



# Calcium and ethylene glycol tetraacetic acid mitigate toxicity and alteration of gene expression associated with cadmium stress in chickpea (*Cicer arietinum* L.) shoots

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## Abstract

In the aim to estimate the protective role of calcium (Ca) and ethylene glycol tetraacetic acid (EGTA) against cadmium (Cd)-induced damage, chickpea (*Cicer arietinum* L.) seeds were exposed to 200  $\mu$ M Cd stress for 6 days or 3 days then subjected to co-treatment of the metal with either 100 mM CaCl<sub>2</sub> or 100  $\mu$ M EGTA for 3 additional days. The addition of Ca and EGTA improved seedling growth. This protecting effect was correlated to the alleviation of the metal-induced oxidative stress, exemplified by the reduction of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) contents. Besides, Ca and EGTA stimulated thioredoxin (Trx) and thioredoxin reductase (NTR) activities (2.75- and 1.75-fold increase when compared to Cd-stressed, respectively) protecting, thereby, protein –SH groups from the Cd-mediated oxidation, and modulated ferredoxin (Fdx) activity to a control level. Moreover, Ca and EGTA reinstated the glutathione redox steady state, mainly via preserving a high level of glutathione reduced form (GSH). This effect coincided with the maintaining of the Cd-stimulated glutathione reductase (GR) activity and the decline of glutathione peroxidase (GPX, 43% lower than Cd-stressed shoots) activity. Ca and EGTA counteracted the inhibitory effect of Cd on the activity and gene expression of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) isoenzyme and modulated the activities of catalase (CAT) and ascorbate peroxidase (APX). Overall, our results provided evidence that Ca and EGTA supplement could be a promising approach in the remediation of Cd-contaminated environment.

**Keywords** Cadmium · *Cicer arietinum* · Glutathione · Redox status · Stress alleviation

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## Introduction

Cadmium (Cd) is classified as a highly toxic heavy metal (HM), mainly resulting from anthropogenic activities (Cheng et al. 2014). Thus, contaminated soil remediation is an increasingly urgent necessity. In this aim, chemical and physiological technologies were carried out to improve plant tolerance to environmental constraints. In this context, germination, being a crucial stage in plant development and highly sensitive to the stress imposed by HM, is considered as a tool of choice to appreciate the changes in the immediate environment of the seed (Bewley 1997). The germinating metabolism and related processes, for instance imbibition capacity, resumption of the respiration, and mobilization of reserves, are affected by metal stress resulting in a reduced germination capacity of seeds and growth delay of the embryonic axis (Nouairi et al. 2019).

Oxidative stress induction is one of the early aspects of HM toxicity (Kharbech et al. 2020). Cadmium, a non-redox

element, generates reactive oxygen species (ROS) mainly through indirect mechanisms (Benavides et al. 2005). Owing to its high affinity for thiol, carboxyl, and amine groups of proteins and its ability to substitute essential ions in protein functional sites, Cd inhibits the suitable functioning of proteins in electron transport chains or antioxidant defense (Štolfa et al. 2015). This dysfunction, in turn, leads to an increased electron leakage and, consequently, an excessive ROS production or/and a decreased cellular capacities of ROS neutralization (Heyno et al. 2008; Srivastava et al. 2018). To cope with Cd-induced oxidative stress, plants trigger “specific” mechanisms involving a variety of molecules to control the levels of ROS present in the cells (Štolfa et al. 2015). Glutathione (GSH) is involved in various biological processes such as cell division and differentiation, senescence, regulation of sulfide transport, signal transduction, metabolite conjugation, and protein and nucleic acid synthesis (Zagorchev et al. 2013). Because of its reducing capacity, GSH is a preponderant player in maintaining redox homeostasis. It is also a precursor of phytochelatins that play a key role in HM sequestration in plants (Yadav 2010). Furthermore, GSH is involved in the recycling of the ascorbate, achieved by the Asada-Halliwell cycle (Potters et al. 2004). On the other hand, plant cells contain numerous protein-disulfide reductases, such as thioredoxin (Trx), NADP-dependent thioredoxin reductase (NTR), glutaredoxin, and peroxiredoxin, implicated, through cellular redox state control, in stress resistance (Zaffagnini et al. 2019). Ferredoxin (Fdx) and Trx systems complement GSH in maintaining the thiol homeostasis by reducing the disulfide bridge of target proteins, protecting, thereby, sulfhydryl (–SH) groups, highly sensitive to ROS-mediated oxidation (Zaffagnini et al. 2019).

Enzymatic and non-enzymatic antioxidative systems act in synergy to reduce the intracellular level of ROS. However, controversial responses of antioxidant enzymes to stressful conditions were noticed. Antioxidative enzymes can be stimulated as they can be, under other conditions, inhibited following post-transcriptional modifications (oxidation, S-thiolation) or even following a down-regulation of involved genes (Rahoui et al. 2016).

Several approaches have been undertaken to reduce the impairment caused by HM pollution. Among these strategies, the exogenous application of certain compounds, including calcium (Ca) and organic and inorganic acids, has proven its effectiveness in ameliorating HM stress (Rafiq et al. 2018; Ben Massoud et al. 2019; Valivand and Amooaghaie 2020).

Calcium is considered as a second messenger involved in plant responses to biotic and abiotic environmental cues and plant cell metabolism regulation (Yang and Poovaiah 2003; Dayod et al. 2010). Recently, the results of Nouairi et al. (2019) showed that pre-treatment of faba bean seeds with  $\text{CaCl}_2$  induced a reduction in Cd accumulation, competing for  $\text{Ca}^{2+}$  channels, improved cell membrane stability, and

increased antioxidant defense systems. Moreover, Ca amendment of Cd-polluted soil ameliorated photosynthetic attributes in *Brassica napus* plants and ameliorated flavonoid and saponin yield in *Panax notoginseng* roots (Wan et al. 2011; Zu et al. 2020). Controversial effects of chelators on HM bioavailability and antioxidant defense system have been reported. Inorganic, i.e., ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and ethylene glycol tetraacetic acid (EGTA), and organic, i.e., malic, oxalic, and citric acids, induced variable responses in plants confronted with HM stress, depending on the chelator and metal concentrations, the duration of exposure to stress, and the plant species (Han et al. 2018; Song et al. 2018; Alzahrani et al. 2020; Soyngbe et al. 2020).

The present investigation aimed to gain insight into the processes by which Ca and EGTA confer Cd tolerance to chickpea seedlings through the study of thiol redox homeostasis and the related enzymes. Also, the antioxidant system was examined.

## Materials and methods

### Germination and treatment conditions

Seeds of chickpea (*Cicer arietinum* L., cv. Beja 1) were sterilized, rinsed with distilled water, and then germinated for 3 days at 25 °C in the dark over two sheets of Whatman filter paper moistened with 20 mL distilled water (first batch) or aqueous solutions of 200  $\mu\text{M}$   $\text{CdCl}_2$  (second batch). Seeds from the first batch continued the germination in distilled water (control condition) or in the presence of 100 mM  $\text{CaCl}_2$  or 100  $\mu\text{M}$  EGTA. The seeds of the second batch continued to germinate in the presence of 200  $\mu\text{M}$   $\text{CdCl}_2$  (stress condition) or were transferred in distilled water (stress abruption) or the combination of 200  $\mu\text{M}$   $\text{CdCl}_2$  with 100 mM Ca (Cd + Ca treatment) or 100  $\mu\text{M}$  EGTA (Cd + EGTA treatment). Applied concentrations were selected based on preliminary studies showing that 200  $\mu\text{M}$  Cd caused almost 50% reduction of seedling growth and 100 mM Ca and 100  $\mu\text{M}$  EGTA caused maximum growth recovery.

