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Effective citric acid and EDTA treatments in cadmium stress tolerance in pepper (*Capsicum annuum* L.) seedlings by regulating specific gene expression

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ABSTRACT

Soil contamination with toxic environmental pollutants [such as cadmium (Cd)] is becoming a serious global problem due to rapid development of social economy. To improve the growth and yield of a plant, various chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and citric acid (CA), can be applied to the soil; such application not only increases plant uptake of metals from the soil but also promotes plant absorption of micronutrient fertilizers from the medium. For this purpose, we have conducted a pot experiment using the exogenous application of CA (2.5 mM) and EDTA (2.5 mM) in pepper (*Capsicum annuum* L.) seedlings grown under the varying levels of Cd (0, 50 and 100 μM) in the soil. M]. Our results depicted that Cd addition to the soil significantly ($P < 0.05$) decreased plant growth and biomass, gas exchange attributes, and mineral uptake by *C. annuum* when compared to the plants grown without the addition of Cd. However, Cd toxicity boosted the production of reactive oxygen species (ROS) by increasing the content of malondialdehyde (MDA), which is the indication of oxidative stress in *C. annuum*, and was also manifested by hydrogen peroxide (H_2O_2) content and electrolyte leakage to the membrane-bound organelles. The results showed that the activities of various antioxidative enzymes, such as superoxidase dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), and their specific gene expression and also the content of non-enzymatic antioxidants, such as phenolic, flavonoid, ascorbic acid, and anthocyanin, initially increased with an increase in the Cd concentration in the soil. The results also revealed that the levels of soluble sugar, reducing sugar, and non-reducing sugar were decreased in plants grown under elevating Cd levels, but the accumulation of the metal in the roots and shoots of *C. annuum*, was found to be increased. The negative impacts of Cd injury were reduced by the application of EDTA and CA, which increased plant growth and biomass, improved photosynthetic apparatus, antioxidant enzymes and their gene expression, and mineral uptake, as well as diminished the exudation of organic acids and oxidative stress indicators in *C. annuum* by decreasing Cd toxicity. Here, we conclude that the application of EDTA and CA under the exposure to Cd stress significantly improved plant growth and biomass, photosynthetic pigments, and gas exchange

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characteristics; regulated antioxidant defense system and essential nutrient uptake; and balanced organic acid exudation pattern in *C. annuum*.

1. Introduction

In recent decades, rapid increases in urbanization and industrialization have caused the excessive release of toxic metals in farmlands with damaging effects on ecosystems (Ashraf et al., 2017; Zainab et al., 2021). Toxic metal accumulation in soils is of great concern in agricultural production due to its adverse effects on food safety and marketability, crop growth due to phytotoxicity, and the environmental health of soil organisms (Madhu and Sadagopan, 2020; Khan et al., 2021). Among various toxic pollutants, cadmium (Cd) is a more prominent toxic pollutant due to its severe toxicity and ability to induce damage to normal growth and development (Adrees et al., 2020; Afzal et al., 2020). Cd, a known toxic metal, possesses properties of water solubility, phytotoxicity, higher rates of relative mobility, and can induce toxicity to membrane-bound organelles (Faizan et al., 2021; Ma et al., 2022c). Furthermore, Cd-toxicity damages plant cells including chloroplasts, cell nuclei, and mitochondria leading to a reduction in chlorophyll (Dad et al., 2021) and also causes the formation of reactive oxygen species (ROS) arises that do not partake in Fenton-type reactions, which ultimately initiates destructive pathways in plants (Heile et al., 2021). The generation of ROS are easily handled by plants through adopting extremely effective endogenous approaches for the mitigation of ROS by implementing enzymatic and non-enzymatic antioxidants of their constituents (Saleem et al., 2020a; Perveen et al., 2021). The principal antioxidant enzymes are catalase (CAT), ascorbate peroxide (APX), and superoxide dismutase (SOD) which were characterized by converter hydrogen peroxide (H_2O_2), and superoxide (O^{-2}) to mitigate toxicity in plants and reduce the concentration of MDA and H_2O_2 (Bashir et al., 2018; Javed et al., 2020). Therefore, it remains a priority to decrease the concentration of Cd in nutritional crops to limit transmission into the food chain.

Different chelating agents have been used to enhance metal solubility in the soil. Ethylenediaminetetraacetic acid (EDTA) is one of the most effective chelating agents for artificially increasing solubility, complexation, and uptake of Cd and several other toxic metals (Farid et al., 2013). Thus, the addition of EDTA into the soil induces uptake and translocation of toxic metals from the roots to shoots of plants (Saleem et al., 2020b) and is especially important for increased uptake and translocation of toxic metals. On the other hand, citric acid (CA) is a is commonly used chelating agent that desorbs metals from soil matrix into soil solution and facilitate their uptake by plants (Najeeb et al., 2011; Maqbool et al., 2018). Although the combined application of EDTA and CA improved plant growth and development and phytoextraction potential regarding various toxic metals (Turgut et al., 2004; Lesage et al., 2005; Mohammadi et al., 2021). Previous studies of the tolerance to and accumulation of toxic metals by pepper (*Capsicum annuum* L.) indicated that this plant has different toxic metal utilization in different growth mediums (Pal et al., 2018; Altaf et al., 2022). In the solanaceous family, *C. annuum* is an important horticultural crop due to its economic significance. Due to its nutritional and economic importance, it is considered a valuable cash crop over the globe. Pepper fruit is an excellent source of antioxidants, vitamins, proteins, carbohydrates, fats, and phenolic compounds (Souri and Sooraki, 2019; Kaya et al., 2020; Mousavi et al., 2021; El-kazzaz et al., 2022). The reports on the physiological and biochemical response under metal toxicity of the *C. annuum* are still low in number, which warrants further investigation.

The present study explored the effects of EDTA and CA on plant growth and biomass, photosynthetic pigments and gas exchange characteristics, oxidative stress indicators and the response of various antioxidants (enzymatic and nonenzymatic) and their gene expression, nutritional status of the plant, organic acid exudation pattern and also Cd accumulation in the roots and shoots of the plants in *C. annuum* seedlings under a high concentration of Cd in the soil. Although a few studies (Turgut et al., 2004; Sinhal et al., 2010) have been conducted on Cd toxicity in different species using chelating agents, i.e., EDTA and CA. The results of this study enhance our knowledge about (i) the enhancement of Cd accumulation in *C. annuum* by using EDTA and CA and (ii) alterations in growth, gas exchange attributes, antioxidants and their gene expression due to EDTA and CA treatment in the presence of a high concentration of Cd in the soil.

2. Materials and methods

2.1. Experimental design and growth condition

The present study was conducted in the botanical garden under a greenhouse environment. The mature and healthy pepper seeds (*Capsicum annum* L. Var. Ca-59) were used in this experiment and experiment was performed in a plant growth room under specific conditions (the overall average day/night temperature was 19 ± 3/10 ± 2 °C, with a relative humidity of 62.0–65.1%, and the day length averaged 10–11 h per day, respectively). The same variety of pepper was used by Altaf et al. (2022) in their experiment under vanadium stress. Before seed sowing, the seeds were carefully washed and sterilized in 0.1% $HgCl_2$ solution for 1 min and then washed thrice with distilled water. All pots (35 cm height × 25 cm width) were covered with plastic bags. For the complete removal of cations and anions, the sand was washed with distilled water several times. After that, in each pot, about 15 seeds were sown and each pot was kept in a greenhouse where they received natural light and air. Each pot was placed in a randomized manner with four replicates per treatment being carried out. The soil used for this experiment was air dried, passed through a 5-mm sieve, and was water saturated twice before being used in pots. The physiochemical properties of the soil used in this study are presented in Supplementary Table 1. Small pots were used in this study, each containing 5 kg of uncontaminated sand. Before starting the pot experiment, the sand was artificially spiked with various levels (0, 50 and 100 μM) of Cd by using $CdCl_2$ salt. All pots have undergone two cycles of water saturation and air drying. The experiment was executed by using a completely randomized design (CRD) with four plants per pot with four replications per treatment. In this experiment, we used an EDTA concentration (2.5 mM) which was followed by Azhar et al. (2006), Habiba et al, (2015) and CA (2.5 mM) concentration was slightly higher than used by Parveen et al. (2020). After four weeks of treatment with $CdCl_2$ alone or with EDTA and/or CA, all plants were harvested for the measurement of morphological traits, gas exchange attributes, antioxidant levels and metal accumulation in different parts of the plant.

