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ORIGINAL PAPER

Calcium and Citrate Protect *Pisum sativum* Roots against Copper Toxicity by Regulating the Cellular Redox Status

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Abstract

The protective effects of exogenous application of calcium (Ca) or citrate against copper (Cu)-induced toxicity in pea seedlings was investigated.

Seeds were germinated in distilled water (control) or aqueous solutions of Cu (200 µM) in combination with Ca (10 mM) or citrate (100 µM) for 6 days.

The exposure of seeds to Cu caused high metal accumulation in tissues associated with increased activities of hydroxyl radical, superoxide anion, and thiol groups as well as the lipoxygenase activity. By contrast, Cu decreased the glutathione and cysteine contents by almost 68% and 27%, respectively. Moreover, one-dimension (1D) and two-dimension (2D) electrophoresis analyses showed that Cu induced a significant increase in the levels of thiol-containing proteins. In contrast, seed treatment with Ca or citrate ameliorated the cellular redox state of roots as reflected by lower levels of oxidative stress biomarkers. This achievement could be attributed to the reduction of Cu accumulation in the tissues. Besides, exogenous Ca and citrate mitigated the Cu-induced disruption of the glutathione and cysteine redox homeostasis via the modulation of the activities of the glutathione reductase, glutathione-*S*-transferase, and glutathione peroxidase enzymes. Moreover, both Ca and citrate modulated the thioredoxin and ferredoxin regeneration systems to a level close to steady-state condition. The current results evidenced that Ca and citrate are effective in imparting Cu tolerance to pea germinating seeds and suggest that Ca or citrate supplementation to Cu-polluted environment could be an economic procedure for the control of heavy metal toxicity and accumulation in crops.

Keywords Calcium · Citrate · Copper · Pea · Proteomics · Redox state

1 Introduction

Legume seeds, in particular pea (*Pisum sativum* L.), is an important legume grown around the world due to their high-quality source of protein and starch (Hedley 2001). This legume is particularly vulnerable to heavy metals toxicity causing physiologic and metabolic damages and leading to crop yield loss (Ben Massoud et al. 2018). Contamination of agricultural soils with copper (Cu) is with increasing interest due to the excessive use of Cu-based fungicides, pesticides, and fertilizers (Alloway 2013). Copper is a micronutrient essential for various plant metabolic processes, including redox metabolism, as an enzyme cofactor, and photosynthesis (Migocka and Malas 2018). Nevertheless, high levels of Cu in soil, as well as in water, cause oxidative stress by accelerating the production of reactive oxygen species (ROS), high metal accumulation in tissues and lipid peroxidation (Adrees et al. 2015; Hossain

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et al. 2020; Zehra et al. 2020). Further, Cu induced oxidative stress which activates antioxidant enzyme system and thioredoxin (Trx) system (Ben Massoud et al. 2018; Sakouhi et al. 2021). Besides, Cu excess altered seeds germination, and post-germination seedlings growth (Ben Massoud et al. 2019; Zehra et al. 2020). Ben Massoud et al. (2019) attributed the growth delay to the perturbation of the reserve mobilization from cotyledons to the embryonic axis and the redox state of nicotinamide. Likewise, Cu stress led to increased levels of hydrogen peroxide, lipoperoxides, carbonylated proteins and enhanced activities of antioxidant enzymes (Ben Massoud et al. 2017). The cell hormonal status and the mineral uptake were also affected (Feil et al. 2020). To avoid Cu-mediated protein thiols oxidation, Trx/NADPH-dependent thioredoxin reductase (NTR) systems are activated (Sevilla et al. 2015). Therefore, the inspection of the response profile of the thioredoxin system is with growing interest.

New agricultural practices such as the exogenous application of biochemical compounds have been adopted to protect crops against heavy metal pollution. These practices aim to restrict metal ion accumulation in plant tissues, enhance its sequestration in non-sensitive organelles and improve defense systems (Kharbech et al. 2020; Betti et al. 2021; Sakouhi et al. 2021).