For each treatment, 6-day-old shoots were harvested, weighed, and frozen in liquid nitrogen for biochemical analyses or dried at 70 °C until weight stabilization for dry weight (DW) estimation. Freshly harvested shoots were used for gene expression studies.

### Hydrogen peroxide content determination

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content was determined according to Sergiev et al. (1997). Shoots were homogenized in trichloroacetic acid (TCA, 0.1% w/v). The homogenate was then centrifuged at 10,000 $\times$ g for 15 min at 4 °C. The resulting supernatant was added to potassium phosphate buffer (25

mM, pH 7.0) and KI (1 M). The absorbance was measured at 390 nm and H<sub>2</sub>O<sub>2</sub> concentration was calculated using a standard curve.

### Determination of the antiradical capacity

The aqueous sample extracts were prepared according to the Dudonné et al. (2009) method and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity of each sample was determined according to the method of Parejo et al. (2003). DPPH radicals have an absorption maximum at 517 nm, which decreases when DPPH• is reduced by a radical scavenging compound. The radical scavenging activity was calculated using the following equation:

$$PI : \%inhibition = [(AB-AE)/AB] \times 100$$

where AB = absorbance of DPPH• solution (blank sample), and AE = absorbance of the shoot extract.

The IC<sub>50</sub> (effective concentration corresponding to 50% inhibition) values, expressed in mg/mL, were calculated for each extract from a curve representing the variation of the free radical scavenging activity depending on the concentration of shoot extracts. The lowest IC<sub>50</sub> value corresponds to the highest antiradical activity.

### Protein extraction and total thiol determination

Shoot samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM Na ascorbate and 0.2 mM ethylenediamine tetraacetic acid in an ice bath. The resulting supernatant, after centrifugation at 10,000×g for 15 min at 4 °C, was used for the determination of protein and total thiol contents, and enzyme activities. Proteins were quantified according to Bradford (1976) and total thiol groups were evaluated according to Ellman's (1959) method.

### Enzyme assays

Superoxide dismutase (SOD; EC. 1.15.1.1) activity was determined by using the protocol of Misra and Fridovich (1972) based on the ability of SOD to inhibit the autoxidation of epinephrine, thus reducing the formation of the adrenochrome. The autoxidation of epinephrine was assessed by the rise in the absorbance at 480 nm of sodium carbonate/bicarbonate buffer (62.5 mM, pH 10.4) containing 1.88 U/mL catalase, 125 μM EDTA, and 10 μL epinephrine (11 mg epinephrine in 2 mL 0.1 N HCl). One unit of SOD was defined as the amount of protein extract that causes a 50% loss in the rate of adrenochrome formation. The addition of 1 mM KCN and 5 mM H<sub>2</sub>O<sub>2</sub> to the reaction medium inhibits Cu/Zn-SOD and Fe-SOD, respectively (Salin and Lyon 1983).

The activity of catalase (CAT; EC 1.11.1.6) was assessed by the decrease in absorbance at 240 nm ( $\epsilon = 0.036 \text{ mM}^{-1} \text{ cm}^{-1}$ ) due to the consumption of H<sub>2</sub>O<sub>2</sub> following the Aebi (1984) method. The reaction medium consisted of 10 mM H<sub>2</sub>O<sub>2</sub> in 25 mM K-phosphate buffer, pH 7.0, and the reaction was triggered by the addition of the protein extract. Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured according to the method described by Nagalakshmi and Prasad (2001) based on the continuous regeneration of glutathione oxidized form (GSSG), which is produced by the action of GPX and catalyzed by glutathione reductase (GR) to the detriment of the NADPH form. Reaction medium contained K-phosphate buffer (50 mM, pH 8.0), 100 mM NaCl, 1 mM GSH, 2.5 mM H<sub>2</sub>O<sub>2</sub>, 0.5 mM NADPH, and 1 U GR. The oxidation of NADPH was monitored by measuring the decrease in absorbance at 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Glutathione reductase (GR; EC 1.6.4.2) was monitored in K-phosphate buffer (50 mM, pH 7.0) containing 0.2 mM NADPH and 0.5 mM GSSG. The oxidation of NADPH was determined by the decrease in the absorbance at 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ; Foyer and Halliwell 1976). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to the protocol of Nakano and Asada (1981). The reaction medium consisted of K-phosphate buffer (25 mM, pH 7.0) containing 0.5 mM Na ascorbate, 5 mM H<sub>2</sub>O<sub>2</sub>, and 0.1 mM EDTA. The decrease of the absorbance at 290 nm, due to the consumption of ascorbate, was monitored ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The Trx activity has been evaluated by the NADMDH test in Tris-HCl buffer (50 mM, pH 8.0), containing 1 mM oxaloacetate, 0.2 mM NADH, 10 μM MDH, and 1 mM DTT. The oxidation of NADH was followed by a decrease of the absorbance at 340 nm ( $\epsilon = 6.22 \text{ M}^{-1} \text{ cm}^{-1}$ ; Jacquot et al. 1994). The NTR (EC 1.8.1.9) activity was measured in the following reaction medium: 50 mM Tris-HCl (pH 8), 100 μM DTNB (5,5-dithiobis-(2-nitrobenzoic acid)), 0.2 mM NADPH, and 30 μg/mL Trx (reduced form). The reduction of DTNB was determined by the increase in the absorbance at 412 nm ( $\epsilon = 13600 \text{ M}^{-1} \text{ cm}^{-1}$ ; Jacquot et al. 1994). The activity of Fdx was assessed by the increase of the absorbance at 550 nm of the reaction mixture containing 50 mM Tris-HCl (pH 7.8), 250 μM NADPH, 40 μM oxidized cytochrome C, 0.1 μM ferredoxin-NADP reductase (FNR), and the protein extract ( $\epsilon = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$ ; Green et al. 1991).

### Quantification of glutathione contents

Thiols were extracted according to the method of Garcia et al. (2008). Briefly, lyophilized shoot tissue was homogenized in 15% TCA at 4 °C. The derivation reaction was realized using 1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB prepared in 0.5 M K-phosphate buffer, pH 8; Katrusiak et al. 2001). After 5 min of incubation in an ice bath, the reaction was stopped

with  $\text{H}_3\text{PO}_4$  (7 M); then, the homogenate was centrifuged at  $12,000\times g$  for 10 min at 4 °C. For total GSH determination, oxidized (GSSG) forms were reduced with dithiothreitol (DTT, 10 mM) before the derivation reaction.

The chromatographic separation was carried out using a reverse-phase column (Agilent, 1100 Series, USA) connected to a UV-visible detector set at 330 nm (Katrusiak et al. 2001). Extracts (20  $\mu\text{L}$ ) were injected into a Zorbax Eclipse Plus C18 column (5  $\mu\text{m}$ ,  $4.6 \times 250$  mm; Agilent, USA). The mobile phase consisted of acetonitrile as mobile phase A and acidified water (pH 3.5) as mobile phase B at a flow rate of 1.2 mL  $\text{min}^{-1}$ . The elution profile was as follows: 0–15 min, 10% A; 15–16 min, 100% A; 16–20 min, 10% A. The comparison of the retention time of the samples with those obtained with corresponding standards permitted to identify the different peaks.