2.2. Plant harvesting and data collection

After four weeks, remaining three seedlings were up rooted and washed gently with the help of distilled water to eliminate the aerial dust and deposition. Functional leaf in each treatment was picked at a rapid growth stage during 09:00–10:30 a.m. The sampled leaves were washed with distilled water, immediately placed in liquid nitrogen, and stored in a freezer at −80 °C for further analysis. All the harvested plants were divided into two parts i.e., roots and shoots to

study different physio-biochemical traits. Leaves from each treatment group were picked for chlorophyll, carotenoid, oxidative stress and antioxidants analysis. Root and shoot lengths were measured straightway after the harvesting by using measuring scale and digital weighting balance to measure fresh biomass. Primary branches were measured by straightway counting it and also fruit length was also measure by measuring scale. Roots were uprooted and immersed in 20 mM Na_2EDTA for 15–20 min to remove Cd adhered to the root surfaces. Then, roots were washed thrice with distilled water and finally once with de-ionized water and dried for further analysis. The different parts of the plant (roots and shoots) were oven-dehydrated at 65 °C for 72 h for Cd determination and the total plant dry weight was also measured. Although this experiment was conducted in pots, for the collection of organic acids, two seedlings were transferred to rhizoboxes which consisted of plastic sheet, nylon net, and wet soil (Javed et al., 2013). After 48 h, plants were taken from the rhizoboxes and the roots were washed with redistilled water to collect the exudates from the root surface. The samples were filtered through a 0.45°μm filter (MillexHA, Millipore, United States) and collected in Eppendorf tubes (Greger and Landberg, 2008). The collected samples were mixed with NaOH (0.01 M) in order to analyze the organic acids. However, the samples used for the analysis of oxalic acid were not treated with NaOH (Javed et al., 2013).

2.3. Determination of photosynthetic pigments and gas exchange characteristics

Leaves were collected for the determination of chlorophyll and carotenoid contents. For chlorophylls, 0.1 g of fresh leaf sample was extracted with 8 mL of 95% acetone for 24 h at 4 °C in the dark. The absorbance was measured by a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan) at 646.6, 663.6, and 450 nm. Chlorophyll content was calculated by the standard method of (Arnon, 1949).

Net photosynthesis (P*n*), leaf stomatal conductance *(Gs)*, transpiration rate (T*s*), and intercellular carbon dioxide concentration (C*i*) were measured from four different plants in each treatment group. Measurements were conducted between 11:30 and 13:30 on days with a clear sky. Rates of leaf *Pn, Gs, Ts*, and *Ci* were measured with a LI-COR gas-exchange system (LI-6400; LI-COR Biosciences, Lincoln, NE, USA) with a red-blue LED light source on the leaf chamber. In the LI-COR cuvette, CO_2 concentration was set as 380 mmol mol^{-1} and LED light intensity was set at 1000 mmol m^{-2} s^{-1}, which was the average saturation intensity for photosynthesis in *C. annuum* (Austin, 1990).

2.4. Determination of oxidative stress indicators

The degree of lipid peroxidation was evaluated as malondialdehyde (MDA) contents. Briefly, 0.1 g of frozen leaves were ground at 4 °C in a mortar with 25 mL of 50 mM phosphate buffer solution (pH 7.8) containing 1% polyethene pyrrole. The homogenate was centrifuged at 10,000 × *g* at 4 °C for 15 min. The mixtures were heated at 100 °C for 15–30 min and then quickly cooled in an ice bath. The absorbance of the supernatant was recorded by using a spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad, United States) at wavelengths of 532, 600, and 450 nm. Lipid peroxidation was expressed as l mol g^{-1} by using the formula: 6.45 (A532-A600)-0.56 A450. Lipid peroxidation was measured by using a method previously published by Heath and Packer (1968).

To estimate H_2O_2 content of plant tissues (root and leaf), 3 mL of sample extract was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and centrifuged at 6000×g for 15 min. The yellow color intensity was evaluated at 410 nm. The H_2O_2 level was computed by the extinction coefficient of 0.28 $mmol^{-1}$ cm^{-1}. The contents of H_2O_2 were measured by the method presented by Jana and Choudhuri (1981).

Stress-induced electrolyte leakage (EL) of the uppermost stretched leaves was determined by using the methodology of Dionisio-Sese and Tobita (1998). The leaves were cut into minor slices (5 mm length) and placed in test tubes having 8 mL distilled water. These tubes were incubated and transferred into a water bath for 2 h prior to measuring the initial electrical conductivity (EC_1). The samples were autoclaved at 121 °C for 20 min and then cooled down to 25 °C before measuring the final electrical conductivity (EC_2). Electrolyte leakage was calculated by the following formula;

$$EL = (EC_1/EC_2) \times 100$$

2.5. Determination of antioxidant enzyme activities and their gene expression

To evaluate enzyme activities, fresh leaves (0.5 g) were homogenized in liquid nitrogen and 5 mL of 50 mmol sodium phosphate buffer (pH 7.0), including 0.5 mmol EDTA and 0.15 mol NaCl. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatant was used for measurement of superoxidase dismutase (SOD) and peroxidase (POD) activities. SOD activity was assayed in 3 mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7), 56 mM nitro blue tetrazolium, 1.17 mM riboflavin, 10 mM methionine, and 100 μL enzyme extract. Finally, the sample was measured by using a spectrophotometer (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad). Enzyme activity was measured by using a method by (Ali et al., 2022) and expressed as U g^{-1} FW.

POD activity in the leaves was estimated by using the method of Sakharov and Ardila (1999) by using guaiacol as the substrate. A reaction mixture (3 mL) containing 0.05 mL of enzyme extract, 2.75 mL of 50 mM phosphate buffer (pH 7.0), 0.1 mL of 1% H_2O_2, and 0.1 mL of 4% guaiacol solution was prepared. Increases in the absorbance at 470 nm because of guaiacol oxidation was recorded for 2 min. One unit of enzyme activity was defined as the amount of the enzyme.

Catalase (CAT) activity was analyzed according to Aebi (1984). The assay mixture (3.0 mL) was comprised of 100 μL enzyme extract, 100 μL H_2O_2 (300 mM), and 2.8 mL 50 mM phosphate buffer with 2 mM ETDA (pH 7.0). The CAT activity was measured from the decline in absorbance at 240 nm as a result of H_2O_2 loss (ε = 39.4 mM^{-1} cm^{-1}).

Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981). The mixture containing 100 μL enzyme extract, 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) was used for measuring APX activity. The oxidation pattern of ascorbate was estimated from the variations in wavelength at 290 nm (ε = 2.8 mM^{-1} cm^{-1}).

The expression profile of the defense genes (i.e., Fe-SOD, POD, CAT, and APX) was carried out through RT q-PCR in rice plants grown after being treated with selected strains in a greenhouse experiment. For this, the selected gene sequences were taken from NCB1, followed by designing primers through the PrimerQuest tool; the primers are listed in Supplementary Materials Table S2. The housekeeping gene elongation factor 1-alpha (ef1) was used in the present study. Briefly, RNA was extracted from fresh rapeseed plant leaves inoculated with selected strains and ddH_2O was used as the control grown under infested and non-infested A. besseyi in greenhouse conditions after 4 days' post-inoculation (dpi) through the TRizole method. Gene-targeting primers were designed based on mRNA or expressed sequence tag (EST) for the corresponding genes as follows: Fe-SOD (F: 5′ ACGGTGTGACCACTGTGACT 3′, R: 5′ GCACCGTGTTGTTTACCATC3′), POD (F: 5′ATGTTTCGTGCGTCTCTGTC3′, R: 5′ TACGAGGGTCCGATCTTAGC 3′), CAT (F: 5′ TCGCCATGCTGAGAAGTATC 3′, R: 5′ TCTCCAGGCTCCTTGAAGTT 3′), APX (F:5′ ATGAGGTTTGACGGTGAGC 3′, R:5′ CAGCATGGGAGATGGTAGG 3′) as an internal control. The Vazyme HiScript II Q RT SuperMix Kit

(Vazyme, Nanjing, China) was used for cDNA synthesis. RT-qPCR was performed to analyze the expression profile of selected genes in rapeseed plants through a ABI 7500 Fast Real-Time PCR Detection System (Thermo Fisher Scientific, San Jose, CA, USA). The PCR machine was programmed using the following steps: initial denaturation at 95 °C for 30 s, including 40 cycles of 95 °C for 5 s, and 34 s at 60 °C. Finally, relative quantification was performed according to the comparative C method of $2-\Delta\Delta$ CT as described in Kong et al. (2021). The threshold cycle (Ct) value of actin was subtracted from that of the gene of interest to obtain the ΔCt value.