Calcium is an important macronutrient with a vital role in plants (Parvin et al. 2019). Recent studies suggested that Ca promoted the growth and development of plants by its involvement in roots elongation (Nedjimi 2018). Calcium plays a crucial role in signal transduction of environmental stimuli and related gene expression, increasing the tolerance of plants against heavy metal stress (Valivand and Amooaghaie 2020; Zu et al. 2020; Sakouhi et al. 2018, 2021). The antagonistic effect of Ca and heavy metals was attributed to the competition on Ca^{2+} channels and the reduction of the membrane surface negativity (Perfus-Barbeoch et al. 2002; Huang et al. 2017). Citrate is also involved in the growth and development of plants as well as in the defense against abiotic stress, such as heavy metals (Gao et al. 2012). The noticed enhancement of plant tolerance to heavy metal poisoning was attributed to a decreased solubility of metal ions. Accordingly, citrate may be involved in the expression of specific proteins related to defense mechanisms.

In this study, the impact of exogenous Cu-chemical effector interactions on pea seedlings metabolism, oxidative stress biomarkers, and antioxidant enzymes have been evaluated. The cellular redox regulation and the involved enzymes were examined in relation with metal toxicity alleviation.

2 Materials and Methods

2.1 Germination and Treatment Conditions

Seeds of pea (*Pisum sativum* var. Douce Provence) were disinfected for 10 min with sodium hypochlorite (2%) then doubly rinsed with distilled water. Seeds were placed on two layers of filter paper, soaked with H_2O or aqueous solution of 200 µM $CuCl_2$, and germinated at 25 °C in the dark up to the 3rd day. The 3-day–Cu-stressed seeds were transferred to control medium ($Cu + H_2O$ treatment; stress cessation) or were co-treated with 200 µM $CuCl_2$ and 10 mM $CaCl_2$ (Ca) or 100 µM citrate-Na (citrate), respectively, for 3 additional days. At day 6, the seedlings were harvested and separated into roots and shoots, weighed, then stored at − 80 °C until use.

The choice of effectors (citrate and calcium) concentrations and treatment duration (6 days) was based on preliminary studies using a range of effectors concentrations in combination with the metal and different exposure times. The seedling analysis was preliminarily performed from the 3rd day until the 9th day of Cu-treatment, and the effectors supply showed significant alleviation of Cu-stress starting from the 6th day of treatment. The lowest concentration of effectors inducing the highest seedling recovery in the presence of metal was selected.

Copper concentration inducing embryo growth decrease by about 50% of control was selected to perform this investigation. For each treatment, embryonic axes aged of 6 days were harvested. Roots were separated and weighed. Samples were stored at − 80 °C for biochemical analysis.

2.2 Copper Content Determination

The root tissues (0.1 g dry weight) were digested with an acid mixture (HNO_3: $HClO_4$, 4:1, v/v) at 100 °C. Cu concentrations were analyzed by an atomic absorption spectrophotometer (Perkin Elmer, Waltham MA, USA) using an air-acetylene flame (Ben Massoud et al. 2018).

2.3 *Estimation of Superoxide Anion ($O_2^{\bullet-}$)*

Levels of $O_2^{\bullet-}$ were evaluated based on the reduction of tetrazolium compounds 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (Dunaevsky and Belozersky 1993). The increase in absorbance at 470 nm in the supernatant was measured using a spectrophotometer (VWR: UV-3100PC).

2.4 *Estimation of Hydroxyl Radical ($OH^{\bullet}$)*

Hydroxyl radical production was detected, using $OH^{\bullet}$-induced oxidative degradation of deoxy-ribose and

the resultant malondialdehyde production, by fluorimetric quantitation of the thiobarbituric acid adduct according to the method of Dunaevsky and Belozersky (1993) with minor modifications. The reaction product was measured fluorimetrically in the clarified sample (excitation: 532 nm, emission: 553 nm) against reagent blanks.

2.5 Quantification of Thiol Groups (–SH)

Proteins extraction was performed by homogenization of samples in K-phosphate buffer (25 mM, pH 7.0) and sodium ascorbate (5 mM). The homogenates were centrifuged at 20,000 g for 15 min at 4 °C and the supernatants were used to determine the levels of –SH groups, according to Ellman (1959). The method requires as substrate 5, 5 dithiobis (2-nitrobenzoic acid), and absorbance reading at 412 nm ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$).