The total (GSH + GSSG) and reduced (GSH) forms of glutathione are quantified using calibration curves made from DTNB-derived glutathione (Sigma) solutions. Concentrations were determined based on the area of the peaks and GSSG contents were calculated as the difference between the total and reduced forms.

## Determination of antioxidant gene expression

### RNA extraction and assay

The total RNA was extracted according to the protocol of Jacquemond et al. (2009) using the Trizol LS Reagent (Trizol RNA stabilization solution, Invitrogen, Life Technologies). One hundred milligrams of fresh plant tissue were ground directly, with tissue lyser II (QIAGEN), in 500  $\mu\text{L}$  of extraction buffer (0.5 M sodium trisodium citrate; 0.1% thioglycolic acid). The ground material was homogenized with 500  $\mu\text{L}$  of Trizol, then vortexed and incubated for 5 min at room temperature before the addition of 100  $\mu\text{L}$  of cold chloroform. The mixture is then incubated for 15 min at room temperature and centrifuged at 14,000 rpm for 15 min at 4 °C. The mixture separates into a red lower phase containing a mixture of phenol and chloroform, an interphase containing the proteins, and a clear aqueous upper phase containing the nucleic acids. The aqueous phase was collected and the nucleic acids were precipitated in the presence of 250  $\mu\text{L}$  of isopropanol for 20 min at  $-20$  °C. The nucleic acids were recuperated by centrifugation at 14,000 rpm for 15 min at 4 °C. The obtained pellet was washed with 250  $\mu\text{L}$  of cold 70% ethanol and centrifuged for 10 min at 14,000 rpm at 4 °C, then dried immediately in a speed-vac for 1 to 2 min before being taken up in 30  $\mu\text{L}$  of RNase-free water.

The quantification of the RNA was carried out by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) with multiple optical sources.

### Synthesis of cDNA: reverse transcription (RT)

For the synthesis of the cDNA, 2  $\mu\text{g}$  of RNA was incubated at 95 °C for 2 min in the presence of 50 ng of reverse primer in a final volume of 10  $\mu\text{L}$ . The mixture was immediately cooled in ice and then added with 4  $\mu\text{L}$  of 5 $\times$  buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl), 1  $\mu\text{L}$  0.1 M DTT, 10 mM dNTP, 0.5  $\mu\text{L}$  RNAsin (RNase inhibitor; 40 U/ $\mu\text{L}$ ), and 40 U of M-MLV reverse transcriptase (200 U/ $\mu\text{L}$ ). The reaction occurred at 37 °C for 60 min.

### Real-time quantitative PCR

Quantitative real-time PCR (qPCR) was performed with a sequence detection system (Applied Biosystems, ABI 7000) in a final volume of 25  $\mu\text{L}$  including 10  $\mu\text{L}$  Igreen qPCR master MixRox (BIOMATIK, USA), 50 ng of cDNA sample as a template, and 400 nM forward and reverse primers. PCR reactions were realized in 96-well optical reaction plates (Applied Biosystems, USA) under the following cycle conditions: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. Genes and the corresponding primers were designed from Chickpea Transcriptome Database (CTBD TC17160) (<http://www.nipgr.res.in/ctdb.html>): CAT (AJ 131046), forward (F) -5'-TCCACAGGGATGAAGAGGTC AATTAC-3' and reverse (R) -5'-AACGATCAGCCTGA GACCAGTATG-3'; APX (AB0 24991), F-5'-TCTA TCAGTTGGGTGGTGTGTTG-3' and R-5'-TTGC CAAACACATCCCTCAAATGG-3'; SOD (AJ 012691), F-5'-ACAAATGGCTGCATATCAACCGGAC-3' and R-5'-TTCCTATGATGGAGTTTGGTCCAG-3'; The actin *Cicer arietinum* gene (ACT) was used as the internal control gene (AJ 012685): F-5'-TCTTGAGTGGTGGTTCTACT ATGTTCC-3' and R-5'-GCTCTCAGTGCTCCATGATT TGAAC-3'.

The relative quantification of the transcripts of each gene was determined by applying the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen 2001):

$$\Delta\text{CT} = \text{CT}_{\text{target gene}} - \text{CT}_{\text{reference gene}}$$

$$\Delta\Delta\text{CT} = (\Delta\text{CT})_{\text{control condition}} - (\Delta\text{CT})_{\text{experimental condition}}$$

### Statistical analysis

Germination was carried out three times in succession to have three independent replications. In each replication, the seeds of 3 crystallizers were subjected to the same treatment. Overall differences were tested by one-way analysis of variance (ANOVA test) and pairwise comparisons between treatments were performed using the Tukey HSD multiple comparisons test. Pearson's correlations were determined to visualize the relationship between the different studied parameters. Also, a

factorial analysis was conducted by principal component analysis (PCA). A probability of  $p < 0.05$  was considered significant in all statistical analyses performed using XLSTAT software.

## Results

### Effects on growth

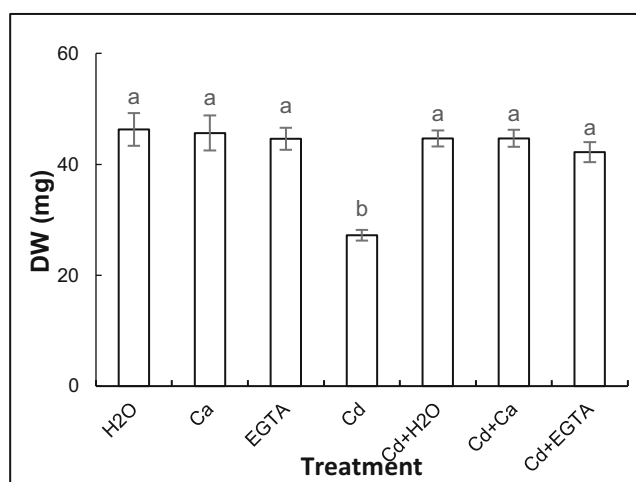
Cadmium reduced chickpea shoot growth by 41% when compared to the control ( $p < 0.05$ ; Fig. 1). However, Ca and EGTA addition to the germinating medium contaminated with Cd suppressed the detrimental effect of the metal on shoot DW (Fig. 1). Interestingly, this protective effect was similar to that of Cd + H<sub>2</sub>O treatment.

### Effects on H<sub>2</sub>O<sub>2</sub> content

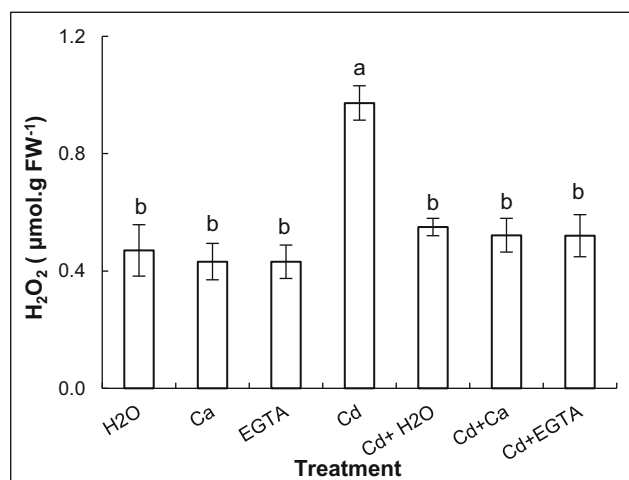
Cadmium exposure led to H<sub>2</sub>O<sub>2</sub> over-accumulation in chickpea shoots by almost 100% over the control (Fig. 2). The application of Ca and EGTA counteracted the Cd-mediated H<sub>2</sub>O<sub>2</sub> accumulation (−46% of the Cd-stressed shoots; Fig. 2). Besides, in the absence of Cd, Ca and EGTA did not influence the accrual of H<sub>2</sub>O<sub>2</sub> in shoot cells. Similar response was observed when the seedlings were transferred to the control condition upon 3-day Cd exposure (Fig. 2).