2.6. Determination of non-enzymatic antioxidants, sugars, and proline contents

Plant ethanol extracts were prepared for the determination of non-enzymatic antioxidants and some key osmolytes. For this purpose, 50 mg of dry plant material was homogenized with 10 mL ethanol (80%) and filtered through Whatman No. 41 filter paper. The residue was re-extracted with ethanol, and the 2 extracts were pooled together to a final volume of 20 mL. The determination of flavonoids (Pękal and Pyrzynska, 2014), phenolics (Bray and Thorpe, 1954), ascorbic acid (Azuma et al., 1999), anthocyanin (Lewis et al., 1998), and total sugars (Dubois et al., 1956) and also free amino acids was performed from the extracts.

Fresh leaf material (0.1 g) was mixed thoroughly in 5 mL aqueous sulphosalicylic acid (3%). The mixture was centrifuged at 10,000×g for 15 min, and an aliquot (1 mL) was poured into a test tube having 1 mL acidic ninhydrin and 1 mL glacial acetic acid. The reaction mixture was first heated at 100 °C for 10 min and then cooled in an ice bath. The reaction mixture was extracted with 4 mL toluene, and test tubes were vortexed for 20 s and cooled. Thereafter, the light absorbance at 520 nm was measured by using a UV–vis spectrophotometer (Hitachi U-2910, Tokyo, Japan). The free proline content was determined on the basis of the standard curve at 520 nm absorbance and expressed as μmol (g FW) $^{-1}$ (Bates et al., 1973).

2.7. Determination of nutrient content

For nutrient analysis, plant roots and shoots were washed twice in redistilled water, dipped in 20 mM EDTA for 3 s, and then, again, washed with deionized water twice for the removal of adsorbed metal on the plant surface. The washed samples were then oven-dried for 24 h at 105 °C. The dried roots and shoots were digested by using a wet digestion method in HNO_3: $HClO_4$ (7:3 V/V) until clear samples were obtained. Each sample was filtered and diluted with redistilled water up to 50 mL. The root and shoot contents of Fe, Ca, Mg, and P and were analyzed by using Atomic Absorption Spectrophotometer (AAS) model Agilent 240FS-AA.

2.8. Determination of root exudates analysis and Cd concentration

In order to determine the concentration of organic acids, freeze-dried exudates were mixed with ethanol (80%), and 20 μL of the solutions were injected into the C18 column (Brownlee Analytical C-183 μm; length 150 mm×4.6 mm^2, USA). Quantitative analysis of organic acids in root exudates was executed with high-performance liquid chromatography (HPLC), having a Flexer FX-10 UHPLC isocratic pump (PerkinElmer, MA, USA). The mobile phase used in HPLC was comprised of an acidic solution of aceto-nitrile containing aceto-nitrile:H_2SO_4:acetic acid in ratios of 15:4:1, respectively, and pH of 4.9. The samples were analyzed at a flow rate of 1.0 mL min^{-1} for a time period of 10 min. The inner temperature of the column was fixed at 45 °C, and quantification of organic acids was carried out at 214 nm wavelength with the help of a detector (UV–vis Series 200, USA) as described by UdDin et al. (2015). Freeze-dried samples were dissolved in redistilled water, and the pH of the exudates was recorded with LL micro-pH glass electrode by using a pH meter (ISTEK Model 4005–08007 Seoul, South Korea).

The ground samples were digested with pure HNO_3 at 190 °C for 45 min (10 min preheating, 15 min heating, 20 min cooling) in a microwave oven (Mars 6, CEM Corporation, Matthews, NC, USA) with the settings described in detail by Jezek et al. (2015). Samples were diluted with 2% HNO_3 and determined by an atomic absorption spectrophotometer (AAS), model Agilent 240FS-AA.

2.9. Statistical analysis

The normality of data was analyzed using IBM SPSS software (Version 21.0. Armonk, NY, USA: IBM Corp) through a multivariate post hoc test, followed by a Duncan's test in order to determine the interaction among significant values. Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. The analysis showed that the data in this study were almost normally distributed. The graphical presentation was carried out using Origin-Pro 2019.

3. Results

3.1. Effect of exogenous application of EDTA and CA on plant growth and photosynthetic pigments under the varying levels of Cd in the soil

In the present study, various growth and photosynthetic parameters were also measured in *C. annuum* grown under the different levels of Cd 0 (no Cd), 50 and 100 μM in the soil which were also supplied with the different exogenous levels of EDTA and CA. The data regarding plant height, primary branches, secondary branches, fruit length, fruit width, fruit fresh weight, number of fruits and fruit dry weight is presented in Fig. 1 and the data regarding the chlorophyll-a, chlorophyll-b, total chlorophyll, carotenoid content, net photosynthesis, stomatal conductance, transpiration rate and intercellular CO_2 is presented in Fig. 2. According to the results, it was noticed that the increasing levels of Cd in the soil significantly ($P < 0.05$) decreased plant growth and biomass and photosynthetic pigments in *C. annuum* without the application of EDTA and CA (Figs. 1, 2). According to the given results, increasing levels of Cd i.e., 50 and 100 μM in the soil significantly ($P < 0.05$) decreased plant height, primary branches, secondary branches, fruit length, fruit width, fruit fresh weight, number of fruits and fruit dry weight, chlorophyll-a, chlorophyll-b, total chlorophyll, carotenoid content, net photosynthesis, stomatal conductance and transpiration rate in *C. annuum*, compared to the plants grown without the treatment of Cd in the soil. The exogenous application of EDTA and CA was also applied to measured various growth (Fig. 1) and photosynthetic attributes (Fig. 2) in *C. annuum* grown under the elevating levels of Cd in the soil. The application of EDTA and CA non-significantly increased plant height, primary branches, secondary branches, fruit length, fruit width, fruit fresh weight, number of fruits and fruit dry weight, chlorophyll-a, chlorophyll-b, total chlorophyll, carotenoid content, net photosynthesis, stomatal conductance and transpiration rate at all levels of Cd in the soil, compared to the plants which were grown without the application of EDTA and CA. We have also noticed that Cd toxicity did not significantly affect intercellular CO_2 and also application of EDTA and CA did not significantly influence intercellular CO_2 in *C. annuum* under all levels of Cd in the soil (Fig. 2H).