2.6 Protein Extraction and Enzyme Activities Assays

Fresh tissues were homogenized in K-phosphate buffer (50 mM, pH 7.0) supplemented with EDTA (1 mM). At 4 °C, the homogenates were centrifuged for 15 min at 10,000 g. The resulting supernatants were used as soluble fractions for measurements of enzyme activities (Ben Massoud et al. 2017).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed according to Misra and Fridovich (1972) with minor modifications. The inhibition of epinephrine auto-oxidation was followed as an increase in absorbance at 480 nm. Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured according to Nagalakshmi and Prasad (2001). The decrease of absorbance indicating the oxidation of NADPH was followed at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$, Navrot et al. 2006). Glutathione reductase (GR, EC 1.6.4.2) activity was assayed according to Foyer and Halliwell (1976) by estimating the rate of NADPH oxidation-decrease in absorbance at 340 nm. Glutathione-*S*-transferase (GST, EC 2.5.1.18) activity was measured according to the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) and GSH as substrates. The absorbance was measured at 340 nm.

The assays of thioredoxin (Trx) and NADPH-thioredoxin reductase (NTR, EC 1.8.1.9) were performed using a mixture containing Tris–HCl (50 mM, pH 8.1), DTNB (100 µM), NADPH (0.2 mM) and 30 µg/mL reduced Trx (NTR assay) or 15 mg/mL NADPH and 0.1 µM NTR (Trx assay). The rate of DTNB reduction was measured at 412 nm ($\varepsilon = 13.6 \times 103\ M^{-1}\ cm^{-1}$; Jacquot et al. 1994).

Ferredoxin (Fdx) and ferredoxin-NADP reductase (FNR, EC 1.18.1.2) activities were measured according to the method described by Green et al. (1991). The protein extract was mixed with 50 mM Tris–HCl (pH 7.8), 250 µM NADPH, 40 µM cytochrome c, 2 µM spinach leaf ferredoxin (FNR assay) or 0.1 µM FNR (Fdx assay). The reduction of cytochrome c was confirmed by the increase of absorbance at 550 nm ($\varepsilon = 19.1 \times 10^{-3}\ M^{-1}\ cm^{-1}$).

Lipoxygenase (LOX, EC 1.13.11.12) was extracted in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1 mM PMSF, 2% (w/v) PVP, 1% glycerol and 0.1% Tween 20. After centrifugation (15,000 g, 4 °C, 20 min), 2.9 mL of the assay solution (1 mM linoleic acid in 0.1 M sodium acetate buffer) was added to 0.1 mL of the plant extract and absorbance was spectrophotometrically measured at 240 nm. The activity was calculated using the extinction coefficient ($\varepsilon = 25\ mM^{-1}\ cm^{-1}$) (Ramakrishna and Rao 2012).

2.7 Glutathione and Cysteine Quantification by HPLC

The samples were homogenized at 4 °C in the presence of K-phosphate buffer (0.2 M, pH 7.5) and immediately subjected to derivation (Chwatko et al. 2014). The derivation reaction was carried out using DTNB (1 mM) prepared in K-phosphate buffer (0.5 M, pH 8) (Katrusiak et al. 2001). After incubation for 5 min at 4 °C, phosphoric acid (7 M) was added to stop the reaction and the mixture was centrifuged at 12,000 g for 10 min at 4 °C. The acquired supernatant was filtered through a 0.45 µm filter and stored at − 20 °C in amber glass vials. For total GSH and cysteine quantification, the oxidized forms (GSSG and CSS) were reduced using 10 mM dithiothreitol (DTT) before the derivation reaction. The concentrations of GSSG and CSS were calculated as the difference in the contents of total and reduced forms. A reverse-phase column (Agilent, 1100 Series), connected to a UV–visible detector set at 330 nm, was used for the chromatographic separation (Katrusiak et al. 2001). The extract (20 µL) was injected into the Zorbax Eclipse Plus C18 column (5 µm, 4.6 × 250 mm) Agilent (USA). The mobile phase consisted of acetonitrile and acidified water (pH 3.5) at a flow rate of 1.2 mL min^{-1}. The quantification of total and reduced forms of thiols was obtained from calibration curves using DTNB-derived glutathione and cysteine (Sigma) solutions. The area of peaks corresponding to GS-TNB and CS-TNB is considered in the calculation of concentrations.

