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ORIGINAL RESEARCH PAPER

EDTA reduces cadmium toxicity in mustard (*Brassica juncea* L.) by enhancing metal chelation, antioxidant defense and glyoxalase systems

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Abstract
To investigate the possible role of EDTA in mitigating cadmium (Cd) toxicity, we treated mustard (*Brassica juncea* L.) seedlings with $CdCl_2$ (0.5 mM and 1.0 mM, 3 days) alone and in combination with 0.5 mM EDTA in a semihydroponic medium. In the absence of EDTA, mustard seedlings accumulated Cd in their roots and shoots in a concentration dependent manner. Overaccumulation of Cd boosted generation of hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$), increased lipoxygenase (LOX) activity, lipid peroxidation, and cytotoxic methylglyoxal (MG) content. It also disturbed components of the antioxidant defense and glyoxalase systems. Furthermore, Cd stress decreased growth, leaf relative water content (RWC) and chlorophyll (chl) content but augmented the proline (Pro) content. On the other hand, EDTA supplemented Cd-stressed seedlings improved the constituents of the AsA-GSH cycle with the upregulated activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT). Moreover, addition of EDTA to the Cd-stressed seedlings notably enhanced Gly I activity in contrast to the stress treatment. Ethylenediaminetetraacetic acid decreased Cd accumulation in the both shoots and roots, as well as increased other nonprotein thiols (NPTs) in leaves, including the phytochelatin (PC) content. It also decreased H_2O_2 and $O_2^{\bullet-}$ generation, lipid peroxidation and MG content but enhanced RWC, chl and Pro contents in the leaves, which confirmed the improved growth of seedlings. The findings of the study suggest that exogenous application of EDTA to the Cd-treated seedlings reduces Cd-induced oxidative injuries by restricting Cd uptake, increasing NPTs concentration and upregulating most of the components of their antioxidant defense and glyoxalase systems.

Keywords
abiotic stress; heavy metals; metal chelation; methylglyoxal; phytoremediation; reactive oxygen species

Introduction

As a result of anthropogenic activities, heavy metal pollution is gradually becoming an increasing problem for world agriculture. Among the heavy metals, cadmium (Cd) is

a serious environmental contaminant. The main sources of Cd are industrial effluents and phosphate fertilizers, from which it enters the environment [1]. Because of its widespread mobility and hydrophilic nature, Cd can easily enter to plant root and be transported to the upper parts and to become a serious threat for living organisms, including humans, by food-chain transfer [2,3].

Numerous studies have confirmed that excess amounts of Cd negatively affect the phenotype and physiology of plants. The general phenotypic signs of Cd-induced stress in plants are chlorosis, necrosis, epinasty of leaves, disturbance of pollen germination and tube growth, as well as overall stunted plant growth. Cadmium hampers plant metabolism because it decreases uptake of nutrients, hinders the photosynthetic process by disturbing the metabolism of chlorophyll (chl) and destroying ultrastructural components of the chloroplast, and alters nitrogen and sulfur metabolism [4–6]. As a redox-inactive metal, Cd indirectly produces more reactive oxygen species (ROS) which include singlet oxygen ($^1O_2$) and superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\bullet$). Cellular macromolecules such as lipids, proteins, and nucleic acids are oxidized by the elevated level of ROS [6,7]. Plants are able to develop numerous methods to combat Cd toxicity to diminish injurious effects by avoiding toxicity through metal-binding on to cell walls, preventing movement across cell membranes, active efflux and excretion processes, compartmentalization, and by internal chelation of the metal [8,9]. Moreover, to diminish ROS-induced oxidative stress, plant cells have well organized antioxidant defense systems which comprise nonenzymatic components including ascorbic acid (AsA), glutathione (GSH), phenolic compounds, alkaloids, α-tocopherol, nonprotein amino acids, etc., and enzymatic components including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) [6,10].

Methylglyoxal (MG), a byproduct of glycolysis, is a cytotoxic component which forms under various environmental stresses including heavy metal toxicity. It can damage cellular ultrastructure, cause mutation and even cell death. However, plants have a MG detoxification system (glyoxalase system) which consists of two vital enzymes, glyoxalase I (Gly I) and glyoxalase II (Gly II) [11,12]. However, the effectiveness of this system varies significantly due to plant type and environmental stimuli.

The exogenous use of protectants, such as plant hormones, organic acids, signalling molecules and trace elements, have become topical in current research because of their demonstrable function in enhancing abiotic stress tolerance of plants. Ethylenediaminetetraacetic acid is a polyamino carboxylic acid, broadly applied as a chemical extractant/chelator for removing heavy/toxic metals from the soil via chelation, particularly where there is little metal bioavailability [13]. Furthermore, EDTA is capable of decreasing metal phytotoxicity [14]. The function of EDTA in Cd uptake and/or decreasing its toxicity has already been studied in several plants [15–18]. In addition, EDTA enhances the solubility of different metals in the soil medium and increases their availability, uptake, and translocation from roots to shoots in most vascular plants [15,16]. Conversely, Evangelou et al. [17] reported that EDTA inhibited Cd accumulation in *Nicotiana tabacum* L. plants. Similarly, this chelating agent was observed to suppress Cd toxicity by altering Cd uptake in several plant species such as *Vigna unguiculata* (L.) [18], *Oryza sativa* L. [19], *Beta vulgaris* L. [20], and *Phaseolus vulgaris* L. [21]. In addition, it has been shown to improve the antioxidant defense as well as overall growth of plants under Cd stress conditions [16,22].

Ethylenediaminetetraacetic acid has been used in several experiments with different species of the Brassicaceae family as a metal chelator for observing a range of physiological and biochemical parameters [16]. But the function of EDTA as to whether it increases or decreases Cd uptake by plants grown in an aqueous medium is still debatable. Moreover, EDTA-dependent harmonized action of the antioxidant defense and glyoxalase systems is still unknown under Cd stress. We therefore investigated the effects of EDTA on metal accumulation, biochemical and physiological parameters, focusing on the antioxidant defense and glyoxalase systems of *Brassica juncea* (L.) Czern. under Cd stress at the early seedling stage.