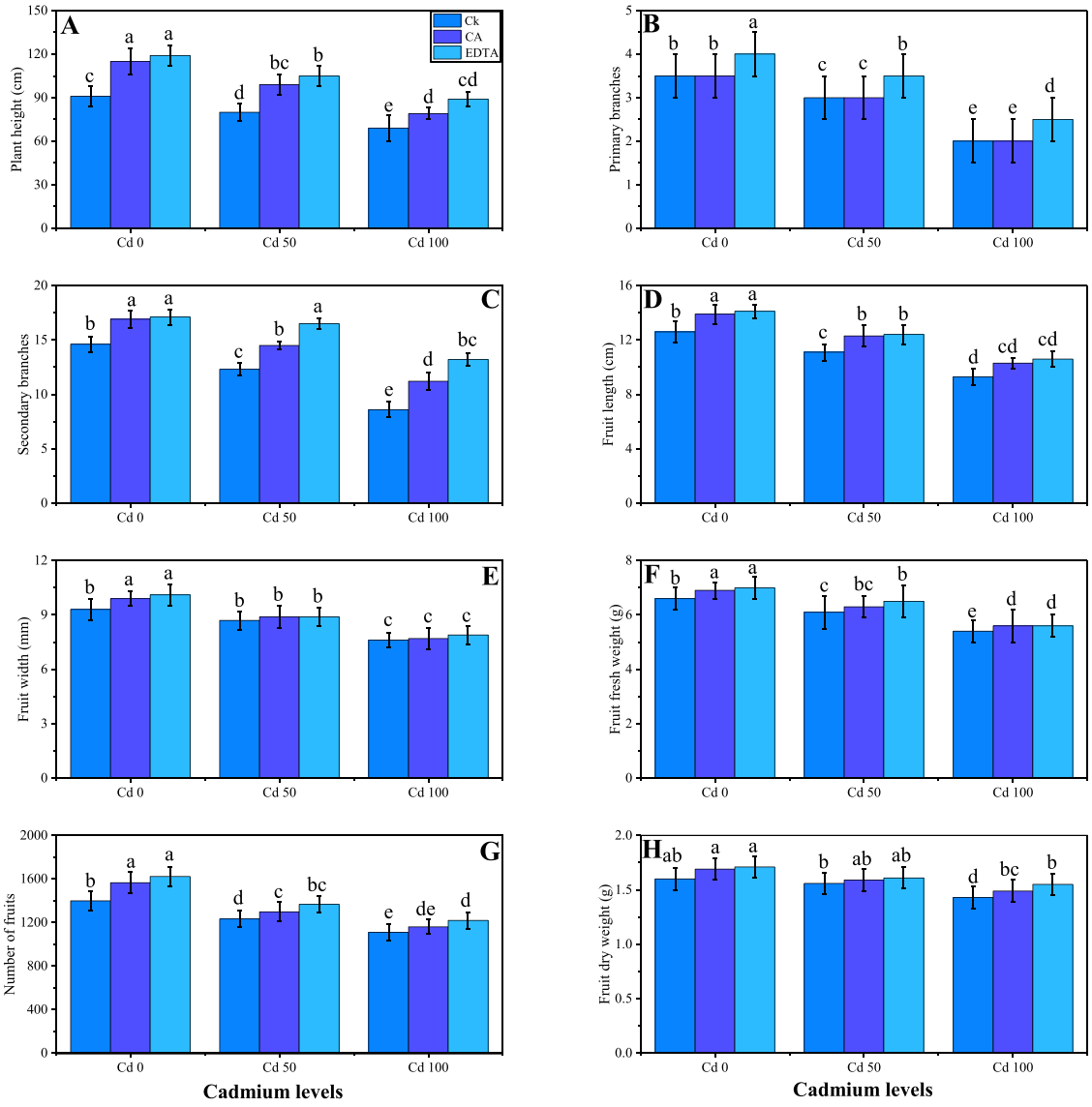

Fig. 1. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on different growth-related attributes i.e., plant height (A), primary branches (B), secondary branches (C), fruit length (D), fruit width (E), fruit fresh weight (F), number of fruits (G) and fruit dry weight (H) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD (n = 4). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

3.2. Effect of exogenous application of EDTA and CA on oxidative stress and antioxidant capacity under the varying levels of Cd in the soil

3.2.1. Oxidative stress indicators

Malondialdehyde (MDA) contents, hydrogen peroxide (H_2O_2) initiation and electrolyte leakage (%) increased in the leaves of *C. annuum* under the increasing Cd i.e., 50 and 100 μM in the soil medium without EDTA and CA as compared to the plants grown in 0 μM of Cd. The data regarding oxidative stress indicators in the leaves of *C. annuum* are presented in Fig. 3. Application of EDTA and CA significantly decreased the contents of MDA, H_2O_2 and EL (%) in the leaves grown with Cd level of 100 μM under EDTA and CA application as compared to those plants grown with 100 μM of Cd without the application of EDTA and CA.

3.2.2. Antioxidant compounds and their relative gene expression

Various antioxidant enzymes such as superoxidase dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) in the leaves of *C. annuum* seedlings and their specific gene expression such as Fe-SOD, POD, CAT, and APX and also the non-enzymatic compounds such as phenolic, flavonoid, ascorbic acid and anthocyanin contents were also measured in the present study. The data regarding the activities of enzymatic antioxidants (SOD, POD, CAT and APX) are presented in Fig. 4, and their specific gene expression such as Fe-SOD, POD, CAT, and APX are presented in Fig. 5 and also the results regarding the compounds of non-enzymatic antioxidants (phenolic, flavonoid, ascorbic acid and anthocyanin) are presented in Fig. 6. The results showed that the activities of enzymatic antioxidants (SOD, POD, CAT and APX) and their specific gene expression such as Fe-SOD, POD, CAT, and APX and the compounds of non-enzymatic antioxidants (phenolic, flavonoid, ascorbic acid and anthocyanin) were increased up to a Cd level of 100 μM in the soil. In addition, compared to the plants grown in 0 μM of Cd in the soil, the activities of enzymatic antioxidants (SOD, POD, CAT and APX) and their specific gene expression such as Fe-SOD, POD, CAT, and APX and also the compounds of non-enzymatic antioxidants (phenolic,

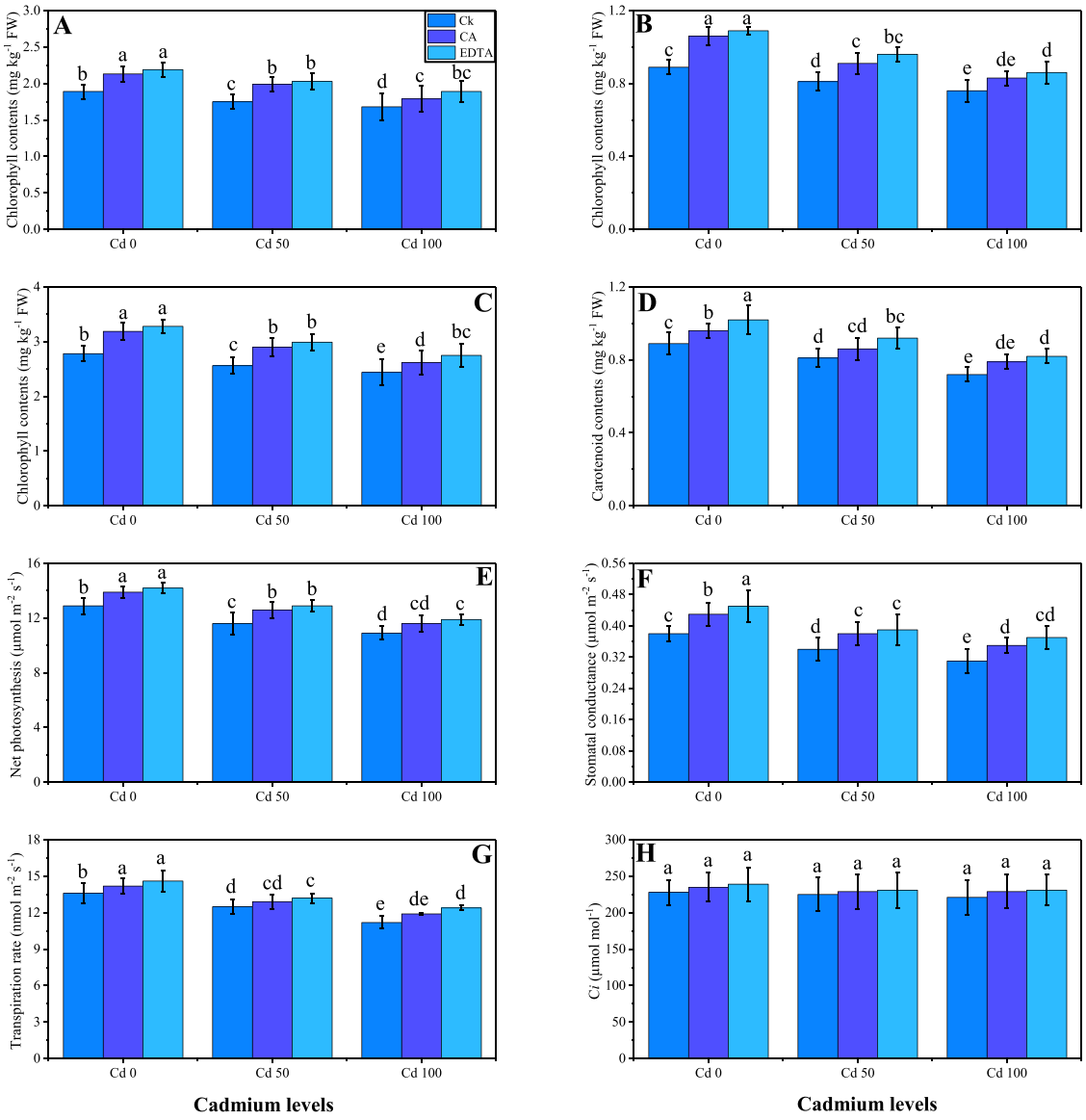

Fig. 2. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on different photosynthetic pigments, i.e., chlorophyll-a content (A), chlorophyll-b content (B), total chlorophyll content (C), carotenoid content (D), net photosynthesis (E), stomatal conductance (F), transpiration rate (G), and intercellular CO_2 (H) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD ($n = 4$). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

flavonoid, ascorbic acid and anthocyanin) were increased significantly ($P < 0.05$) in the plants grown in the Cd concentration of 100 μM in the soil. Results also showed that the exogenous application of EDTA and CA non-significantly increased the activities of enzymatic antioxidants (SOD, POD, CAT and APX) and their specific gene expression such as Fe-SOD, POD, CAT, and APX and also the compounds of non-enzymatic antioxidants (phenolic, flavonoid, ascorbic acid and anthocyanin) at all levels of Cd (no Cd), 50 and 100 μM in the soil, compared to the plants which were not applied by the EDTA and CA.