2.8 Protein Thiols Labeling

The proteins were extracted in 10 mM of Tris–HCl buffer (pH 7.2) containing: saccharose (500 mM), KCl (150 mM), EDTA (1 mM) and PMSF (1 mM). The homogenate was centrifuged at 4 °C for 1 h and the supernatant was recovered. After incubation at 37 °C for 150 min in the dark in the presence of 5'-iodoacetamide fluorescein (IAF, 0.2 mM), proteins were precipitated with TCA (20%) then centrifuged at 20,000 g for 3 min at 4 °C. For SH groups, the pellets

were recovered and resuspended in acetone (96%). The obtained pellets were resuspended in a mixture containing: Tris–HCl (0.5 M, pH 6.8), glycerol (10%), SDS (0.5%) and bromophenol blue, then applied to 1D SDS-PAGE gels (12%) (Laemmli 1970) (Mini-PROTEAN system, Bio-Rad). 1D Gels were scanned in a Typhoon Trio Scanner 9400. The analysis of protein-associated fluorescence intensity (arbitrary units, AU) was performed using Quantity One image analysis software (BioRad, Hercules, CA, USA). The gels were stained with Colloidal Coomassie Brilliant Blue G250 (Dyballa and Metzger 2009), and the total OD was measured and normalized by a calibrated densitometer GS-800 (BioRad, Hercules, CA, USA). For 2D gels elaboration, proteins were separated according to their pI (first dimension: isoelectric focusing IEF), then according to their molecular weight (second dimension: SDS-PAGE). Proteins were first rehydrated in thiourea (2 M), urea (5 M), CHAPS (2%), ampholyte (4%) (Pharmalyte 3–10, Amersham-Pharmacia Biotech, Little Chalfont, Bucks, UK), Destreak reagent (1%) (Amersham-Pharmacia Biotech), and bromophenol blue, and then immobilized in 7 cm IPG strips (pH 3–10) of dimension 70 × 3 × 0.5 mm and linear-gradient (NL) (GE Healthcare ImmobilineTM Dry Strip IPG, Bio-Sciences AB, Bio-Rad, Hercules, CA, USA). For the separation, the proteins were focused on a Protean IEF Cell (Bio-Rad) for at least 15 h at room temperature starting by a linear voltage increase until 250 V for 15 min, then 10,000 V for 2 h (50 µA/strip), followed by focusing at 20,000 V, and finally hold at 500 V. Strips were incubated for 20 min in equilibration buffer: Tris (0.375 M, pH 8.8), urea (6 M), SDS (2%), and glycerol (20%) containing 2% DTT), and then for supplementary 20 min in equilibration buffer containing 2.5% iodoacetamide. For protein separation, IPG strips were loaded onto 12% SDS-PAGE gels (PROTEAN Plus Dodeca Cell Bio-Rad). Then, the gels were scanned for fluorescence and stained with Colloidal Coomassie Brilliant Blue R-250 followed by densitometry scanning. Normalization of IAF-labeled protein spots and Coomassie-staining intensity was performed using Progenesis Same Spots Software (Ref: S/No.62605/3787; Nonlinear USA Inc/2530 Meridian Parkway/3rd Floor Durham/NC 27,713/USA). For the same gel, fluorescence spots were normalized to protein intensity revealing increased fluorescence.

2.9 Statistical Analysis

Germination and the above-cited experiments were performed in triplicate. Significances were tested by ANOVA followed by Tukey's (HSD) test. Principal component analysis (PCA) was undertaken to depict the interplay between the different studied parameters and to visualize the response profile of root cells to different treatments. Landmarking alignment (3D Gaussian distribution) was used for protein spots of 2D gels, and their intensities were corrected by background subtraction and normalized. Then, the images were subjected to quantitative and qualitative analyses. A probability of $p < 0.05$ was considered significant in all statistical analyses performed using IBM SPSS Statistics version 26.0.0.0 Software.

3 Results

3.1 Effects of Ca or Citrate on Cu Accumulation and Oxidative Stress Biomarkers

The roots of pea seedlings exposed to $CuCl_2$ accumulated high levels of Cu (2.5-fold compared to control); while Cu content decreased around half when the seedlings were treated simultaneously with combinations Cu and Ca or citrate (Fig. 1a). Cu raised the $O_2^{\bullet -}$ content by 67%, $OH^{\bullet}$ by 50% and non-protein thiols (-SH) by 47%, compared to untreated samples (Fig. 1b, c, d). Subsequent to exogenous Ca or citrate addition, the over-accumulation of ROS and –SH groups in Cu-treated roots was significantly ($p < 0.05$) reduced (Fig. 1). Indeed, Ca and citrate decreased the $O_2^{\bullet -}$ content by 37% and 58%, $OH^{\bullet}$ by 45% and 22% and -SH by 51% and 50%, respectively with respect to Cu-treated roots (Fig. 1b, c, d). Besides, when the seedlings were transferred to control condition after 3 days of exposure to Cu ($Cu + H_2O$), the contents of oxidative stress biomarkers were close to that of Ca and citrate (Superoxide anions content was reduced by 50%, $OH^{\bullet}$ by 34% and –SH groups by 45%, compared to Cu treatment; Fig. 1b, c, d).