Material and methods

Plant material and stress treatments

Indian mustard seeds (*Brassica juncea* L. 'BARI Sharisha-11') were sorted and sterilized with 70% ethanol, and washed several times with sterilized water. Seeds were then planted on six layers of filter paper inside 9-cm Petri plates with 10 mL of distilled water and incubated for 2 days. Following this, the Petri plates containing the mustard seedlings were transferred to a cultivation chamber, keeping 60 healthy seedlings and maintaining standard growing conditions (temperature: 25 ±2°C, relative humidity: 65–70%, and irradiance: 350 µmol photons m^{-2} s^{-1}). The seedlings were grown for 10 days, nourishing with a 5,000-fold diluted Hyponex nutrient solution (Hyponex, Japan), which contained 8% N, 6.43% P, 20.94% K, 11.8% Ca, 3.08% Mg, 0.07% B, 0.24% Fe, 0.03% Mn, 0.0014% Mo, 0.008% Zn, and 0.003% Cu. After 10 days, EDTA (0.5 mM) and Cd (0.5 mM and 1.0 mM $CdCl_2$) were applied individually and in combination, considering $CdCl_2$ 0.5 mM and 1 mM as mild and severe stressors, respectively. The treatment period was for 3 consecutive days. Although 0.5 mM and 1.0 mM $CdCl_2$ doses are not environmentally and ecologically realistic, they were selected to produce quick physiological responses within a short time (3 days). After 3 days, samples were collected randomly from each of the Petri plates for data collection on various morphophysiological characteristics. The experiment was performed in a completely randomized design (CRD) with six treatments and three replications.

Measurement of lipid peroxidation

Fresh leaves (0.5 g) of *B. juncea* seedlings were taken to measure lipid peroxidation and expressed as the thiobarbituric acid reactive substances (TBARS) content and quantified from thiobarbituric acid (TBA) according to the method of Heath and Packer [23] with a minor amendment by Hasanuzzaman et al. [24]. The content of TBARS was estimated by monitoring the absorbance at 532 nm (for correction 600 nm) spectrophotometrically.

Determination of hydrogen peroxide

The concentrations of H_2O_2 was determined from 0.5 g leaves of *B. juncea* seedlings according to Yu et al. [25]. This determination was based on the reaction of H_2O_2 with H_2SO_4 and $TiCl_4$ forming an H_2O_2–titanium complex which was monitored optically at 410 nm.

Histochemical detection of hydrogen peroxide and superoxide

Following the method Nahar et al. [26], H_2O_2 and $O_2^{\bullet-}$ formation inside *B. juncea* leaves was confirmed by histochemical staining, observing the brown and dark blue spots/patches from the reaction of 3-diaminobenzidine (DAB) and nitroblue tetrazolium chloride (NBT), respectively.

Measurement of photosynthetic pigments content

Leaf photosynthetic pigments were extracted with acetone (80%, v/v) and the amounts of chl *a* and chl *b* in the extract were determined spectrophotometrically according to the method of Arnon [27].

Measurement of growth parameters

A metric scale was used to determine shoot length in cm. Ten fresh seedlings were weighed and the mean was expressed as the fresh weight (FW) (g plant^{-1}). After that, the seedlings were oven-dried for 48 h and again weighed to establish the dry weight (DW) (g plant^{-1}).

Measurement of leaf relative water content

Relative water content (RWC) was determined gravimetrically [26]. For measuring RWC, 0.5 g of leaves were weighed as FW and dipped in Petri plates employing 20 mL distilled water inside a dark chamber. Leaves attained a constant turgid weight (TW) after 12 h, and the leaves were then oven-dried for taking DW. Finally, RWC was calculated as: $\%\ RWC = (FW - DW)/(TW - DW) \times 100$.

Measurement of proline content

The amount of free proline (Pro) present in leaf tissue was quantified with the method previously adopted by Bates et al. [28]. Fresh leaf samples were extracted with sulfosalicylic acid and mixed with acetic acid and acid ninhydrin in a ratio of (1:1:1), incubated at 100°C for 60 min, chilled in ice, and next toluene was added to separate the chromophore which was read optically at 520 nm.

Measurement of methylglyoxal level

Methylglyoxal content measurement was performed according to Wild et al. [29]. Leaf samples were extracted using perchloric acid, decolorized with charcoal and then neutralized with saturated Na_2CO_3. *N*-acetyl-L-cysteine was next added to develop an intermediate [*N*-α-acetyl-*S*-(1-hydroxy-2-oxoprop-1-yl) cysteine], which was read at 288 nm optically and calculated using a standard curve.

Extraction and measurement of ascorbate and glutathione

Harvested fresh leaf samples were extracted with meta-phosphoric acid, and the collected supernatants were employed to determine the AsA and GSH contents. The extract was neutralized (pH 7.0) with potassium-phosphate (K-P) buffer.

Total AsA and reduced AsA were determined enzymatically according to Huang et al. [30] and Hasanuzzaman et al. [24] by reading the absorbance at 265 nm and calculated using a known AsA standard curve. Before determining total AsA, 0.1 M dithiothreitol was used to reduce the oxidized AsA fraction. Dehydroascorbate (DHA) was obtained by deducting the AsA from total AsA.

The enzymatic recycling principle was employed to determine total GSH and GSSG following Yu et al. [25] and Nahar et al. [26] by reading the absorbance at 412 nm and calculated using known GSH and GSSG standard curves. Before measuring GSSG; 2-vinylpyridine masking was done to GSH. Afterwards, GSH was calculated by deducting GSSG from total GSH.

Extraction and measurement of protein content and assay of enzyme activity

Freshly harvested leaves were extracted with a commonly used enzyme extraction buffer [50 mM pH 7.0 K-P buffer, 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and (10%, v/v) glycerol] maintaining 0–4°C temperature. The collected supernatants were then used to determine free protein content and enzyme activity.

We determined free protein content following Bradford [31], where bovine serum albumin (BSA) was considered as the protein standard.

Lipoxygenase (LOX; EC: 1.13.11.12) activity was determined following the method of Doderer et al. [32] by monitoring the increase in absorbance at 234 nm using linoleic acid, and the activity was calculated using the extinction coefficient 25 mM^{-1} cm^{-1}.

Superoxide dismutase (EC: 1.15.1.1) activity was read at 560 nm by the xanthine-xanthine oxidase principle [33]. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 2.24 mM NBT, catalase (0.1 U), xanthine oxidase (0.1 U), xanthine (2.36 mM), and enzyme extract. The activity of SOD was expressed as units (amount of enzyme required to inhibit NBT reduction by 50%) min^{-1} mg^{-1} protein.

Catalase (EC: 1.11.1.6) activity was assayed by monitoring absorbance at 240 nm [24]. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution. The activity was calculated using extinction coefficient 39.4 M^{-1} cm^{-1}.

Ascorbate peroxidase (EC: 1.11.1.11) was measured with a solution mixture of K-P buffer (pH 7.0), AsA, H_2O_2, EDTA, and enzyme extract. The activity was measured by monitoring the decrease in absorbance at 290 nm for 1 min and calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} [34].

Monodehydroascorbate reductase (EC: 1.6.5.4) activity was determined following the method Hossain et al. [35] with a solution mixture of K-P buffer (pH 7.0), AsA, H_2O_2, EDTA, and enzyme extract. The activity was measured by monitoring the decrease in absorbance at 290 nm for 1 min and calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1}.

Dehydroascorbate reductase (EC: 1.8.5.1) activity was assayed following the procedure of Nakano and Asada [34]. The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM glutathione (GSH), 0.1 mM EDTA, and 0.1 mM dehydroascorbate (DHA). The activity was measured after observing the change in absorbance at 265 nm for 1 min using the extinction coefficient 14 mM^{-1} cm^{-1}.