### Effects on antiradical capacity, thiol content, and thioredoxin system

As illustrated in Fig. 3, Cd resulted in a substantial increase (2.5-fold increase compared to the control; Fig. 3) of the IC<sub>50</sub> in shoot cells. This finding reflected the loss of the antiradical



**Fig. 1** Chickpea shoot dry weight (DW) after seed imbibition in H<sub>2</sub>O or 200  $\mu$ M CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100  $\mu$ M EGTA. Values ( $\pm$  SE,  $n = 3$ ) followed by a common letter are not different at the 0.05 level of significance according to Tukey's test

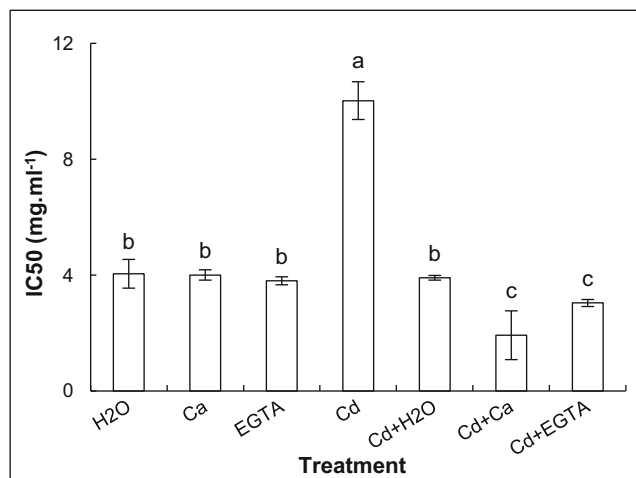


**Fig. 2** H<sub>2</sub>O<sub>2</sub> content in chickpea shoots after seed imbibition in H<sub>2</sub>O or 200  $\mu$ M CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100  $\mu$ M EGTA. Each measurement was performed in an extract obtained from several seedlings. Values ( $\pm$  SE,  $n = 3$ ) followed by a common letter are not different at the 0.05 level of significance according to Tukey's test

capacity under metal distress. The combinations Cd + Ca and Cd + EGTA improved the cell performance to reduce free radicals by approximately 80% and 50% as compared to the Cd-stressed and the control, respectively (Fig. 3). Unlike Ca and EGTA treatments, Cd and the combination Cd + effectors increased −SH groups content with an increment rate of 43% and 27% of control, respectively (Table 1). Besides, the thioredoxin system displayed a treatment-dependent response profile. Cd declined markedly Trx and NTR activities (3.8- and 1.8-fold lesser than the control, respectively; Fig. 4a, b) and enhanced the Fdx one by 1.4-fold of control (Fig. 4c). The effectors improved Trx activity by 175% over the Cd-treated samples but without reaching the control level (Fig. 4a). Nevertheless, the co-application of Cd and effectors corrected NTR and Fdx activities (Fig. 4b, c). In the absence of Cd, Ca and EGTA did not influence the antiradical capacity nor the −SH group content and the thioredoxin system (Figs. 3 and 4; Table 1).

### Effects on glutathione homeostasis and related enzymes

Cadmium significantly ( $p < 0.05$ ) raised the glutathione redox status (GSH to GSSG ratio) by 36% over the control (Table 1). Concomitantly, a decreased content of the reduced form (GSH; 37% decrease versus control; Table 1) and unchanged level of the oxidized form (GSSG) were observed. Likewise, GR and GPX activities were enhanced by 75% and 71% of control, respectively (Fig. 5). Calcium and EGTA maintained the up-regulated GR activity and reduced the GPX one to a control level (Fig. 5). Thus, the effectors completely suppressed the disrupting effect of Cd on the GSH/GSSG ratio



**Fig. 3** Antiradical capacity, evaluated by the determination of the concentration corresponding to 50% inhibition of DPPH free radical (IC<sub>50</sub>, mg mL<sup>-1</sup>), of aqueous extracts obtained from chickpea shoots after seed imbibition in H<sub>2</sub>O or 200 μM CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100 μM EGTA. Values (± SE, *n* = 3) followed by a common letter are not different at the 0.05 level of significance according to Tukey's test

and the GSH content, and kept the level of GSSG unchanged (Table 1).

### Effects on antioxidative enzymatic and transcriptional systems

As illustrated in Fig. 6a, activities of SOD isoforms exhibited various responses under Cd stress. The Mn-SOD activity was enhanced by 56%, whereas the Cu/Zn-SOD activity was reduced by 85% of control, and the Fe-SOD activity was maintained at the control level (Fig. 6a). Furthermore, the investigation of the transcriptional system revealed a down-regulation of the Cu/Zn-SOD gene by 1.73-fold of the control (Fig. 6b). Besides, Cd stimulated APX and CAT activities by 127% and 355% over the control, respectively (Fig. 6c, e), but declined the expression of APX gene by 2.6-fold compared to the unstressed samples and did not significantly (*p* > 0.05) affect CAT gene transcripts (Fig. 6d, f). The exogenous effectors counteracted the stimulating effect of Cd on APX activity (Fig. 6c) and partially impeded the impact on CAT activity (Fig. 6e). Simultaneously, Ca mitigated the Cd-elicited

repression on APX gene expression (Fig. 6d), but EGTA was the most effective with a 160% increase versus Cd treatment (Fig. 6d). The sensitivity profile of the CAT transcriptional system revealed significant variability (Fig. 6f). Calcium did not affect CAT gene expression, while EGTA, Cd + Ca, and Cd + EGTA induced its up-regulation by more than 3.6-, 2.4-, and 1.9-fold, respectively (Fig. 6f).

### Pearson's correlation and principal component analysis

The statistical analysis (Table 2) revealed that the redox stress biomarker, H<sub>2</sub>O<sub>2</sub> content, was negatively correlated with shoot DW (*r* = -0.973, *p* < 0.01), Trx (*r* = -0.878, *p* < 0.01), NTR (*r* = -0.815, *p* < 0.05), GSH content (*r* = -0.939, *p* < 0.01), GSH/GSSG ratio (*r* = -0.887, *p* < 0.01), and APX and Cu/Zn-SOD gene expressions (*r* = -0.762 and -0.756, respectively, *p* < 0.05). Contrariwise, a positive correlation associated the contents of H<sub>2</sub>O<sub>2</sub> to -SH groups (*r* = 0.790, *p* < 0.05), IC<sub>50</sub> (*r* = 0.893, *p* < 0.01), Fdx (*r* = 0.785, *p* < 0.05), GPX (*r* = 0.957, *p* < 0.01), APX (*r* = 0.980, *p* < 0.01), and CAT (*r* = 0.904, *p* < 0.01) activities (Table 2).