3.2 Effects of Ca or Citrate on Activities of Antioxidant Enzymes under Cu Stress

The activities of antioxidant enzymes in roots increased considerably under Cu stress showing an increase of 40% in SOD, 41% in GST, and 55% in LOX as compared to control (Fig. 2a, b, c). With Ca or citrate, the activities of SOD, GST, and LOX were decreased significantly under Cu stress by − 30% and − 35% in SOD, − 36% and − 35% in GST and − 47% and − 51% in LOX, respectively as compared to Cu treatment (Fig. 2a, b, c). The treatment ($Cu + H_2O$) induced a decrease in antioxidant activities close to that of Ca and citrate by − 37% in SOD, − 32% in GST and − 33% in LOX in comparing with Cu treatment only (Fig. 2a, b, c). Simultaneously, Cu induced a significant increase in GPX and GR activities (+ 50% and + 43%, respectively compared to control) (Fig. 2d, e). Application of Ca and citrate decreased these activities in pea roots − 39% and − 38% for GPX and − 35% and − 33% for GR, respectively compared to Cu treatment (Fig. 2d, e). Similarly, GPX and GR activities

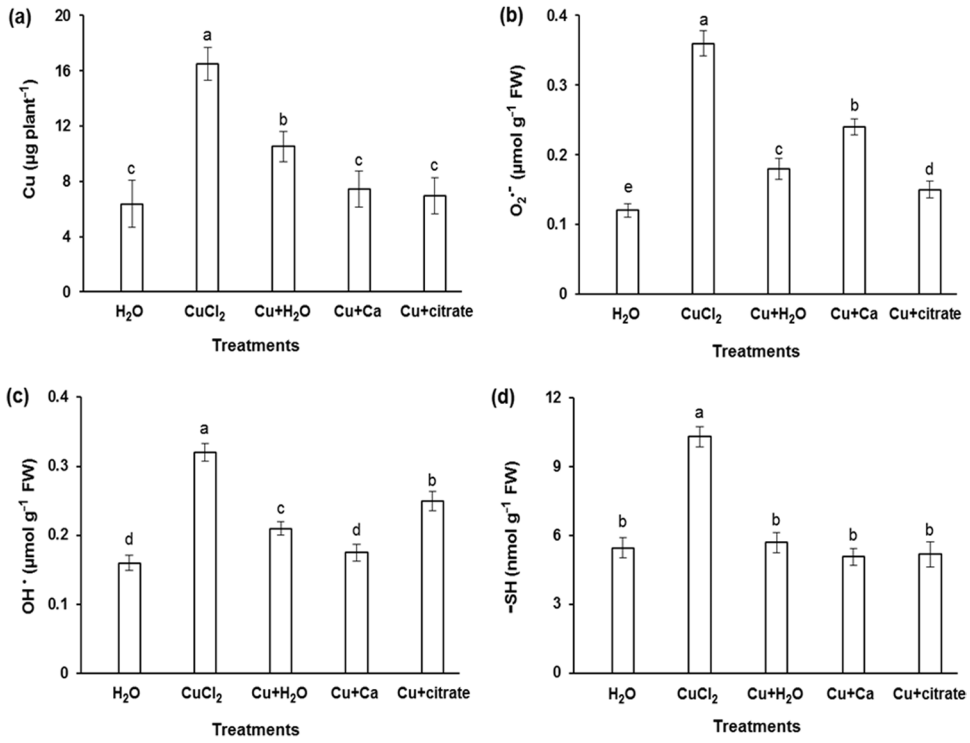

Fig. 1 Copper accumulation (**a**), contents of $O_2^{\bullet-}$ (**b**), $OH^{\bullet}$ (**c**) and protein thiols (**d**) in roots of pea seedlings. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Values ± SE ($n = 5$) followed by a common letter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test

decreased by 45% and 38% when Cu is eliminated during the last three days of germination (Cu + H_2O; Fig. 2d, e).