Glutathione reductase (EC: 1.6.4.2) was assayed from the optical absorbance change at 340 nm [24]. The reaction mixture contained K-P buffer (pH 7.0), EDTA, GSSG, NADPH, and enzyme extract. The activity was calculated using the extinction coefficient 6.2 mM^{-1} cm^{-1}.

Glutathione peroxidase (EC: 1.11.1.9) activity was assayed from the absorbance change due to oxidation of NADPH at 340 nM using the extinction coefficient 6.62 mM^{-1} cm^{-1} [24,36]. The assay mixture consisted of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.12 mM NADPH, 2 mM GSH, 1 U GR, 0.6 mM H_2O_2 (as a substrate), and 20 mL of sample solution.

Glyoxalase I (EC: 4.4.1.5) activity was determined following the method of Hasanuzzaman et al. [24]. The reaction mixture contained 100 mM K-P buffer (pH 7.0), 15 mM $MgSO_4$, 1.7 mM GSH, and 3.5 mM MG. The change in absorbance was read at 240 nm activity was calculated using the extinction coefficient 6.62 mM^{-1} cm^{-1}.

Glyoxalase II (EC: 3.1.2.6) activity was estimated using the extinction coefficient 6.62 mM^{-1} cm^{-1} and observing the absorbance change at 412 nm [37]. The assay mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM *S*-D-lactoylglutathione (SLG).

Measurement of Cd content, biological concentration factor, biological accumulation coefficient, and translocation factor

To measure the root and shoot Cd concentrations, 0.1 g of dry sample was digested in 5 mL of HNO_3:$HClO_4$ (4:1, v/v) mixture. The Cd concentration in the digestate was measured by atomic absorption spectrophotometry from a standard curve of known Cd concentrations. Following Nahar et al. [26], the biological concentration factor (BCF), translocation factor (TF) and biological accumulation coefficient (BAC) were computed using the following equations:

BCF = Metal (roots)/Metal (growing media)
TF = Metal (shoots)/Metal (roots)
BAC = Metal (shoots)/Metal (growing media).

Measurement of other nonprotein thiols content (total NPTs-GSH content)

The content of nonprotein thiol was quantified according to Ellman [38] by homogenizing fresh leaves of *B. juncea* in sulfosalicylic acid (3%, w/v) using Ellman's reagent. Afterwards, other NPT contents, which includes phytochelatin (PC), were estimated by subtracting the amount of GSH from the amount of total NPTs.

Statistical analysis

There were three replications for all parameters studied. The results obtained are presented as mean ±*SE*. For each parameter, significant differences between the means were tested by one-way ANOVA and the mean differences were compared using Fisher's LSD test with the assistance of XLSTAT ver. 2017 software at $p \leq 0.05$ [39].

Results

Lipid peroxidation level, H_2O_2 content, and LOX activity

In comparison to the control plants, the TBARS level enhanced by 46% and 78% under mild and severe stress, respectively (Tab. 1). The rise in H_2O_2 content and activity of LOX was accountable for the enhancement of TBARS content or lipid peroxidation under Cd stress. Nevertheless, application of EDTA reduced the H_2O_2 level and activity of LOX, which diminished peroxidation of lipids in the *B. juncea* seedlings under Cd stress, compared to the stress treatment alone (Tab. 1).

Tab. 1 TBARS and H_2O_2 content, LOX activity, and chl content of *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress.

Treatment	TBARS content (μmol g^{-1} DW)	H_2O_2 content (nmol g^{-1} FW)	LOX activity (μmol min^{-1} mg^{-1} protein)	Chl *a* content (mg g^{-1} FW)	Chl *b* content (mg g^{-1} FW)
Control	36.5 ±1.3 ^{e}	8.68 ±0.6 ^{c}	20.9 ±1.8 ^{d}	0.73 ±0.04 ^{a}	0.30 ±0.03 ^{a}
EDTA	35.7 ±0.8 ^{e}	8.58 ±0.9 ^{c}	21.0 ±1.7 ^{d}	0.73 ±0.06 ^{a}	0.31 ±0.04 ^{a}
Cd1	53.2 ±1.6 ^{b}	13.22 ±0.4 ^{b}	33.0 ±2.8 ^{b}	0.36 ±0.02 ^{d}	0.16 ±0.02 ^{c}
Cd1+EDTA	42.4 ±2.3 ^{d}	9.34 ±1.1 ^{c}	25.0 ±1.8 ^{c}	0.66 ±0.01 ^{b}	0.26 ±0.02 ^{b}
Cd2	63.1 ±3.2 ^{a}	16.13 ±1.3 ^{a}	39.4 ±2.2 ^{a}	0.24 ±0.01 ^{e}	0.10 ±0.01 ^{d}
Cd2+EDTA	46.9 ±1.9 ^{c}	11.92 ±0.6 ^{b}	28.5 ±1.9 ^{c}	0.55 ±0.01 ^{c}	0.16 ±0.01 ^{c}

EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

Histochemical detection of H_2O_2 and $O_2^{\bullet-}$

Production of ROS, for example, H_2O_2 and $O_2^{\bullet-}$, were detected using DAB and NBT staining of leaves. In contrast to control treatments, Cd stress augmented H_2O_2 and $O_2^{\bullet-}$ production, which could be seen in the leaves of the Cd-stressed *B. juncea* seedlings. Applying EDTA to Cd-stressed plants reduced the spots on the *B. juncea* leaves generated by H_2O_2 and $O_2^{\bullet-}$ (Fig. 1).

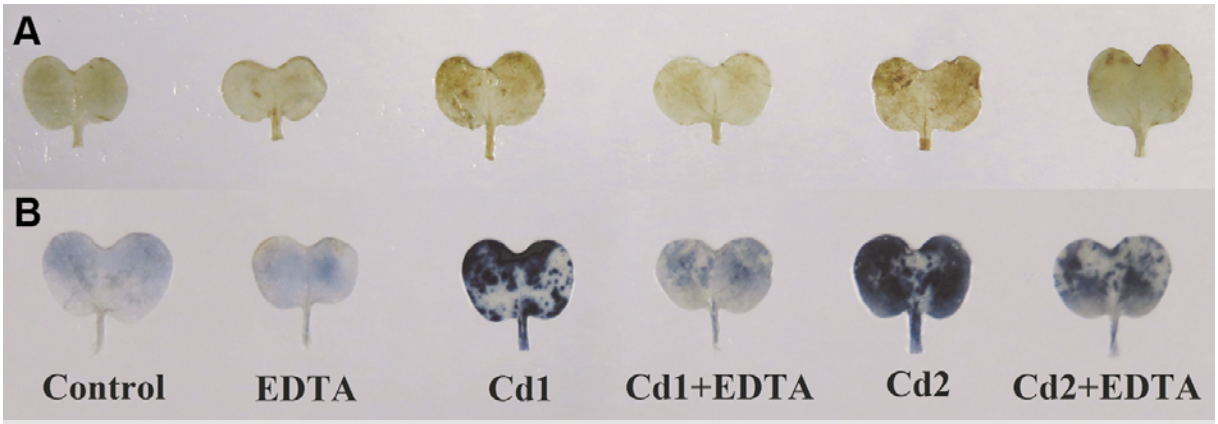

Fig. 1 Histochemical detection of H_2O_2 (**A**) and $O_2^{\bullet-}$ (**B**) in the leaves of *B. juncea* L. seedlings. EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively.