3.2.3. Sugar, proline and nutrients uptake by the plant parts

Soluble sugar, reducing sugar, non-reducing sugar, proline and various nutrients such as calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{2+}) and phosphorus (P) contents from the roots and shoots of *C. annuum*, were also measured in the present study under the different levels of Cd 0 (no Cd), 50 and 100 μM in the soil which were also supplied with the application of EDTA and CA. The data regarding the content of soluble sugar, reducing sugar, non-reducing sugar, proline is presented in Fig. 7 and the data regarding the content of Ca^{2+}, Mg^{2+}, Fe^{2+}, and P from the roots and shoots of the plants are presented in Fig. 8. Results from the present study is showing that the increasing levels of Cd in the soil significantly ($P < 0.05$) decreased the contents of nutrients (Ca^{2+}, Mg^{2+}, Fe^{2+}, and P) in the roots and shoots of the plants and also decreased the sugar content (soluble sugar, reducing sugar, non-reducing sugar), compared to the plants which were grown in the soil which was not treated with Cd. However, the content of proline was increased by increasing the levels of Cd in the soil, compared to the plants which were not treated with Cd (Fig. 6H). The application of EDTA and CA was also applied to the plants exogenously and determined various sugar (Fig. 6), phenolic and nutrient content (Fig. 7) from the shoots of the plants. Results from the present study suggested that the application of EDTA and CA non significantly increased sugar content (soluble sugar, reducing sugar, non-

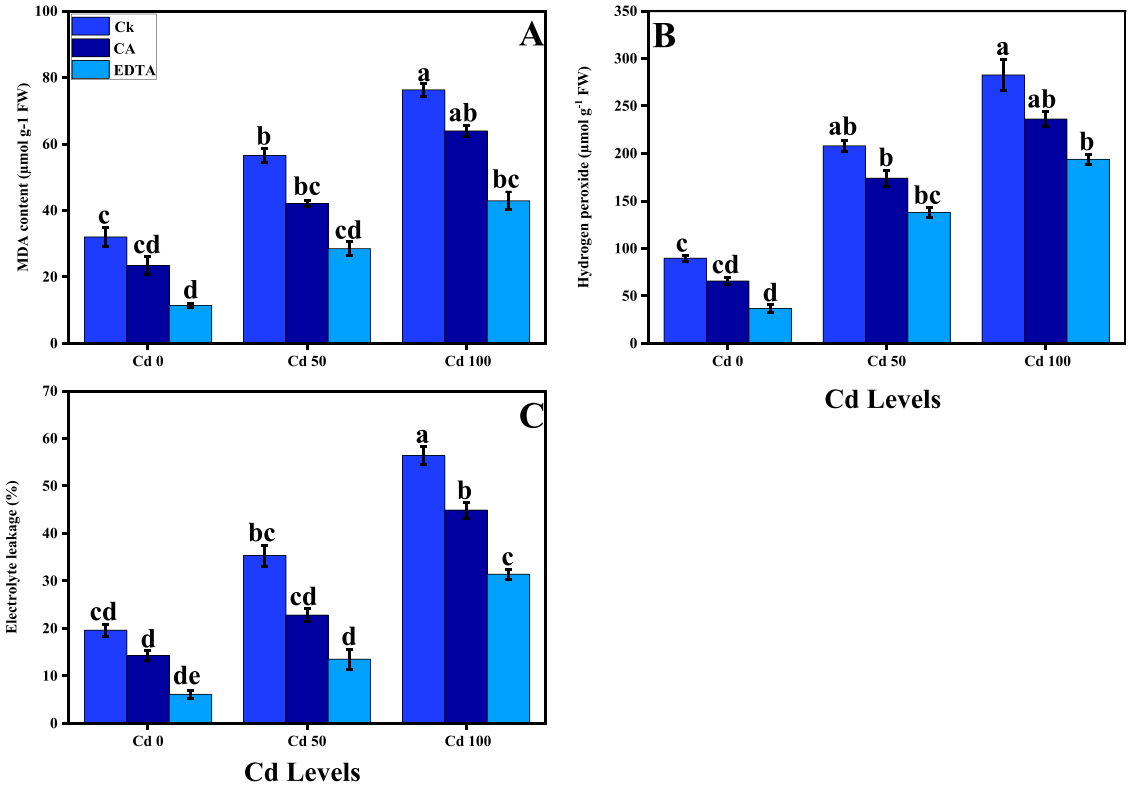

Fig. 3. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on oxidative stress indicators, i.e., MDA content in the leaves (A), H_2O_2 content in the leaves (B), and EL percentage in the leaves (C) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD ($n = 4$). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

reducing sugar), proline in the shoots and significantly increased nutrients (Ca^{2+}, Mg^{2+}, Fe^{2+}, and P) in the roots and shoots of the plants, compared to the plants grown without the treatment of EDTA and CA, at all the levels of Cd in the soil.

3.3. Effect of exogenous application of EDTA and CA on organic acids exudation and Cd uptake under the varying levels of Cd in the soil

The contents of fumaric acid, formic acid, acetic acid, citric acid, malic acid, and oxalic acid in the roots and Cd concentration in the roots and shoots of *C. annuum* grown under toxic levels of Cd in the soil, with or without the application of EDTA and CA are presented in Fig. 8. According to the given results, we have noticed that increasing the concentration of Cd in the soil (50 and 100 μM) induced a significant ($P < 0.05$) increased in the content of fumaric acid, formic acid, acetic acid, citric acid, malic acid, and oxalic acid in the roots and also Cd concentration in the roots and shoots of *C. annuum*, compared to those plants, which were grown in Cd level of 0 μM in the soil. Results also illustrated that the application of EDTA and CA decreased the contents of fumaric acid, formic acid, acetic acid, citric acid, malic acid, and oxalic acid in the roots while increased Cd concentration in the roots and shoots of *C. annuum*, compared with those plants, which were grown without the exogenous application with EDTA and CA.

4. Discussion

Metal contamination of soil may pose risks and hazards to humans and the ecosystem through: direct ingestion or contact with contaminated soil, the food chain (soil-plant-human or soil-plant-animal human), drinking of contaminated ground water, reduction in food quality (safety and marketability) via phytotoxicity, reduction in land usability for agricultural production causing food insecurity, and land tenure problems (Rana et al., 2020; Kamal et al., 2022; Ma et al., 2022a). Contamination of agricultural soils with Cd has become one of the most toxic and widespread environmental problems (Afzal et al., 2020; Ma et al., 2022c) and Cd typically causes direct or indirect inhibition of various physiological processes, such as respiration, transpiration, photosynthesis, oxidative stress, cell elongation, nitrogen metabolism and uptake of mineral nutrition, finally resulting in growth retardation, leaf chlorosis and reduced biomass (Rizwan et al., 2019; Ma et al., 2022b). In addition, Cd reduces the photosynthetic capacity of plants by devastating the enzymes of Calvin cycle and carbohydrate metabolism and, also, modulates the plant's antioxidant machinery which were found to be similar in our study the varying levels of Cd in the soil significantly ($P < 0.05$) decreased plant growth and biomass (Fig. 1) and also affects the photosynthetic machinery (Fig. 2). Reduction in growth and biomass accumulation could also be attributed to alterations in the ultrastructure of various plant components under toxic levels of Cd in the soil that have direct impacts on plant growth and yield (Singh et al., 2020; Zainab et al., 2021).

Stress conditions can disturb the dynamic equilibrium of reactive oxygen species (ROS) production and elimination under normal growth in plants (Yaseen et al., 2020; Rehman et al., 2021), which promote ROS accumulation and membrane lipid peroxidation, and disrupt the structure and function of cell membrane system (Ahmad et al., 2018; Zafar-ul-Hye et al., 2020). High concentration of Cd in the soil induced oxidative damage by increasing the contents of MDA, initiation of H_2O_2, and increased percentage of EL which was observed in *Brassica napus* (Jung et al., 2020), *Arabidopsis thaliana* (Zhu et al., 2012), and *Brassica juncea* (Alam et al., 2020). This ROS accumulation in plants is removed by a variety of antioxidant enzymes such as SOD, POD, CAT, and APX (Fig. 4) and non-enzymatic antioxidant (Fig. 6). However, the expression of specific genes, such