Moreover, a significant stimulation of the Trx, NTR, Fdx, and FNR capacities was observed after treatment with Cu (+ 73% in Trx, + 57% in NTR, + 52% in Fdx and + 70% in FNR) compared to control (Fig. 3). Calcium and citrate decreased these activities with respect to Cu-stressed samples, respectively by 48% and 44% for Trx, 57% and 56% for NTR, 37% and 32% for Fdx and 52% and 53% for FNR (Fig. 3). Interestingly, exogenous effectors were efficient at modulating the Trx and Fdx systems to Cu + H_2O level where the metal stress has been ceased after 3 days of exposure (Fig. 3).

3.3 Ca and Citrate Modulate Non-enzymatic Antioxidants Levels under Cu Stress

The content of GSH, GSSG, and GSH/GSSG ratio in the roots of pea subjected to different Cu treatments was shown (Fig. 4a, b). Total GSH levels decreased significantly (about four times relative to control) in roots exposed to Cu only. However, the application of effectors induced regeneration of total glutathione (GSH + GSSG) (Fig. 4a). Generally, the content of reduced forms (GSH) was much higher than that of the oxidized forms (GSSG), so the variation of GSH content was significant between the different treatments whereas the content of GSSG remained relatively stable and not significant between the control and the other treatments (Fig. 4a).

The depletion of GSH was accompanied by a strong perturbation of the tripeptide redox status in roots, whose GSH/GSSG ratio is shifted to the oxidized form (Fig. 4b). The GSH/GSSG ratio in roots treated with Cu decreased by almost 68% compared to control. The correction was more evident in the GSH/GSSG ratio, which showed that Ca and citrate restored the reduced GSH content (Fig. 4b).

Free cysteine is a limiting factor in the biosynthesis of glutathione. Moreover, the implication of its redox status in cellular homeostasis in plant cells is with increasing interest. Under Cu stress, the cysteine oxidation state (Fig. 5a) was similar to that of glutathione (Fig. 4a). Application of Ca and citrate reversed the negative effect of Cu on CSH/CSS balance (Fig. 5b). The effector-induced correction of the redox status was more prominent than that observed when stress was abolished (Cu + H_2O treatment; Fig. 5).

3.4 Calcium and Citrate–Induced Changes in Protein Thiols under Cu Stress

The 1D SDS PAGE results (Fig. 6) showed that the total spot intensity of free thiols decreased approximately threefold in the roots after Cu exposure (− 68% of control), while a slight decrease was registered with Ca or citrate treatments (respectively; − 30% and − 34% of control) (Fig. 6c). The 2D SDS PAGE results showed different spots after IAF-labeling and CBB staining (Fig. 7). The results (Table 1) showed significant differences (increased or decreased spot numbers) occurring within the thiols of proteins. Moreover,