Photosynthetic pigments

Mild and severe stress decreased the chl *a* content by 51% and 67%, and chl *b* content by 47% and 66%, respectively, in contrast to the control (Tab. 1). In comparison with Cd stress alone, application of EDTA with Cd was efficient in reinstating and raising the level of chl *a* and chl *b* contents in the Cd-affected *B. juncea* seedlings (Tab. 1).

Growth parameters

In the present study, compared to the control the *B. juncea* seedlings exhibited reduced growth including shoot length, FW, and DW under Cd-induced stress (Tab. 2). Adding EDTA to the Cd-treated plants suppressed the growth inhibitory events and enhanced shoot length by 5% under both levels of stress; fresh weight by 16% and 12% for mild and severe stress, respectively; and dry weight by 12% and 9% for mild and severe stress, respectively (compared to stress treatment alone) (Tab. 2).

Proline and leaf RWC

The Cd-affected plants also suffered from water deficit stress as indicated by decreased RWC of the leaves. Enhanced proline content in the Cd-stressed seedlings further established the water deficit stress. Addition of EDTA with mild and severe stress enhanced the proline levels by 9% and 29%, respectively, which corroborates the increase in leaf RWC (Tab. 2).

Tab. 2 Growth and water status of *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress.

Treatment	Shoot length (cm)	Fresh weight (mg seedling^{-1})	Dry weight (mg seedling^{-1})	Leaf RWC (%)	Pro content (µmol g^{-1} FW)
Control	4.17 ±0.05 ^{a}	63 ±1.2 ^{a}	6.3 ±0.2 ^{a}	90.7 ±0.8 ^{a}	1.4 ±0.1 ^{c}
EDTA	4.18 ±0.10 ^{a}	62 ±1.1 ^{a}	6.3 ±0.1 ^{a}	90.9 ±0.6 ^{a}	1.4 ±0.1 ^{c}
Cd1	3.86 ±0.04 ^{cd}	52 ±1.4 ^{d}	5.5 ±0.1 ^{cd}	84.3 ±0.6 ^{c}	1.9 ±0.2 ^{b}
Cd1+EDTA	4.02 ±0.06 ^{b}	60 ±1.4 ^{b}	6.1 ±0.2 ^{ab}	87.3 ±1.1 ^{b}	2.1 ±0.2 ^{b}
Cd2	3.78 ±0.04 ^{d}	50 ±0.8 ^{d}	5.3 ±0.2 ^{d}	80.2 ±1.0 ^{d}	2.2 ±0.2 ^{b}
Cd2+EDTA	3.95 ±0.04 ^{bc}	56 ±0.9 ^{c}	5.8 ±0.1 ^{bc}	86.1 ±0.6 ^{b}	1.9 ±0.2 ^{a}

EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

Ascorbate–glutathione pool

The ascorbate content was reduced by 20% and 37% under mild and severe stress, respectively, whereas the DHA content was enhanced by 241% and 630% under mild and severe stress, respectively (Fig. 2A,B). Accordingly, the ratio of AsA/DHA declined under both mild and severe stress, in contrast to that of the control plants. The ascorbate content was restored and increased, whereas the DHA level decreased with the application of EDTA under Cd stress, causing an increase in the AsA/DHA ratio, in contrast to Cd stress alone (Fig. 2A–C). Cadmium stress at both levels resulted in an increase in the GSH level and a large increase in the GSSG level, compared with the control. Adding EDTA with Cd caused an 11% increase in the GSH level at either concentration of Cd compared with Cd stress without EDTA (Fig. 2D). In contrast, due to the addition of EDTA, GSSG levels reduced by 21% and 18% under mild and severe stress conditions, respectively, in comparison with Cd stress alone (Fig. 2E). The change in the GSH and GSSG levels resulted in a declined GSH/GSSG ratio under Cd stress, compared to the control plants. However, the GSH/GSSG ratio increased when EDTA was added to the growing medium with Cd (compared to the Cd stress treatment without EDTA) (Fig. 2F).