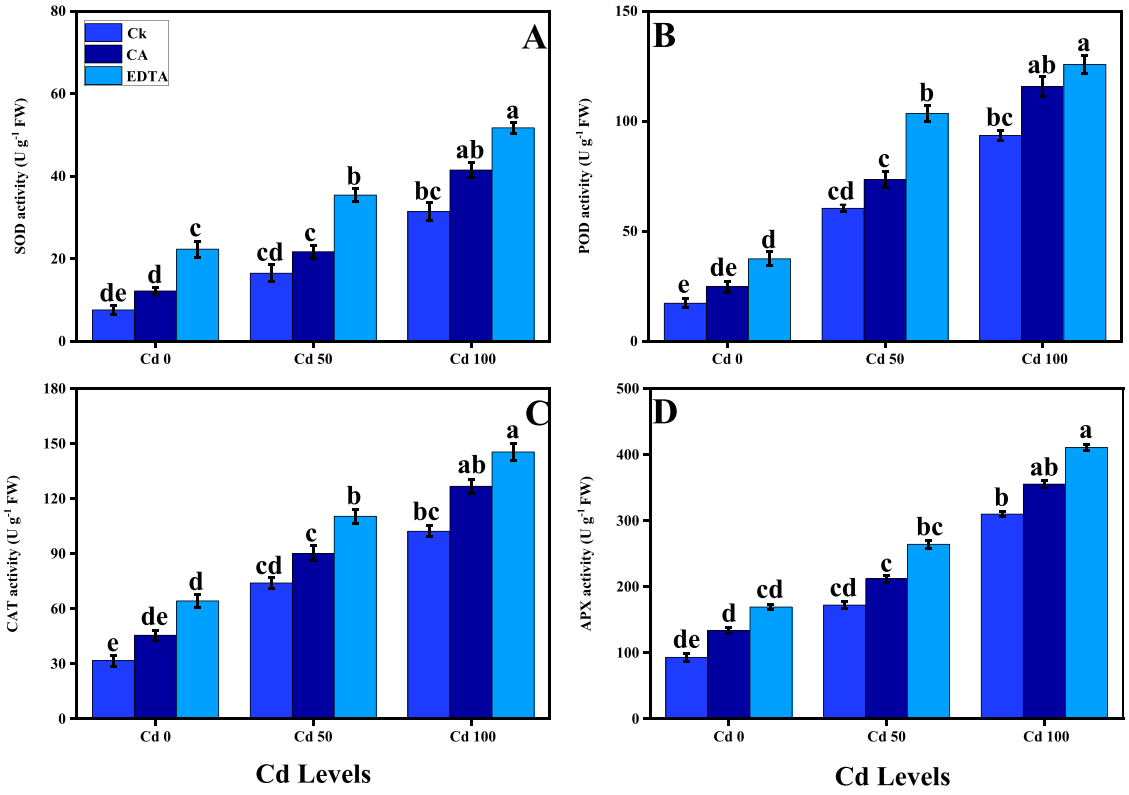

Fig. 4. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on enzymatic antioxidative enzymes, i.e., SOD activity in the leaves (A), POD activity in the leaves (B), CAT activity in the leaves (C), and APX activity in the leaves (D) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD (n = 4). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

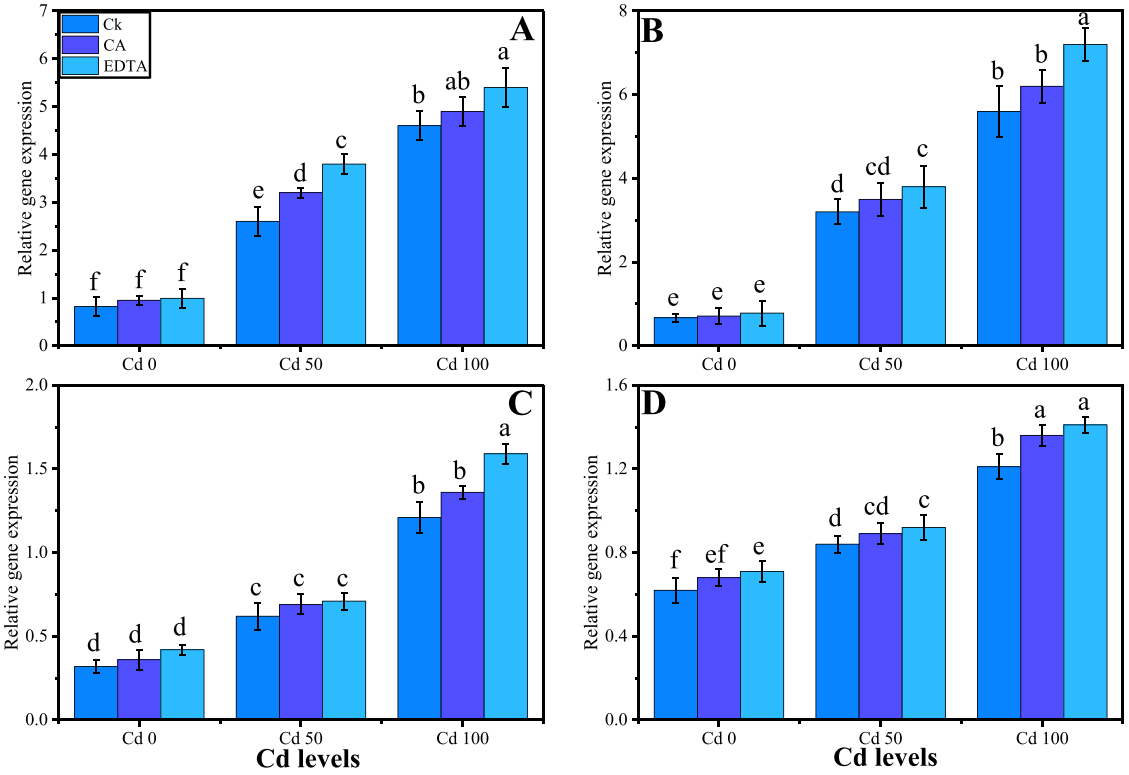

Fig. 5. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on relative gene expression, i.e., Fe-SOD activity in the leaves (A), POD activity in the leaves (B), CAT activity in the leaves (C), and APX activity in the leaves (D) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD (n = 4). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

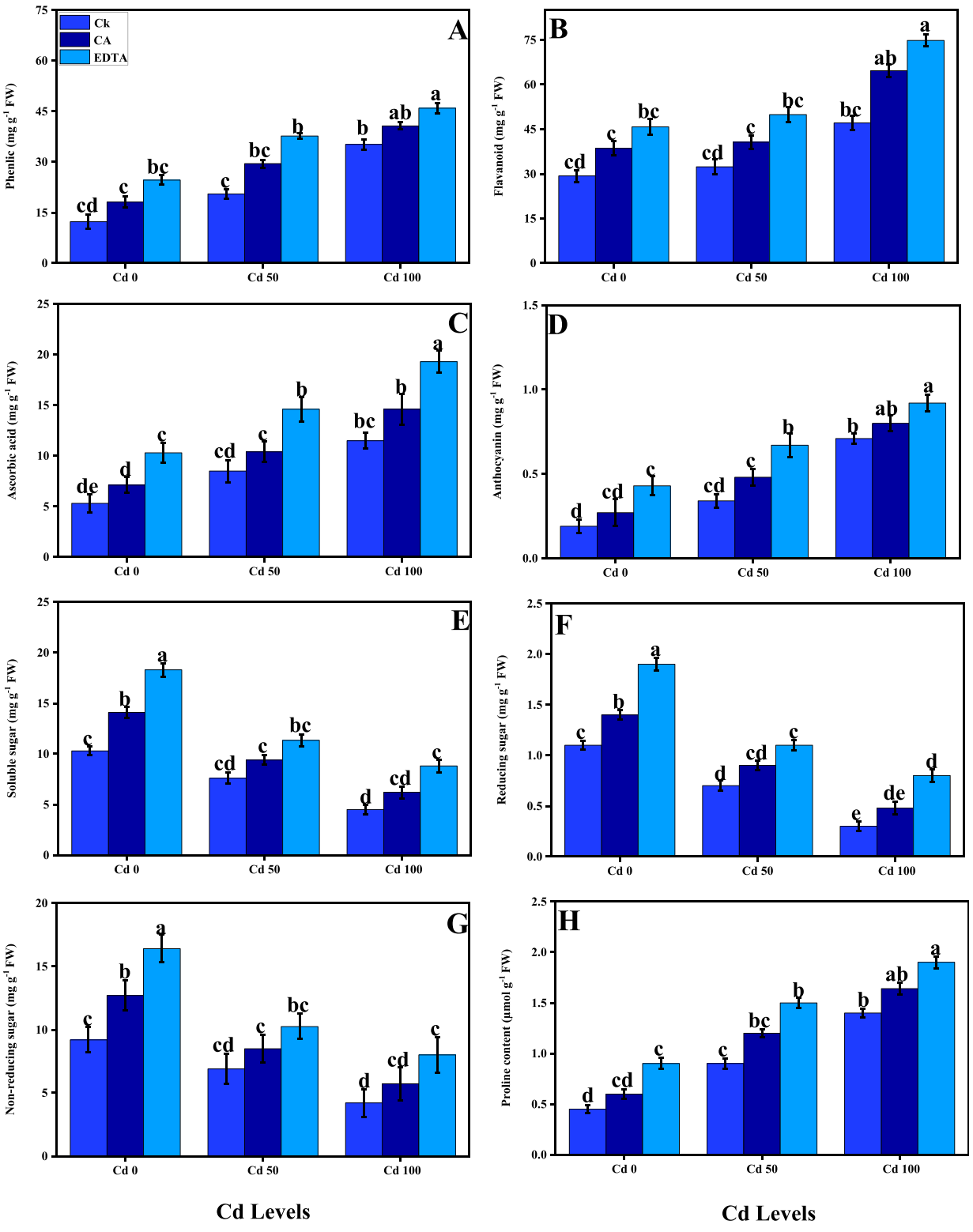

Fig. 6. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on osmolytes and sugars, i.e., phenolic content (A), flavonoid content (B), ascorbic acid content (C), anthocyanin content (D), soluble sugar content (E), reducing sugar content (F), non-reducing sugar content (G), and proline content (H) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD (n = 4). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

as Fe-SOD, POD, CAT, and APX under Cd stressed environment plays a significant role in reducing Cd toxicity, which was reported in a number of studies under various plant species (Jan et al., 2019; El-Esawi et al., 2020; Imran et al., 2020). Plants produce a variety of secondary metabolites such as proline, flavonoids, and phenolics that improve tolerance against metal toxicity. Although, proline accumulation in plant tissue/organs is a response to metal toxicity, which might be associated with signal transduction and prevents membrane