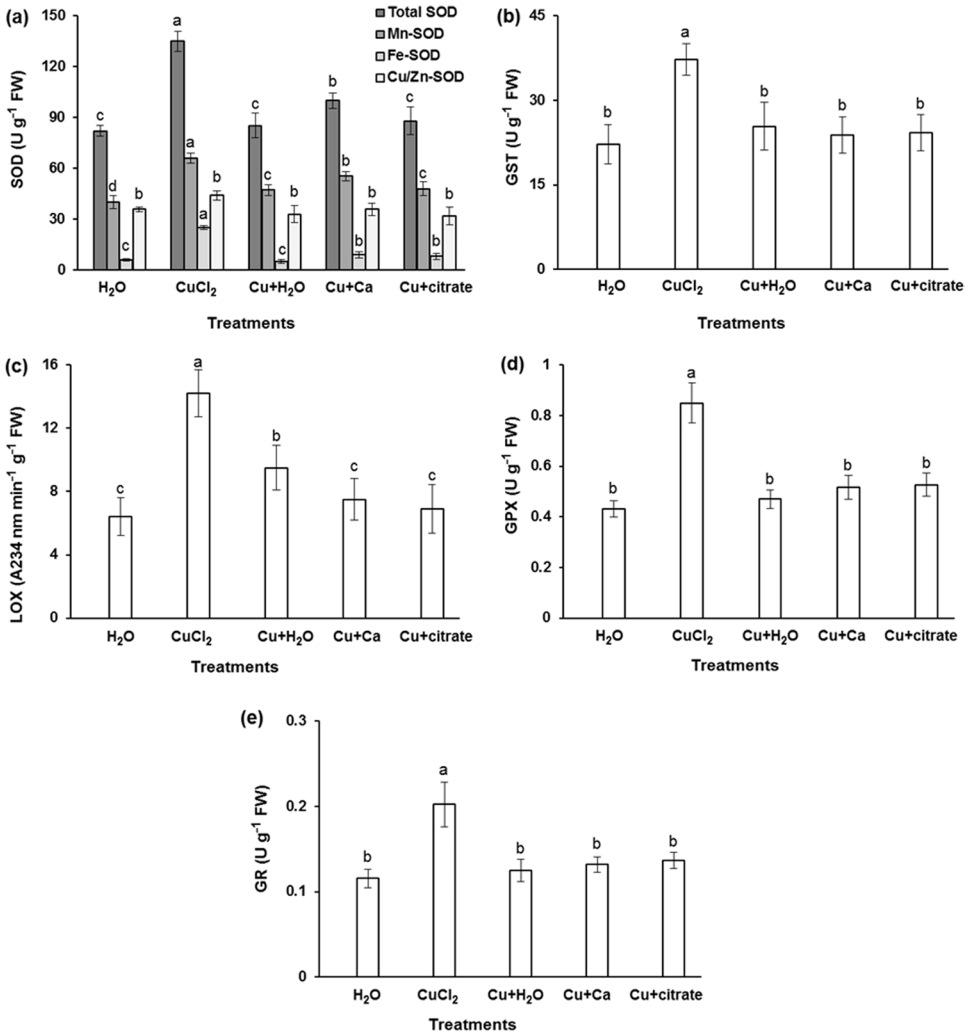

Fig. 2 Activities of antioxidants enzymes SOD (**a**), GST (**b**), LOX (**c**), GPX (**d**) and GR (**e**) in roots of pea seedlings. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Values ± SE ($n = 5$) followed by a common letter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test

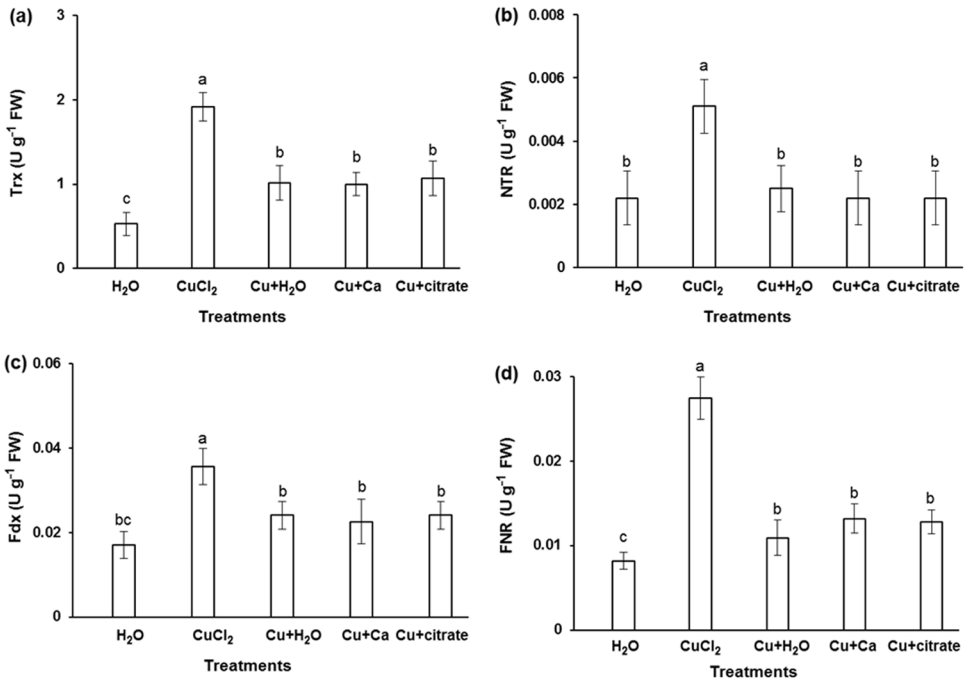

Fig. 3 Activities of Trx (**a**), NTR (**b**), Fdx (**c**) and FNR (**d**) in roots of pea seedlings. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Values ± SE ($n = 5$) followed by a common letter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test