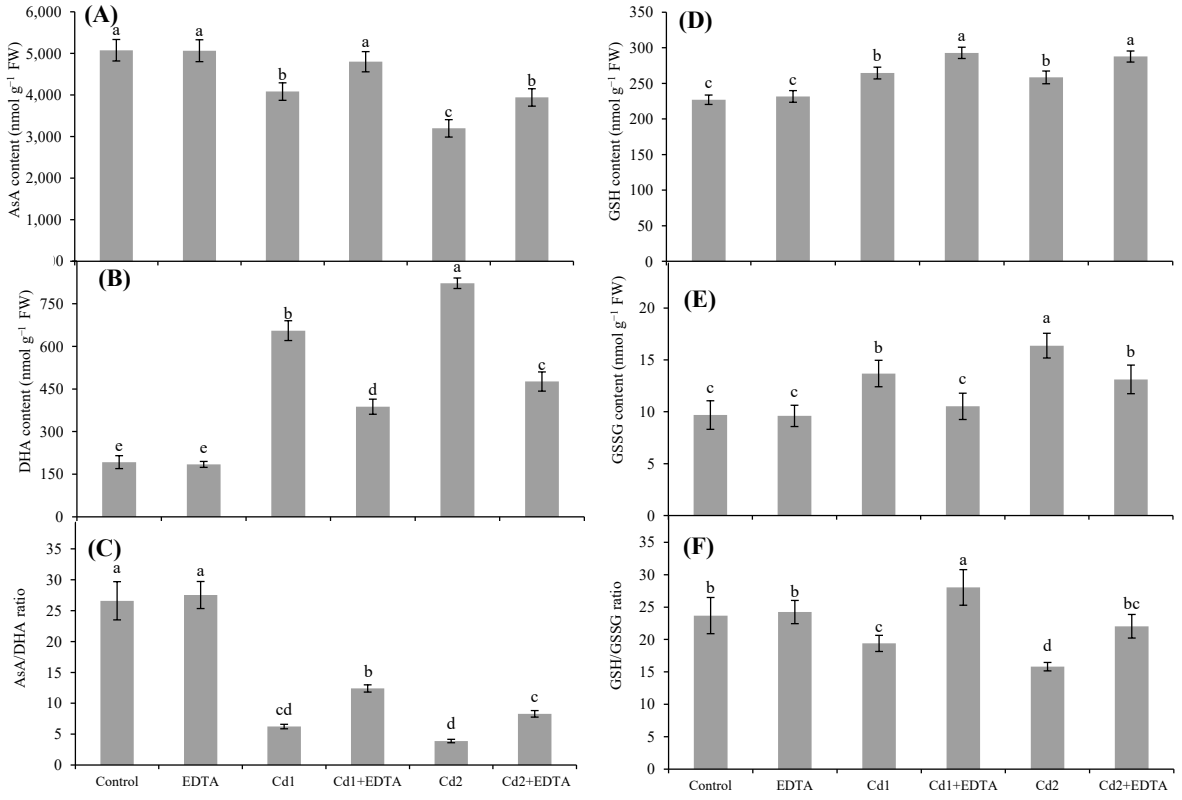

Fig. 2 AsA content (**B**), DHA content (**B**), AsA/DHA ratio (**C**), GSH content (**D**), GSSG content (**E**), and GSH/GSSG ratio (**F**) in *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress. EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

Antioxidant enzymes

The activity of APX was enhanced in a concentration-dependent manner. Its activity increased under mild stress by 16% and severe stress by 35%, in contrast to the control plants. After exogenous application of EDTA, APX activity increased again by 11% and 13% under mild and severe stress, respectively, compared to Cd stress alone (Fig. 3A). Activity of MDHAR decreased after Cd application (in contrast to the control treatment) but enhanced after adding EDTA with Cd (compared with Cd stress alone) (Fig. 3B).

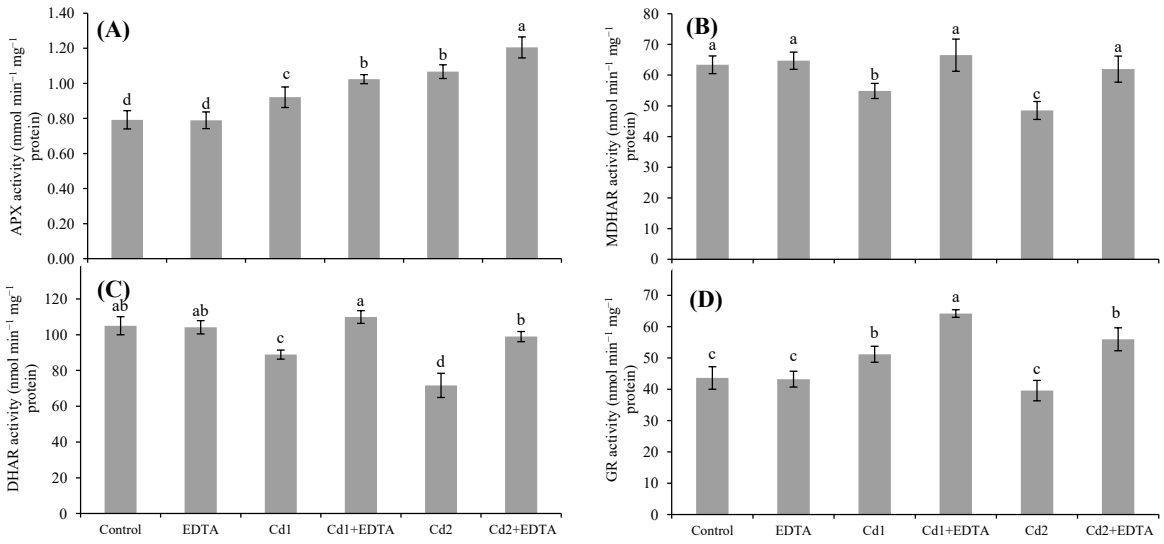

Fig. 3 Activity of APX (**A**), MDHAR (**B**), DHAR (**C**), and GR (**D**) in *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress. EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

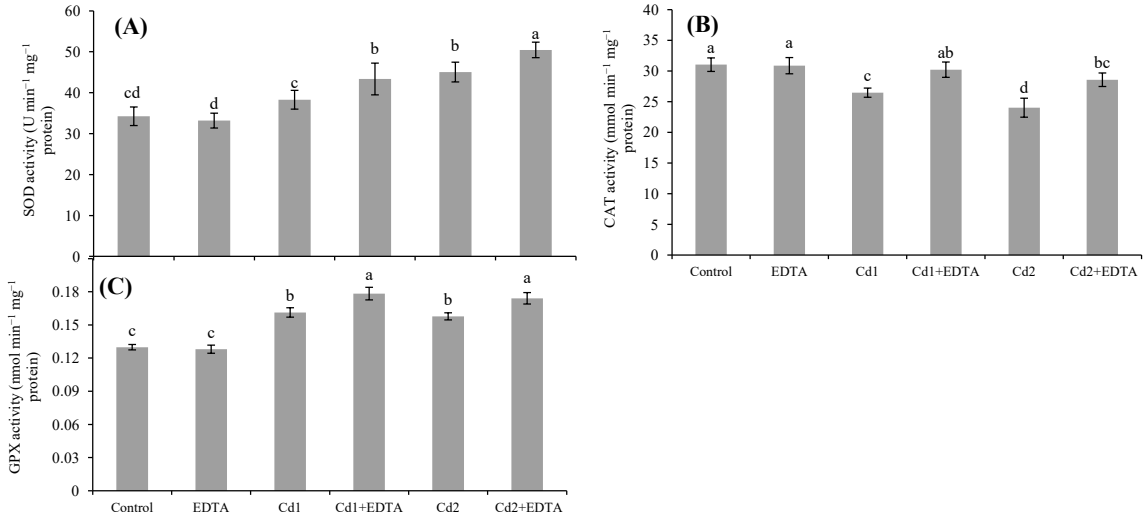

Fig. 4 Activity of SOD (**A**), CAT (**B**), and GPX (**C**) in *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress. EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

Both mild and severe stress decreased activity of DHAR by 15% and 32%, respectively (in contrast to the control). In contrast, the DHAR activity increased when EDTA was added to the Cd-treated seedlings, compared with the Cd stress alone (Fig. 3C). Activity of GR enhanced significantly under severe stress only. Applying EDTA to the Cd-treated *B. juncea* seedlings the enhanced activity of GR by 25% and 41% under mild and severe stress, respectively, compared with Cd stress alone (Fig. 3D). Mild stress was unable to enhance SOD activity significantly, but the activity sharply increased upon exposure to severe stress. Exogenous EDTA supplementation to the Cd-affected plants raised activity of SOD further, in comparison with Cd stress alone (Fig. 4A). Cadmium stress increased GPX activity compared to the control plants. An enhancement in activity of 11% was observed when the Cd-affected seedlings were supplemented with EDTA (at both levels of Cd), compared to the Cd-treated plants without EDTA (Fig. 4B). Catalase activity declined with the rise in the levels of Cd in comparison with the control

seedlings. Compared to Cd stress alone, exogenous EDTA application to the Cd-affected seedlings reversed and reinstated the CAT activity (Fig. 4C).