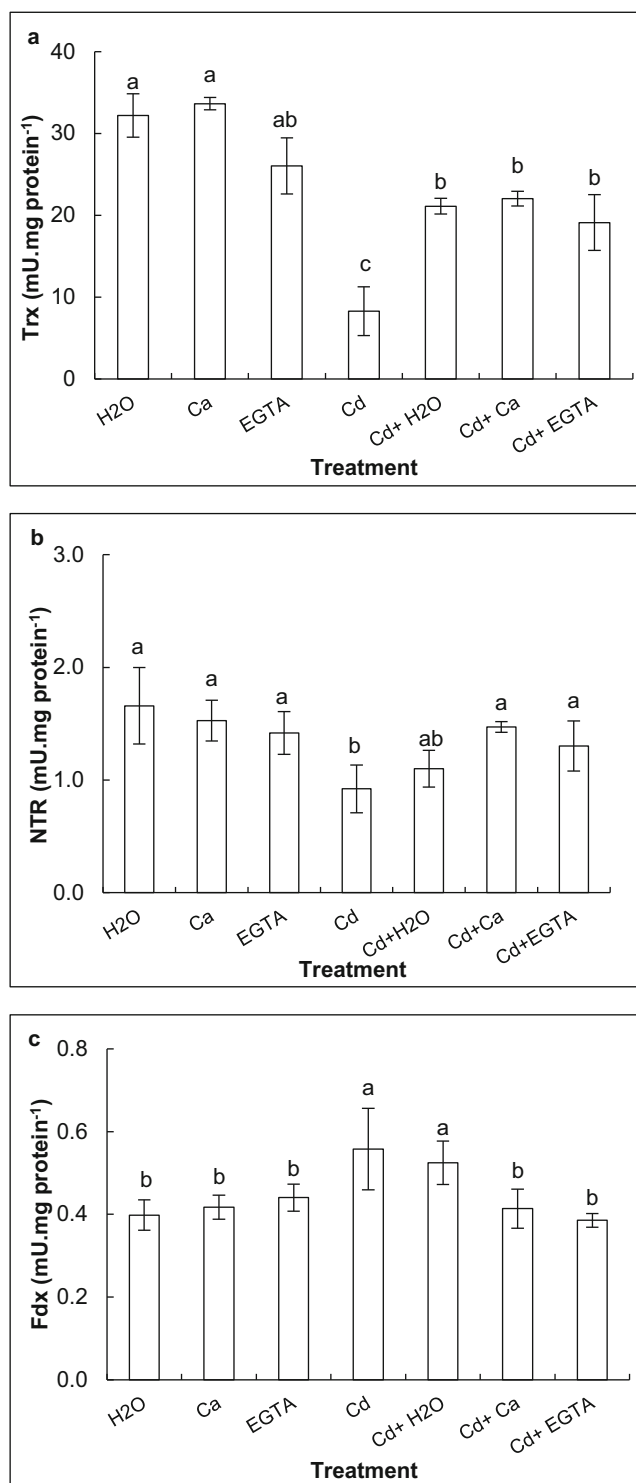
These findings were further confirmed by PCA illustration. As shown in Fig. 7a, H<sub>2</sub>O<sub>2</sub> and -SH contents and the activities of Fdx, GPX, APX, and CAT were loaded in the positive side of the first component (PC1, 65% of the variance). By contrast, GSH, GSH/GSSG ratio, Trx, NTR, and the gene expressions of APX and Cu/Zn-SOD were loaded in the opposite direction (Fig. 7a). As judged by Pearson's correlation analysis, there was no significant correlation (at *p* < 0.05) between enzymatic activities (Cu/Zn-SOD, APX, and CAT) and the expression of related genes (*r* values were 0.120, -0.702, and 0.072, respectively; Table 2).

Applied treatments were mapped in the space spanned by the first two principal components, PC1 versus PC2 (15.39% of inertia; Fig. 7b). This score plot illustrated a visible clustering depending on germination conditions. The PC1 separated the Cd treatment from the control (H<sub>2</sub>O), Ca and EGTA treatments that exhibited a distinct cluster. On the other hand, Cd stress abruption (Cd + H<sub>2</sub>O) and co-treatments (Cd + effectors) had low PC1 scores (Fig. 7b).

**Table 1** Thiols (-SH) and glutathione (GSH and GSSG) contents and the glutathione redox ratio (GSH/GSSG) in chickpea shoots after seed imbibition in H<sub>2</sub>O or 200 μM CdCl<sub>2</sub> in the presence and absence of

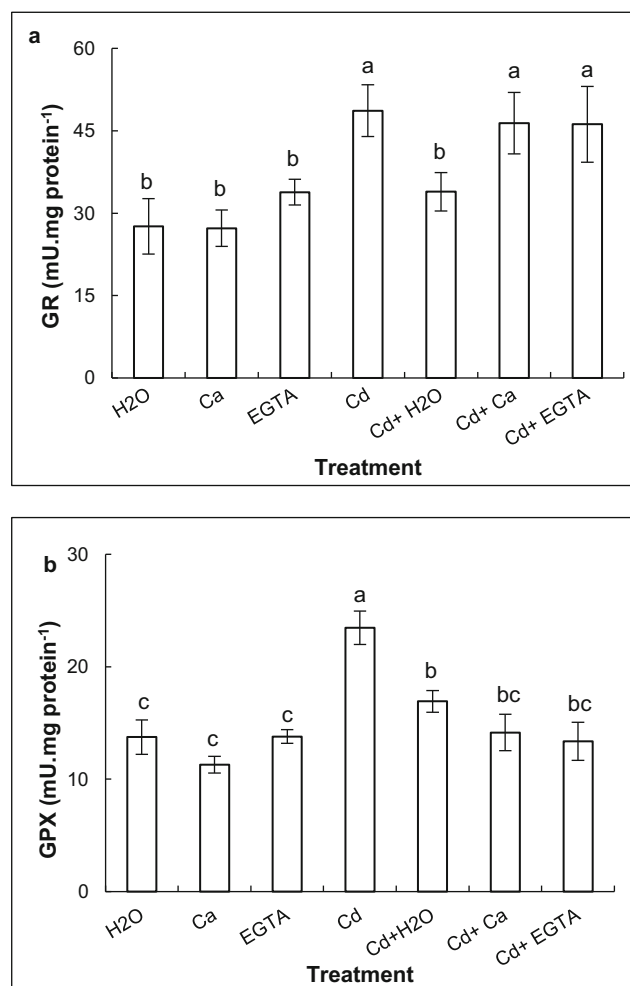
10 mM CaCl<sub>2</sub> and 100 μM EGTA. Values (± SE, *n* = 3) followed by a common letter within the same line are not different at the 0.05 level of significance using ANOVA followed by Tukey's test

Treatment	H <sub>2</sub> O	Ca	EGTA	Cd	Cd + H <sub>2</sub> O	Cd + Ca	Cd + EGTA
SH nmol g FW <sup>-1</sup>	87.48 ± 2 <sup>b</sup>	89.75 ± 9 <sup>b</sup>	88.77 ± 7 <sup>b</sup>	125.22 ± 9 <sup>a</sup>	80.10 ± 7 <sup>b</sup>	88.97 ± 8 <sup>ab</sup>	111 ± 9 <sup>b</sup>
GSH nmol g FW <sup>-1</sup>	140 ± 9 <sup>b</sup>	150 ± 11 <sup>b</sup>	160 ± 11 <sup>b</sup>	88.02 ± 12 <sup>a</sup>	140.75 ± 11 <sup>b</sup>	136.95 ± 12 <sup>b</sup>	160.67 ± 10 <sup>b</sup>
GSSG nmol g FW <sup>-1</sup>	44.66 ± 7 <sup>a</sup>	43.50 ± 3 <sup>a</sup>	43.12 ± 2 <sup>a</sup>	43.77 ± 6 <sup>a</sup>	40.63 ± 4 <sup>a</sup>	37.42 ± 3 <sup>a</sup>	41.80 ± 4 <sup>a</sup>
GSH/GSSG	3.13 ± 0.5 <sup>a</sup>	3.44 ± 0.6 <sup>a</sup>	3.71 ± 0.2 <sup>a</sup>	2.01 ± 0.2 <sup>b</sup>	3.46 ± 0.2 <sup>a</sup>	3.65 ± 0.3 <sup>a</sup>	3.82 ± 0.3 <sup>a</sup>



**Fig. 4** Trx (a), NTR (b), and Fdx (c) activities in chickpea shoots after seed imbibition in H<sub>2</sub>O or 200  $\mu$ M CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100  $\mu$ M EGTA. Values ( $\pm$  SE,  $n = 3$ ) followed by a common letter are not different at the 0.05 level of significance according to Tukey's test

Overall, the biochemical results were confirmed by the statistical analysis and provided further clues of the effectiveness of Ca and EGTA in alleviating Cd stress in chickpea shoots.

