicity and phytochelatins production in a rooted-submerged macrophyte Vallisneria spiralis exposed to low concentrations of cadmium. Environ Toxicol 24:271–278

Wei S, da Silva JAT, Zhou Q (2008) Agro-improving method of phytoextracting heavy metal contaminated soil. J Hazard Mater 150:662–668

Yadav SK (2010) Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S Afr J Sci 76:167–179

Yang XE, Long XX, Ye HB, He ZL, Calvert DV, Stoffella PG (2005) Cadmium tolerance and hyperaccumulation in a new Zn hyper accumulating plant species (*Sedum alfredii Hance*). Plant Soil 259:181–189

Yoon J, Cao XD, Zhou QX, Ma LQ (2006) Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. Sci Total Environ 368:456–464

Zhang J, Kirkham MB (1994) Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant Cell Physiol 35:785–791

Zhang H, Li YH, Hu LY, Wang SH, Zhang FQ, Hu KD (2008) Effects of exogenous nitric oxide donor on antioxidant metabolism in wheat leaves under aluminum stress. Russ J Plant Physiol 55:469–474

Zhang FQ, Zhang HX, Wang GP, Xu LL, Shen ZG (2009) Cadmium-induced accumulation of hydrogen peroxide in the leaf apoplast of Phaseolus aureus and Vicia sativa and the roles of different antioxidant enzymes. J Hazard Mater 168:76–84

Zhuang P, Yang QW, Wang HB, Shu WS (2007) Phytoextraction of heavy metals by eight plant species in the field water, air, and soil pollution. Int J Environ Pollut 12:234–324