Methylglyoxal content and glyoxalase system

Compared to control treatments, the MG level enhanced by 94% under mild stress and 155% under severe stress resulting from exposure of *B. juncea* seedlings to Cd. Glyoxalase I activity was enhanced, but Gly II activity was reduced in the Cd-affected plants in a concentration-dependent fashion, in contrast to the control seedlings. Applying EDTA to the Cd-affected plants enhanced Gly I and Gly II activity with GSH content, which was involved in decreasing the MG content (compared to the stress alone) (Fig. 5A–C).

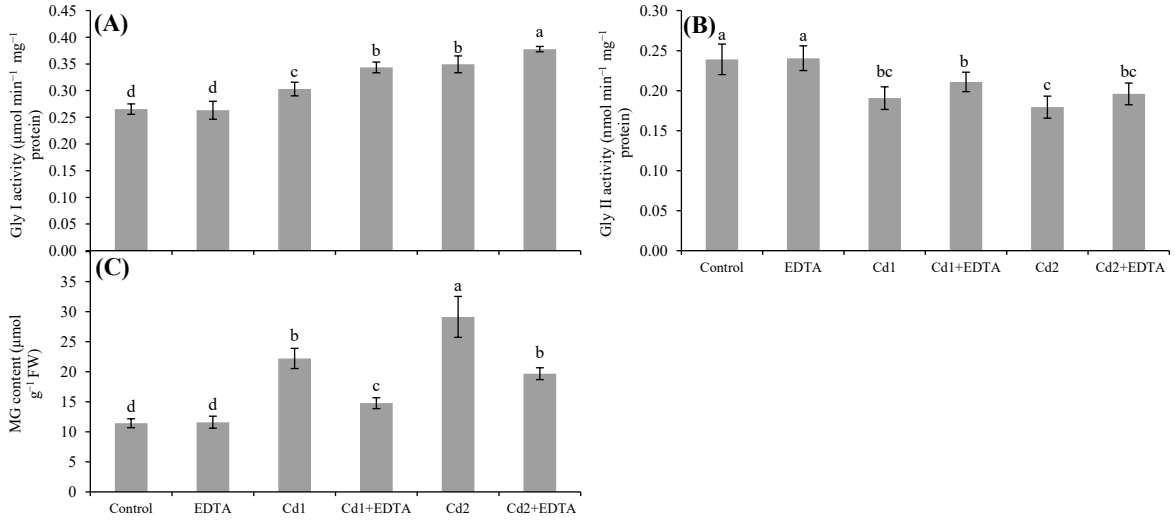

Fig. 5 Activity of Gly I (**A**), Gly II (**B**), and MG content (**C**) in *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress. EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

Cadmium content, BCF, BAC, TF, and other NPTs (NPTs-GSH) content

The Cd concentration of the *B. juncea* seedlings was determined in the roots and shoots. Exogenous Cd application resulted in Cd accumulation inside the plants where a higher Cd content was observed in the roots, compared to the shoots (Tab. 3). Adding EDTA with Cd to the plant growth medium reduced the root and shoot Cd content. In contrast to Cd stress alone, after adding EDTA, the Cd content in the shoot was reduced by 77% and 74% under mild and severe stress, respectively. On the other hand, the root Cd content was reduced by 95% and 51% under mild and severe stress, respectively (Tab. 3). The concentration of Cd in the plant growth medium, transport and accumulation of Cd from the substrate to the plant parts were revealed from the measured biochemical parameters including BCF, BAC, and TF (Tab. 3). Both levels of Cd in the substrate of seedlings showed higher BCF, BAC, and TF values (except TF under mild stress) and EDTA supplementation decreased these values (except TF under mild stress) (Tab. 3). Compared to the control, other NPT (NPTs-GSH) contents were augmented by 47% and 110% in the plants exposed to mild and severe stress, respectively. Nevertheless, supplementation of EDTA to the Cd-affected seedlings further enhanced other NPTs (total NPTs-GSH) contents by 23% and 11% under mild and severe stress, respectively, compared with the stress treatment alone (Tab. 3).

Tab. 3 Cadmium content in the shoots and roots, BCF, BAC, TF, and other nonprotein thiols content (total NPTs-GSH content) of *B. juncea* L. seedlings induced by exogenous EDTA under Cd stress.

Treatment	Cd content in shoots (mg g^{-1} DW)	Cd content in roots (mg g^{-1} DW)	Biological concentration factor (BCF)	Biological accumulation coefficient (BAC)	Translocation factor (TF)	Total NPTs-GSH content (nmol g^{-1} FW)
Control	ND	ND	-	-	-	117 ±10 [e]
EDTA	ND	ND	-	-	-	126 ±7 [e]
Cd1	7.4 ±0.2 [b]	56.8 ±1.1[b]	0.106 ±0.002 [b]	0.04 ±0.001 [b]	0.13 ±0.001 [c]	172 ±12 [d]
Cd1+EDTA	1.7 ±0.1 [d]	3.0 ±0.3 [d]	0.006 ±0.001 [d]	0.01 ±0.000 [d]	0.56 ±0.031 [a]	211 ±9 [c]
Cd2	20.2 ±0.5 [a]	79.1 ±1.0 [a]	0.143 ±0.001 [a]	0.10 ±0.003 [a]	0.26 ±0.003 [b]	245 ±7 [b]
Cd2+EDTA	5.3 ±0.2 [c]	38.6 ±0.7 [c]	0.076 ±0.001 [c]	0.03 ±0.001 [c]	0.14 ±0.003 [c]	272 ±9 [a]

EDTA, Cd1, and Cd2 denote 0.5 mM EDTA, 0.5 mM $CdCl_2$, and 1.0 mM $CdCl_2$, respectively. Mean (±*SD*) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

Discussion

Overproduction of H_2O_2, $O_2^{\bullet-}$ and TBARS contents in the *B. juncea* seedlings confirmed the oxidative stress in the current experiment. It is also evident from the photographs of the histochemical detection of H_2O_2 and $O_2^{\bullet-}$ in the leaves. Being a nonessential heavy metal, Cd is not involved in generating oxidative stress through the Fenton or Haber-Weiss reactions. Nevertheless, Cd can block the photosynthetic electron transport chain, which is the key reason for increased ROS generation. Cadmium hinders the activation of photosystem II by inhibiting electron transfer and leads to the generation of ROS [7]. Modulation of antioxidants (GSH or the GSH/GSSG ratio) by Cd is another reason for overproduction of ROS [4]. The oxidative stress markers were $O_2^{\bullet-}$, H_2O_2 and TBARS, which were enhanced in a dose-dependent fashion in the present study. Lipoxygenase activity also increased under Cd stress, which enhanced lipid peroxidation. Similar results were observed in previous experiments on Cd stress [26,40,41]. Conversely, EDTA supplementation notably decreased ROS generation and the activity of LOX as well as the TBARS level in the present study. Farid et al. [16] demonstrated that adding EDTA to Cd-stressed *Brassica napus* L. seedlings significantly prevented lipid peroxidation and H_2O_2 production compared to a Cd treatment alone. It was also reported that EDTA is involved in reducing metal-induced membrane damage in *Vetiveria zizanioides* (L.) [22], *O. sativa* [19], and *V. unguiculata* [18]. Our findings also suggest that EDTA may lessen Cd bioavailability in the nutrient solution and uptake of Cd, which reduces the Cd-induced oxidative stress.