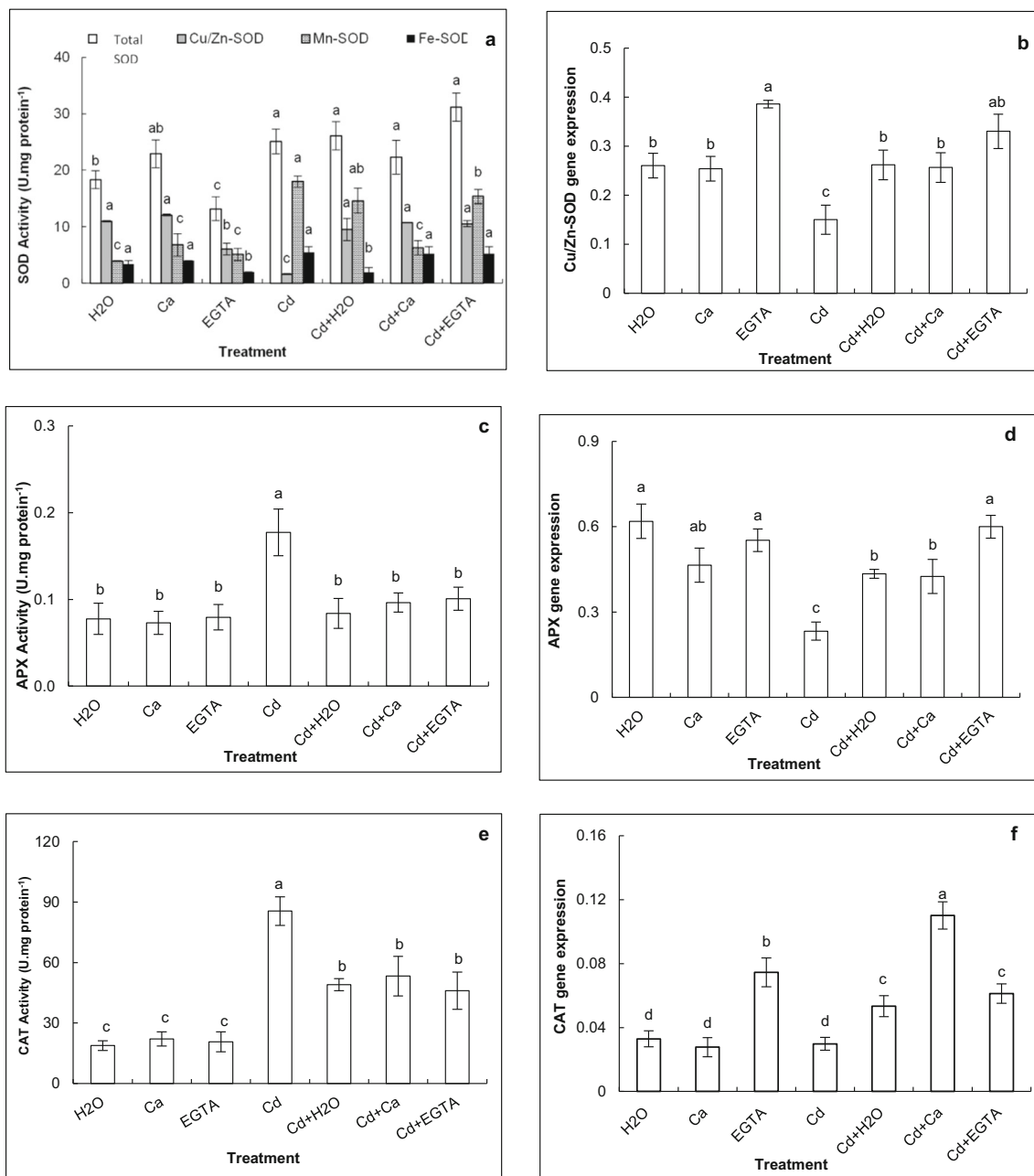


**Fig. 5** GR (a) and GPX (b) activities in chickpea shoots after seed imbibition in H<sub>2</sub>O or 200  $\mu$ M CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100  $\mu$ M EGTA. Values ( $\pm$  SE,  $n = 3$ ) followed by a common letter are not different at the 0.05 level of significance according to Tukey's test

## Discussion

### Ca and EGTA protect growth

Stunted growth represents the early symptom of Cd toxicity (Ci et al. 2009), as illustrated in Fig. 1. The adverse effect of Cd on biomass production was attributed to the failure of the reserve mobilization-related processes (Rahoui et al. 2015). In addition to the induction of apoptosis, inhibition of cell elongation, peroxidation of membrane lipid, protein carbonylation, and binding of the metal ions to the DNA were reported (Romero-Puertas et al. 2002; Štolfa et al. 2015). Interestingly, Ca and EGTA addition to the Cd-contaminated medium induced growth recovery evidenced by DW correction (Fig. 1). The protective impact of Ca and chelators against HM-induced damage on growth has been largely reviewed (Huang et al. 2017). It was suggested that exogenous Ca competes with Cd ions against Ca channels,



**Fig. 6** Activities of SOD (a), APX (c), and CAT (e) enzymes and expression of Cu/Zn-SOD (b), APX (d), and CAT (f) genes, estimated in chickpea shoots after seed imbibition in H<sub>2</sub>O or 200  $\mu$ M CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100  $\mu$ M EGTA. *Cicer*

*arietinum* actin gene was used as reference. Values ( $\pm$  SE,  $n = 3$ ) followed by a common letter are not different at the 0.05 level of significance according to Tukey's test

whereas EGTA acts as an exogenous chelator, leading in both cases to the restriction of Cd accumulation in plant cells (Sakouhi et al. 2016). In concordance with our results, recently, Li et al. (2021) reported that Ca(OH)<sub>2</sub> and CaCl<sub>2</sub> treatments decreased Cd availability in soils and Cd contents in all parts of *Panax notoginseng* plant. The authors noticed that ATPase activity correlated positively with the Ca to Cd content ratio, suggesting the involvement of ATPase in the uptake of Ca<sup>2+</sup> and Cd<sup>2+</sup> by root cells. Furthermore, Ca application decreased the frequency of

the Cd-mediated chromosomal aberration, ameliorated the mitotic index, and modulated plant growth regulators, such as auxin (El-Ashry and Mohamed 2012; Li et al. 2016).