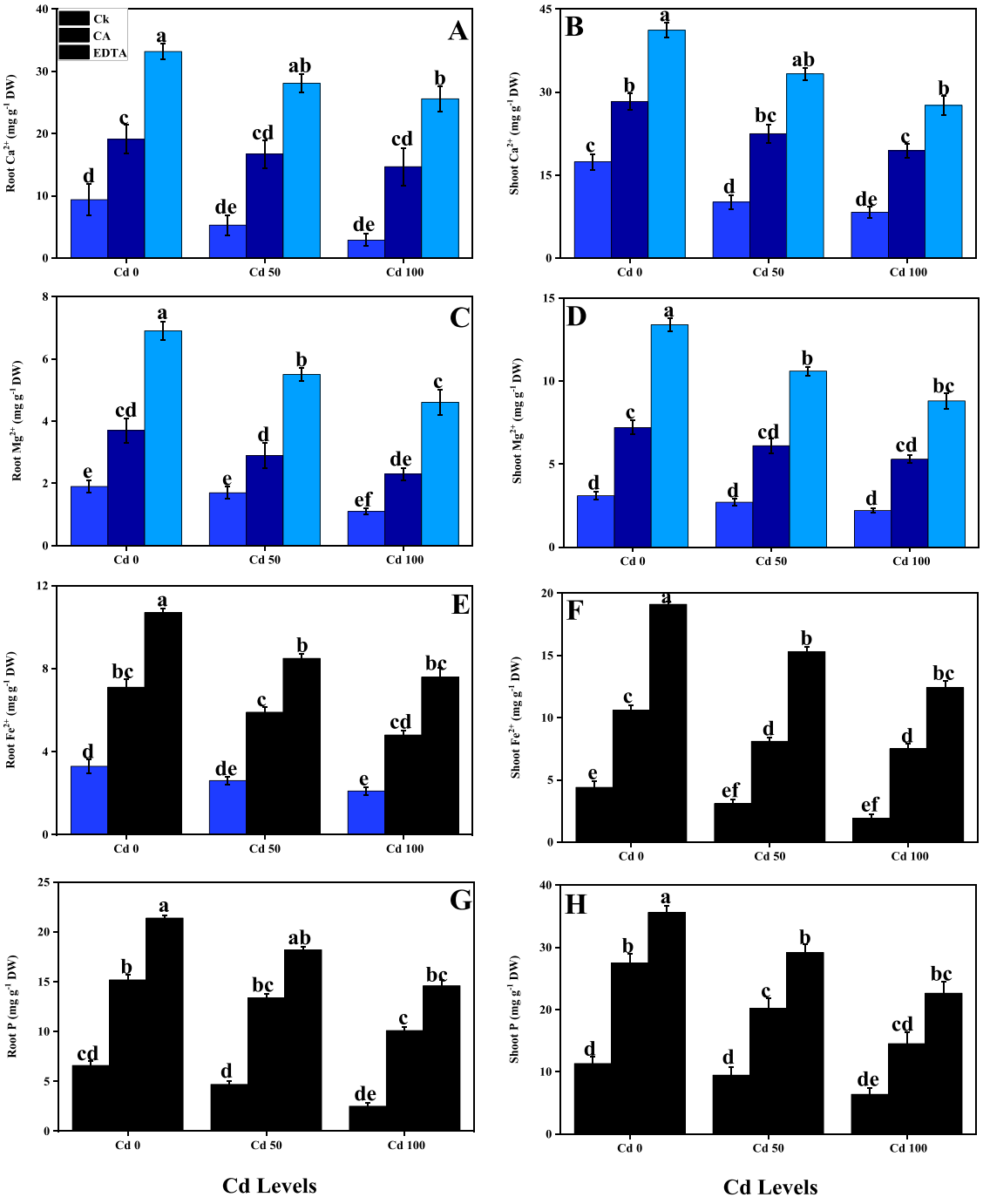

Fig. 7. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on nutritional status, i.e., calcium content in the roots (A), and calcium content in the shoots (B), magnesium content in the roots (C), magnesium content in the shoots (D), iron content in the roots (E), iron content in the shoots (F), phosphorus content in the roots (G) and phosphorus content in the shoots (H) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD (n = 4). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

distortion, which has been observed in many plant species (REHMAN et al., 2020; Sakya and Prahasto, 2020).

With increasing concentrations of Cd (50 and 100 μM) in the soil, the contents of Ca^{2+}, Mg^{2+}, Fe^{2+}, and P in the roots and shoots of *C. annuum* seedlings were decreased significantly ($P < 0.05$) when compared to those plants grown without Cd addition (Figs. 7). The decrease in essential ions accumulation in different organs of *C. annuum* seedlings under varying Cd concentrations might also be due to the alteration in the physiological processes such as the failure of biosynthesis of chlorophyll and carotenoid contents (Tanwir et al., 2015; Afzal et al., 2018). Under Cd toxicity, reduction in the contents of Fe^{2+} (Bashir et al., 2018), Mg^{2+} (Javed et al., 2017), P (Javed et al., 2017) and Ca^{2+} (Anwar et al., 2017) were recorded. Increasing Cd levels (50 and 100 μM) in the soil exerted a strong influence on exudation of organic acids from roots of *C. annuum* which probably is an adoptive mechanism for plant survival under Cd stressed conditions (Fig. 8). The increasing contents of organic acids in the root exudates of *C. annuum* are likely to protect the plants against Cd stress and limit the uptake of metal from roots to aboveground plant parts due to metal-organic acid anions-complex formation (Javed et al., 2018; Saleem et