High concentrations of metal ions in the plant growth medium may reduce water uptake through adjusting osmotic potential which is also a strategy for preventing water uptake and reducing entrance of toxic metal ions within the plant. Damage in the roots by heavy metals/Cd is another reason for inhibiting water uptake and lowering tissue the water content of plants [42]. In our present study, seedlings under Cd stress showed a reduction of tissue water content as specified by the decreased leaf RWC with a concomitant enhancement of proline content, which is well supported by a previous experiment [43]. Free Pro functions as an osmoprotectant and antioxidant, and aids in chelation of metal ions [10]. After adding EDTA, the Pro content significantly increases in Cd-stressed plants to reduce oxidative stress and cellular toxicity of Cd in rice plants [19]. Here, the data we report are similar to earlier results, in which exogenous EDTA addition with mild and severe stress enhanced the Pro levels, corroborated by the increase in leaf RWC compared to Cd stress alone.

Cadmium stress is often associated with oxidative stress and changes in metabolism such as alteration of enzyme activity, chlorophyll biosynthesis, and pigment content [40,44]. Cadmium-induced oxidative stress also damages chl. Therefore, chlorosis of leaves is a common deleterious effect of Cd stress [40]. Correspondingly, the reduction of chl content in our study resulted in chlorosis in the leaves of the seedlings. The intensity of injurious effects was dose-dependant. However, EDTA application improved the chl

content of the Cd-treated plants. Our results are in agreement with those of Niakan and Kaghazloo [45] and Farid et al. [16], who reported that EDTA increases the content of chl in *Helianthus annuus* L. and *B. napus* L. plants.

Cadmium stress is also associated with other changes in metabolism including water and nutrient uptake, inhibition of enzyme activity, reduction of photosystem efficiency and photosynthetic rate, which all result in growth inhibition [36,42,44]. In our study, the growth of the Cd-affected seedlings (shoot length, fresh and dry weight) decreased significantly, in comparison to control seedlings, which is similar to earlier reports [41,44]. Ethylenediaminetetraacetic acid significantly restores the growth and biomass of *B. napus* by reducing Cd-induced inhibition and increasing plant resistance towards Cd stress [16]. Similarly, Agbadah et al. [18] exposed *Zea mays* L. and *V. unguiculata* plants to Cd in combination with EDTA and found that EDTA considerably increased the stem length and fresh weight of Cd-stressed plants. In the present study, EDTA suppressed the inhibitory events of growth and enhanced shoot length, FW, and DW of the *B. juncea* plants. This result indicates that EDTA is responsible for reducing oxidative stress because of a reduced Cd uptake by the plants.

Ascorbate scavenges a range of ROS. The cadmium-affected seedlings showed a reduced AsA level because of a higher generation of H_2O_2 under stress conditions. Decreased AsA content is also related in upregulation of APX and downregulation of DHAR and MDHAR (enzymes which recycle DHA to AsA) activity under Cd stress, for which Cd-stressed seedlings also show elevated DHA content, increased ROS generation and oxidative stress [10]. The GSH level increases due to Cd stress, which is a general characteristic in plants under heavy metal stress [24]. The content of GSSG also increases considerably under Cd stress. Seedlings show decreased ratios of GSH/GSSG and AsA/DHA under Cd stress, which are steady with high ROS generation and oxidative stress [26,40]. The lower GSH/GSSG ratio occurs because of higher production of GSSG (indicative of oxidative stress) and lower regeneration of GSH (from GSSG), which is accomplished by GR activity [10]. Thus, higher activity of GR is advantageous to recycle GSH and enhance its content. Adding EDTA alone to the *B. juncea* seedlings slightly affected the components of antioxidant defense system. On the other hand, adding exogenous EDTA to the Cd-stressed seedlings increased the AsA/DHA ratio by increasing the AsA content and decreasing the DHA content. Similarly, the GSH/GSSG ratio also increased after applying EDTA to the Cd-stressed seedlings, which is responsible for the elevated GSH and less GSSG content. Supplementation of EDTA to the Cd-stressed plants also enhanced the enzyme activity of the AsA-GSH cycle (APX, MDHAR, DHAR, and GR). These results are almost the same as those of previous studies [16,19,22]. Farid et al. [16] recorded that treating Cd-stressed plants with EDTA notably increased protein content and antioxidant enzyme activity, whilst it reduced TBARS and H_2O_2 contents by scavenging ROS. In addition, GSH content reduction under Cd toxicity was observed in *V. zizanioides*, but applying EDTA to Cd-stressed plants showed a drastic improvement in the GSH level [22]. Therefore, EDTA may contribute a positive function to the enzymes of the AsA-GSH pool in providing resistance to Cd stress. Superoxide dismutase works as a vital enzyme which converts toxic $O_2^{\bullet-}$ to H_2O_2 [10]. Under mild Cd stress, SOD activity did not increase significantly, but under severe Cd stress it increased considerably in the *B. juncea* seedlings. However, $O_2^{\bullet-}$ and H_2O_2 generation increased under Cd stress. Catalase eradicates H_2O_2 from cell by converting it to O_2 and H_2O [46]. Reduced CAT activity caused a greater generation of H_2O_2 in the seedlings, in contrast to control plants. Moreover, GPX plays a key role in protecting components of the cell from oxidative injury by scavenging peroxides and different electrophiles [10]. Its activity upregulated in the *B. juncea* seedlings under Cd stress. Cadmium stress-induced changes in SOD, CAT, and GPX activities in our experiment similar to the results of earlier experiments on Cd [26,41]. However, adding EDTA generally increases the activity of SOD in Cd-affected plants in contrast to Cd treatment only [16,18,19]. In our experiment, EDTA decreased the $O_2^{\bullet-}$ content by increasing the SOD activity of Cd-stressed *B. juncea* seedlings and provided the foremost protection in opposition to ROS. Adding EDTA also augmented the activity of CAT and reduced the formation of H_2O_2. Similar findings were demonstrated in *B. napus* [16] and *Z. mays* L. [18] under Cd stress by the action of EDTA. Conversely, GPX activity further increased in the Cd-stressed *B. juncea* seedlings by the action of EDTA, compared to Cd stress without EDTA. These findings may suggest that EDTA

not only decreases metal uptake but also upregulates the antioxidant enzymes to protect *B. juncea* seedlings from Cd stress.