### **Ca and EGTA ameliorate antiradical capacity and maintain redox homeostasis**

Oxidative burst, evidenced by ROS overproduction, particularly the H<sub>2</sub>O<sub>2</sub> signaling molecule, is among the early aspects of the toxicity induced by environmental constraints (Cuypers



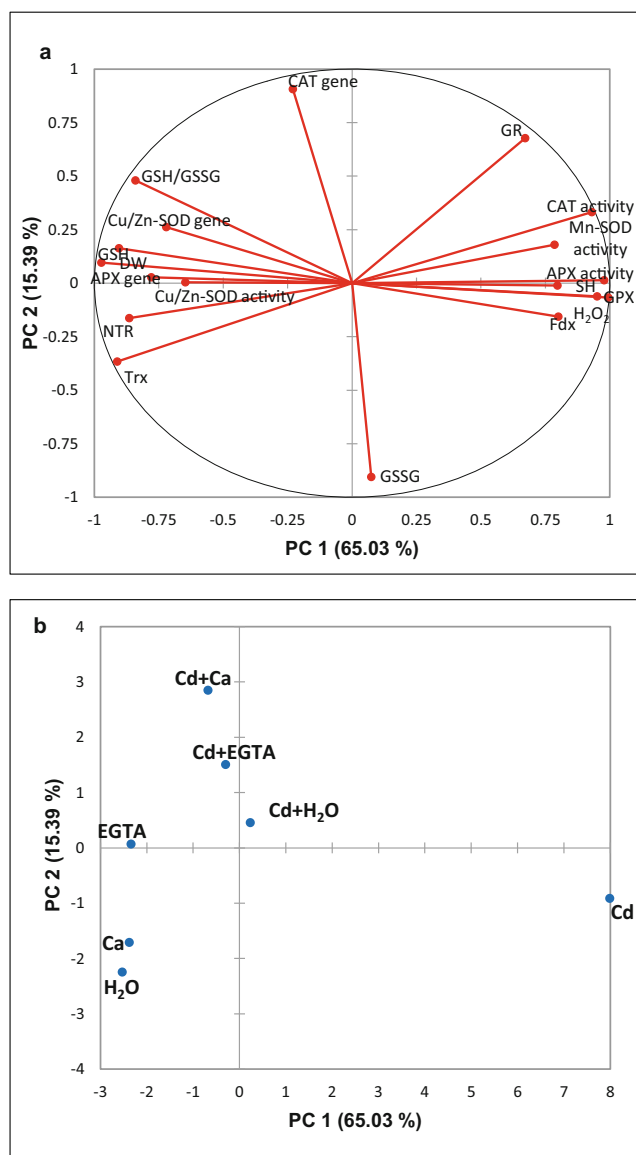
**Table 2** Pearson's correlation analysis ( $n = 3$ ) between studied parameters

Variables	SH	DW	IC <sub>50</sub>	Trx	NTR	Fdx	GSSG	GSH	GSH/GSSG
SH	1								
DW	-0.869*	1							
IC <sub>50</sub>	0.715	-0.866*	1						
Trx	-0.752	0.873*	-0.621	1					
NTR	-0.584	0.785*	-0.685	0.891**	1				
Fdx	0.344	-0.709	0.785*	-0.672	-0.874**	1			
GSSG	0.264	-0.134	0.522	0.214	0.063	0.107	1		
GSH	-0.596	0.872*	-0.846*	0.725	0.645	-0.756*	-0.440	1	
GSH/GSSG	-0.626	0.832*	-0.948**	0.563	0.558	-0.723	-0.482	-0.466	1
GR	0.701	-0.692	0.273	-0.870*	-0.606	0.266	0.102	-0.935**	-0.236
GPX	0.629	-0.899**	0.864*	-0.858*	-0.857*	0.887**	0.127	-0.922**	-0.852*
H <sub>2</sub> O <sub>2</sub>	0.790*	-0.973**	0.893**	-0.878**	-0.815*	0.785*	0.105	-0.939**	-0.887**
Mn-SOD activity	0.674	-0.700	0.566	-0.857*	-0.912**	0.654	-0.039	-0.505	-0.444
APX activity	0.874*	-0.993**	0.837*	-0.892**	-0.768	0.678	0.105	-0.890*	-0.833*
CAT activity	0.699	-0.881**	0.631	-0.962**	-0.850*	0.694	-0.283	-0.813*	-0.633
Cu/Zn-SOD activity	-0.872*	0.775	-0.652	0.617	0.450	-0.282	-0.327	0.476	0.518
APX gene	-0.396	0.740*	-0.687	0.603	0.687	-0.800*	0.119	0.781	0.701
CAT gene	-0.264	0.210	-0.593	-0.086*	0.135	-0.304	-0.874*	0.253	0.556
Cu/Zn-SOD gene	-0.417	0.658	-0.674	0.484	0.447	-0.592	-0.051	0.867*	0.829*

Variables	GR	GPX	H <sub>2</sub> O <sub>2</sub>	Mn-SOD activity	APX activity	CAT activity	Cu/Zn-SOD activity	APX gene	CAT gene	Cu/Zn-SOD gene
SH										
DW										
IC <sub>50</sub>										
Trx										
NTR										
Fdx										
GSSG										
GSH										
GSH/GSSG										
GR	1									
GPX	0.538	1								
H <sub>2</sub> O <sub>2</sub>	0.628	0.957**	1							
Mn-SOD activity	0.637	0.700	0.732	1						
APX activity	0.721	0.905**	0.980**	0.705	1					
CAT activity	0.836*	0.860*	0.904**	0.805*	0.900**	1				
Cu/Zn-SOD activity	-0.601	-0.557	-0.647	-0.401	-0.498	-0.498	1			
APX gene	-0.390	-0.728	-0.762*	-0.507	-0.702	-0.751	0.238	1		
CAT gene	0.443	-0.224	-0.270	-0.218	-0.187	0.072	0.102	0.148	1	
Cu/Zn-SOD gene	-0.262	-0.675	-0.756*	-0.412	-0.677	-0.672	0.120	0.800*	0.371	1

\* $p \leq 0.05$ ; \*\* $p \leq 0.01$



**Fig. 7** Principal component analysis (PCA) of various parameters (a) and treatments (b) applied to chickpea shoots after seed imbibition in H<sub>2</sub>O or 200  $\mu$ M CdCl<sub>2</sub> in the presence and absence of 10 mM CaCl<sub>2</sub> and 100  $\mu$ M EGTA. The first component (PC1) represents 65.03% of the inertia and the second component (PC2) explains 15.39% of the inertia

et al. 2010). Our results showed the same trend of response, as testified by the H<sub>2</sub>O<sub>2</sub> over-accumulation in chickpea shoots (Fig. 2), the negative correlation between H<sub>2</sub>O<sub>2</sub> content and seedling growth (Table 2), and the opposite direction of these two parameters in the PCA illustration (Fig. 7a). The enhanced production of ROS can be the result of the disruption of the balance between prooxidant and antioxidant systems, the oxidation of proteins, and the disruption of mitochondrial respiration (Grafão et al. 2015).

As predicted, the tested effectors (Ca and EGTA) annihilated, completely, the Cd-imposed rise in H<sub>2</sub>O<sub>2</sub> content, indicating the recovery of the cellular redox state (Fig. 2). This

protective role should be assigned, at least in part, to the restriction of the accumulation of Cd ions in cell tissues as judged by our previous investigation (Sakouhi et al. 2016). Furthermore, growing evidence suggests that competitive (Ca) and chelating (EDTA) agents are effective in reducing ROS levels in plant tissues by improving ROS-scavenging activities (Rafiq et al. 2018).