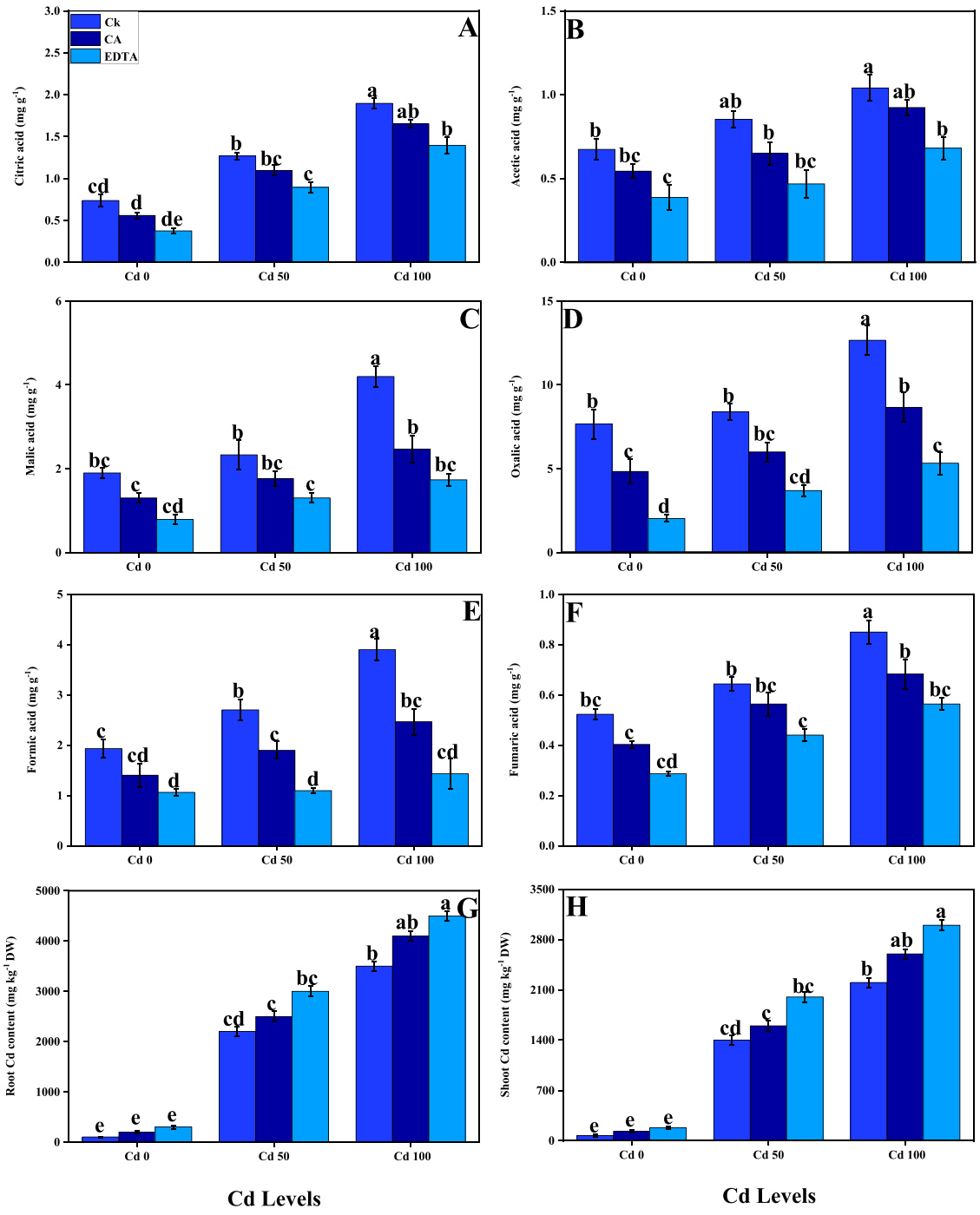

Fig. 8. Impact of exogenous application of citric acid (CA) and ethylenediaminetetraacetic Acid (EDTA) on organic acids and Cd uptake, i.e., citric acid content (A), acetic acid content (B), malic acid content (C), oxalic acid content (D), formic acid content (E), and fumaric acid content (F) in the roots, Cd content in the roots (G), and Cd content in the shoots (H) of pepper (*Capsicum annuum* L.) seedlings grown under the various levels of Cd contaminated soil i.e., 0 (no Cd), 50 and 100 μM. Values in the figures indicate just one harvest. Mean ± SD (n = 4). Thus, the differences between treatments were determined by using ANOVA, and the least significant difference test ($P < 0.05$) was used for multiple comparisons between treatment means where significant, Tukey's HSD post hoc test was used to compare the multiple comparisons of means. Different lowercase letters on the error bars indicate significant differences between the treatments.

al., 2022). Outcomes of this study revealed that higher Cd levels (50 and 100 μM) resulted in a significant ($P < 0.05$) increase in the root and shoot Cd contents *C. annuum*. Previously, increasing Cd contents in soil caused a significant ($P < 0.05$) increase in Cd contents of plants organs of *Pfaffia glomerata* (Pereira et al., 2018), *Zea mays* (Anwar et al., 2017), *Boehmeria nivea* (Tang et al., 2015) and *Linum usitatissimum* (Ali et al., 2015).

Recently, the use of organic chelators has been reported widely acceptable because of their low cost and high degradability as compared to expensive and highly leachable synthetic chelators (Chen et al., 2019; Zaheer et al., 2020). Many studies documented the chelating potential and plant growth promoting role of CA under different toxic elements such as Cr (Afshan et al., 2015), Cd (Ehsan et al., 2014), Pb (Shakoor et al., 2014), Cu (Zaheer et al., 2015) and As (Bhat et al., 2022). The promotive role of CA in plants exposed to toxic metals stress is well recognized. The application of CA improved the growth and biomass and photosynthetic pigments of *C. annuum* seedlings under Cd stress (Figs. 1 and 2). Although the application of CA is

independent to metal stress as it increased plant growth and biomass (even under normal condition), this might be due to increased nutrient uptake and/or CA induced chelation of metals decreasing free metal ions in plants as suggested by Zaheer et al. (2015). Moreover, the CA application also increased photosynthetic pigments, gaseous exchange attributes, and ultrastructure of chloroplast which is linked with the improvement in plant growth and biomass as suggested by Parveen et al. (2020). As suggested by Mallhi et al. (2019), improvement in plant growth and biomass under toxic metal stress condition might be due to the chelating role of CA, which helps to increase nutrient uptake by the plant. Improvement in plant growth and biomass might be accredited to the ability of CA to enhance the uptake of essential nutrients by the formation of complexes with nutrients (Afshan et al., 2015). In numerous studies the application of chelating agents increased the activities of antioxidative enzymes and reduced oxidative stress by decreased the generation of ROS production (Najeeb et al., 2011; Maqbool et al., 2018; Saleem et al., 2020b). This might be due to the growth promotive character of CA in assisting the plant to recover fast from oxidative damage (Zaheer et al., 2015). Although, the increase in Cd uptake by plants might be due to chelation of Cd with CA and CA application also increased plant growth and biomass and, consequently, the accumulation and uptake of metals in plants. This relative increase in Cd contents might be due to CA-induced increase transpiration rate, which in turn increased Cd translocation to shoot through water movement and/or due to Cd chelation (Ehsan et al., 2014; Hassan et al., 2016).

EDTA significantly increased plant growth and biomass compared to Cd-stressed plants (Fig. 1). Application of EDTA increased plant growth and development in *Brassica napus* (Habiba et al., 2015), *Helianthus annuus* (Azhar et al., 2006) and *Cicer arietinum* (Ali et al., 2017). The conversion of light energy into photochemical reactions is more efficient under the application of EDTA in Cd-stressed plants may also play a key role in improving growth and development (Jiang et al., 2019; Mousavi et al., 2021). (Kanwal et al., 2014) also reported that application of EDTA reduced oxidative stress under high concentration of Pb as indicated by decrease in MDA contents which showed similar trends with our study (Fig. 3). Moreover, increase in photosynthetic rate may be due to the protective role of EDTA on photosynthetic machinery by reducing the metal free ions and increase the activities of antioxidants which ultimately reduced oxidative stress in *C. annuum* (Chen and Cutright, 2001; Lu et al., 2017). In the present experiment, increasing growth, biomass, gaseous exchange attributes (Figs. 1, 2), and alleviates oxidative stress (Fig. 4) is directly linked with the activities of antioxidants (Figs. 4–6) with the application of EDTA under elevating levels of Cd in the nutrient solution.

5. Conclusion

On the basis of these findings, it can be concluded that the negative impact of Cd toxicity can be overcome by the external application of EDTA and CA. Our results depict that Cd toxicity induced severe metal toxicity in *C. annuum* by increasing the generation of ROS in the form of oxidative stress and also increasing the concentration of Cd in the roots and shoots of the plants. Furthermore, Cd toxicity also increased organic acid exudation and imbalance in the nutritional status of the plants, which ultimately decrease plant growth, yield, and photosynthetic efficiency. Hence, Cd toxicity was eliminated by the external application of EDTA and CA, which also decreased the Cd concentration in the plant tissues, degenerated ROS, and organic acid exudation, but increased the activities of antioxidants and their gene expression and essential nutrients in the plants. Therefore, long-term field studies should be executed to draw parallels amongst plants/crops root exudations, metal stress, nutrient fertigation regimes, nutrient mobility patterns, and plant growth in order to gain insights into the underlying mechanisms.

Ethical approval

Not applicable.

CRediT authorship contribution statement

Rana M. Alshegaihi: Conceptualization, Writing – original draft, Writing and review & editing. **Manar Fawzi Bani Mfarrej:** Writing – original draft, Software, Data curation, Writing – review & editing, Validation. **Muhammad Hamzah Saleem:** Supervision, Writing – original draft, Software, Data curation, Writing – review & editing. **Abida Parveen and Khawaja Shafique Ahmad:** Formal analysis, Data curation, Writing – review & editing. **Baber Ali:** Writing – original draft, Data curation, Formal Analysis, Software, Writing – review & editing. **Amany H.A. Abeed, Dikhnah Alshehri, Sameera A. Alghamdi, Suliman M.S. Alghanem, Tarek M.A. Soliman, Fathia A. Soudy:** Software, Data curation, Writing – review & editing, Funding Acquisition. **Javeed Ahmad Lone:** Formal Analysis, Software, Writing – review & editing.

Consent to participate

Not applicable.

Consent to publish

Written consent was sought from each author to publish the manuscript.

Funding

Teaching & Research Practice Field for School of Public Administration provided the pot experiment site.

Availability of data and materials

Data and material is available for research purpose and for reference.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2023.06.024.

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