A positive improvement of the glyoxalase system is vital for plants to develop stress resistance against MG-induced oxidative damage [11,12]. Toxic MG can be detoxified efficiently by the Gly I and Gly II enzymes of the glyoxalase system in a two-step reaction. Firstly, MG is converted to SLG by Gly I, where GSH works as a cofactor, and in the second step, Gly II transforms SLG to D-lactate where GSH is recycled [11,12]. The concentration of MG in the Cd-treated *B. juncea* plants increased markedly in a concentration-dependent fashion, which was associated with upregulation of Gly I and downregulation of Gly II activity. Cadmium-induced differential responses of the glyoxalase system enzymes with high MG content were reported in an earlier study in *V. radiata* L. [26]. In our study, EDTA application in the Cd-stressed plants enhanced Gly I and Gly II activities which accelerated MG detoxification. Therefore, EDTA might have an efficient function in detoxifying MG by upregulation of the GSH level, and the activity of Gly I and Gly II.

When Cd was supplemented in the growth medium, its uptake by roots and transport into shoots of *B. juncea* plants increased in parallel with stress intensity. Cadmium accumulation was far higher in the root tissues of the *B. juncea* seedlings, in comparison with the shoot tissues. Increased Cd concentrations led to a reduction in seedling elongation as well as growth and biomass of the seedlings. Zheng et al. [41] stated that adding Cd to the growth medium resulted in higher Cd accumulation in root tissues, compared to those of the shoot, which was been substantiated by a higher reduction in root growth, compared to shoot growth. Applying EDTA to soil decreases free Cd^{2+} ions around the root surface and thereby decreases the metal uptake by plants [47]. Additionally, Jiang et al. [48] demonstrated that EDTA probably reduces solubility and bioavailability of Cd in the soil and thus decreases uptake by plants. However, a role of EDTA in increasing metal uptake including Cd has been reported in many studies [15,16]. In the present study, EDTA addition reduced metal uptake by the roots of the *B. juncea* seedlings, which was confirmed by the lower root and shoot Cd content in plants. Similar findings were observed in rice and *V. zizanioides* [19,22].

Cadmium-affected *B. juncea* seedlings show an increased rate of BCF, BAC, and TF in a concentration-dependent fashion [26]. Najmanova et al. [43] also demonstrated similar findings under Cd stress. In our study, BCF and BAC increased in a dose-dependent manner and EDTA supplementation decreased these values. However, TF increased markedly in the *B. juncea* plants after adding EDTA under mild stress and the reverse result was observed in the case of severe stress. Phytochelatin, oligomers of the γ-Glu-Cys unit, is created from GSH with the assistance of the phytochelatin synthase enzyme and attaches to the metal and transfers metal into the cell vacuole as an inert form which confirms the reduction of metal toxicity [49,50]. In our present study, Cd stress notably increased other NPTs (total NPTs-GSH) contents, probably due to higher PC content, which agrees with the findings of Nahar et al. [26]. Under elevated levels of metal toxicity, cellular PC content increases, which assists the plant to grow well [49]. Applying EDTA to the Cd-treated seedlings further enhanced the other NPTs (NPTs-GSH) containing PC content.

Our study suggested that Cd stress significantly decreased the growth, photosynthetic pigments and water status of the test plant. It also altered the antioxidative machinery due to overgeneration of ROS, MG production, and lipid peroxidation. Furthermore, applying EDTA to the Cd-treated seedlings reduced Cd accumulation and upregulated the different nonenzymatic metabolites (AsA and GSH), antioxidant enzymes activity (SOD, CAT, GPX, APX, DHAR, GR), and glyoxalase enzymes (Gly I and Gly II) which scavenged the toxic ROS (H_2O_2 and $O_2^{\bullet-}$) and MG from the plant. Thus, EDTA can be a potent candidate for conferring Cd stress but the underlying molecular mechanism to Cd stress should be further elucidated.

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EDTA obniża toksyczność kadmu w gorczycy sarepskiej (*Brassica juncea* L.) poprzez zwiększenie chelatowania metalu oraz poprawę działania systemu antyoksydacyjnego i glioksalazowego

Streszczenie

W celu określenia roli EDTA (kwas edetynowy, acidum edeticum) w obniżaniu toksyczności kadmu (Cd), siewki gorczycy (*Brassica juncea* L.) traktowano $CdCl_2$ (w stężeniach 0,5 i 1 mM przez 3 dni) w kombinacjach z lub bez dodatku 0,5 mM EDTA do semi-hydroponicznego podłoża. Rośliny uprawiane w obecności Cd bez EDTA akumulowały metal w korzeniach i częściach nadziemnych proporcjonalnie do zastosowanego stężenia Cd. Akumulacja Cd indukowała produkcję nadtlenku wodoru (H_2O_2) i anionu ponadtlenkowego ($O_2^{\bullet-}$), zwiększała aktywność lipoksygenazy (LOX), peroksydację lipidów i poziom cytotoksycznego metyloglioksalu (MG). Kadm zakłócał również funkcjonowanie składników systemu antyoksydacyjnego i glioksalazowego. Ponadto, Cd negatywnie wpływał na wzrost roślin, względną zawartość wody w liściach (RWC) i stężenie chlorofilu, ale stymulował akumulację proliny (Pro). Dodatek EDTA do pożywki zawierającej Cd pozytywnie wpływał na funkcjonowanie cyklu glutationowo-askorbinianowego, zwiększając aktywność

peroksydazy askorbinianowej (APX), reduktazy monodehydroaskorbinianowej (MDHAR), reduktazy dehydroaskorbinianowej (DHAR), reduktazy glutationowej (GR), peroksydazy glutationowej (GPX), a także dysmutazy ponadtlenkowej (SOD) i katalazy (CAT). Ponadto, dodanie EDTA do środowiska wzrostu roślin traktowanych Cd zwiększyło aktywność glioksalazy I (Gly I) w roślinach, czego nie stwierdzono w roślinach traktowanych wyłącznie Cd. EDTA ograniczył akumulację Cd zarówno w korzeniach, jak i w częściach nadziemnych, zwiększając jednocześnie poziom niebiałkowych tioli (NPT), w tym fitochelatyn (PC). Dodatkowo, w roślinach traktowanych Cd w obecności EDTA stwierdzono niższy poziom H_2O_2, $O_2^{\bullet-}$ i MG oraz obniżenie peroksydacji lipidów, ale wzrost RWC, a także stężenia chlorofilu i Pro, co wpłynęło na lepszy wzrost roślin. Uzyskane wyniki sugerują, że zastosowanie egzogennego EDTA razem z Cd obniża toksyczność metalu związaną z generowaniem stresu oksydacyjnego, poprzez ograniczenie pobierania Cd, zwiększenie stężenia NPT i pozytywny wpływ na aktywność większości składników systemu antyoksydacyjnego i glioksalazowego.