The application of Ca and EGTA reversed the substantial depressive effect of Cd on the antiradical capacity of chickpea shoot cells (Fig. 3). Thus, the growth recovery implemented by Ca and EGTA (Fig. 1) could be the achievement of the improved cellular antioxidant capacity, resulting from the interaction between the effectors and the metal.

Since thiols play a central role in plant adaptation to environmental stress, we focused on the examination of thiol homeostasis. Herein, the increased content of -SH groups may suggest protein protection against Cd-induced oxidation, which could be the result of the activation of thiol compound synthesis and/or disulfide group reduction involved systems. Given the above considerations, the thioredoxin system, involved in thiol redox state, was examined with interest.

Thioredoxins are small proteins with an extremely reactive site able to reduce the disulfide bridges of the target proteins (Zaffagnini et al. 2019). Besides, they modulate plant cell oxidative stress response and are involved in signaling pathways (Zaffagnini et al. 2012). The depressive impact of Cd on Trx should be the result of protein oxidation as well as the decreased NTR activity (Fig. 4b), known for its recycling role of the Trx reduced forms. Exogenous Ca and EGTA counteracted the adverse effect of Cd on both Trx and NTR activities (Fig. 4a, b) and restored control Fdx activity (Fig. 4c). We suppose that effectors alleviated the Cd-inhibiting effect on the Trx system, which, in turn, catalyzed the reduction of H<sub>2</sub>O<sub>2</sub> (Fig. 2), preventing, thereby, oxidative stress and apoptosis occurring, as previously reported under the same conditions (Sakouhi et al. 2016). This assumption is strengthened by the negative correlation between Trx and H<sub>2</sub>O<sub>2</sub> content (Table 2) and the factorial analysis (Fig. 7a). Glutathione, owing to its sulfhydryl group and its high intracellular content level, is considered the main redox buffer maintaining cellular redox homeostasis (Foyer and Noctor 2011). Current results give evidence that Cd critically perturbs the redox status of glutathione (Table 1) sustaining, thereby, previous studies (Singh et al. 2016). The shift of the glutathione redox balance in favor of GSSG is caused by a depletion of the reduced forms of glutathione (Table 1) without altering the content of GSSG, despite the drastic increase of GR activity (Fig. 5a). The negative correlation between GSH, GPX, and H<sub>2</sub>O<sub>2</sub> (Table 2) suggested that the noted depletion of GSH in Cd-stressed seedlings resulted from the enhancement of GPX activity, using GSH as a reducing agent for H<sub>2</sub>O<sub>2</sub>. This fact was further confirmed by PCA results since PC1 opposed GPX and H<sub>2</sub>O<sub>2</sub> to GSH and the GSH/GSSG ratio (Fig. 7a).

Moreover, the negative correlation between glutathione redox ratio (GSH/GSSG) and  $IC_{50}$ , which is inversely proportional to the antiradical capacity, evinced the important role of glutathione homeostasis in the cell ability to reduce free radicals (Table 2).

Exogenous Ca and EGTA reversed the disruptive effect of Cd on GSH redox state as in the case of stress abruption (Cd + H<sub>2</sub>O treatment), mainly through the restoration of a steady-state level of GSH (Table 1). The efficiency of Ca and EGTA in the restoration of glutathione redox homeostasis coincides with the up-regulated GR activity (Fig. 5a) and the declined consumption of GSH by GPX activity (Fig. 5b). These data are in agreement with the assumption that Ca efficiency in Cd stress alleviation is mainly related to its ability to maintain GSH optimal level and, thereby, increasing phytochelatin biosynthesis, as suggested by López-Climent et al. (2014).

### Ca and EGTA modulate the antioxidant system

Under Cd stress, divergent responses of the enzymatic and transcriptional systems have been reported (Gratão et al. 2019). This effect was explained by metal ion interference with transcription mechanisms by the displacement of Zn ions by Cd in transcription factors, owing to the chemical similarity of the two ions (Di Toppi and Gabbrielli 1999). Interestingly, the current results corroborated our previous study, suggesting a differential response to Cd stress according to SOD isoform (Fig. 6a) as well as to the seedling part (Sakouhi et al. 2018). The substitution of Zn by Cd might cause Cu/Zn-SOD enzyme inactivation and protein denaturation by oxidation (Cuypers et al. 2010; Semane et al. 2010). However, no direct link was revealed by the results of Pearson's correlation between the response of this activity and H<sub>2</sub>O<sub>2</sub> contents (Table 2). On the other hand, Cd exposure caused a gene expression inhibition, stated by the decreased Cu/Zn-SOD gene transcript accumulation (Fig. 6b). Khraiweh et al. (2010) suggested that the post-transcriptional regulation of this isoform might be attributed to the binding of the microRNAs to the enzyme mRNA, resulting in either its degradation or protein biosynthesis inhibition.

The modulating effect of Ca on the antioxidative system was connected to its role in signal transduction, in which Ca acts as a second messenger (Dayod et al. 2010). Exogenous Ca corrected the Cd-induced perturbation of the cytosolic Ca content (Farzadfar et al. 2013). This fact is perceived by Ca<sup>2+</sup> sensors, such as calmodulin, calmodulin-like protein, calcineurin B-like protein, and Ca-dependent protein kinase, which trigger downstream signaling responses, resulting in modification of antioxidant activities and target protein-related gene expression at an appropriate threshold (Hashimoto and Kudla 2011). For instance, Ca-calmodulin complex occurrence regulated gene expression and metabolism involved in plant tolerance to Cd stress (Yang and Poovaiah 2003). This finding

could explain the noticed up-regulation of APX and CAT gene expressions following co-treatment Cd + Ca (Fig. 6d, f), whereas Ca, when applied in the absence of Cd, caused no significant change in the investigated gene expression (Fig. 6b, d, e). Besides, emerging concepts suggest the involvement of crosstalk between Ca and H<sub>2</sub>O<sub>2</sub> signaling pathways in antioxidant system regulation to improve plant resistance to oxidative stress (Steinhorst and Kudla 2014).

The effects of the chelating agents on the transcriptional system are still lacking. Our results suggested an indirect effect, through the reduction of H<sub>2</sub>O<sub>2</sub> accumulation (Fig. 2) that, in turn, controls the level of target gene expression. However, the enhancing impact of EGTA, when added alone, on Cu/Zn-SOD and CAT gene expressions (Fig. 6b, f) confirmed the occurrence of a regulating pathway mediated by EGTA.

The absence of a significant correlation between enzymatic activities and the expression pattern of related genes (Table 2) sustains the hypothesis that the noted modulation of enzyme activities was mainly resulting from post-translational modifications.

### Conclusions

The present investigation provided new insight that Ca and EGTA addition, even after the initiation of the germination, could be a promising alternative in Cd stress alleviation. Exogenous Ca and EGTA counteracted the detrimental effect of Cd on seedling growth and prevented oxidative stress via the restoration of thiol homeostasis and the modulation of antioxidative enzymes at both transcriptional and post-transcriptional levels. However, further physiological and molecular studies are needed to determine the most appropriate treatment condition and to elucidate the entire mechanism by which Ca and EGTA act to improve plant Cd tolerance.

**Authors' contributions** LS performed all laboratory experiments, statistically analyzed the data, and wrote the manuscript; OK and MBM helped in manuscript drafting and revised the language; CG performed PCR analysis; SBH performed HPLC analysis; SM designed the primers and revised the manuscript; YM revised critically the manuscript; AC supervised the experiments and helped in the physiological analyses and revision step. All authors have read and approved the final manuscript.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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