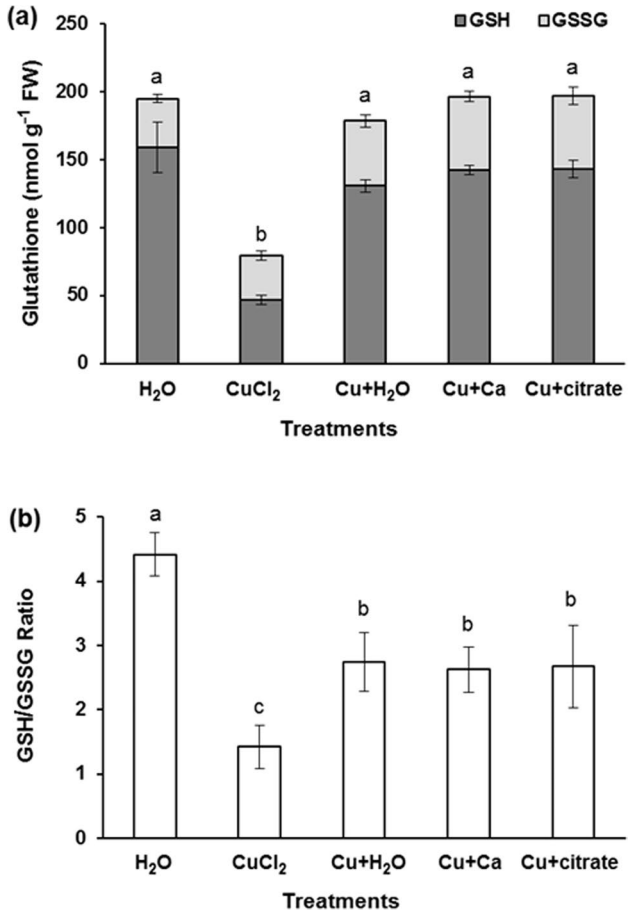

Fig. 4 Contents of the glutathione pool (GSH and GSSG) (**a**), and glutathione redox ratio (GSH/GSSG) (**b**) in roots of pea seedlings The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Values ± SE ($n = 5$) followed by a common letter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test

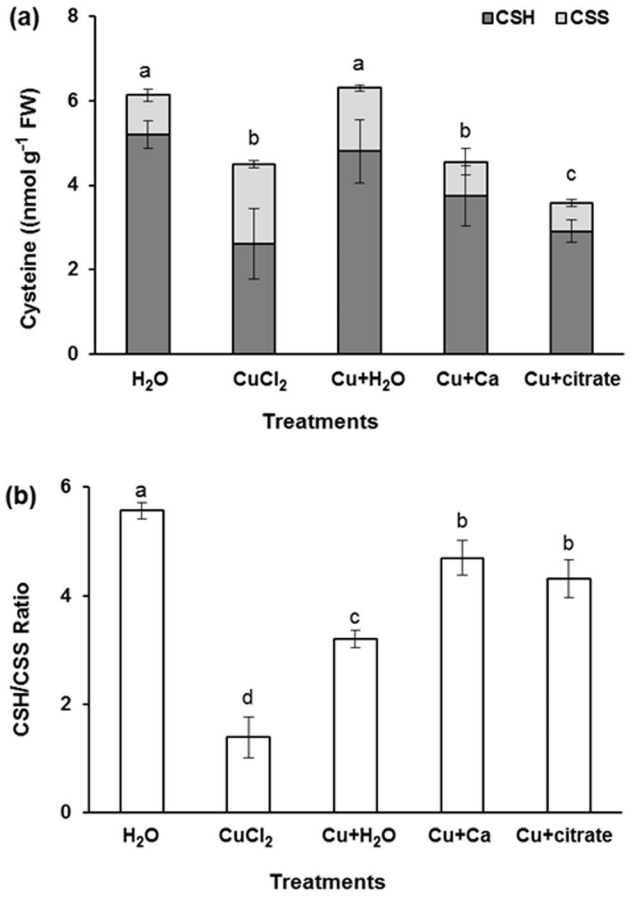

Fig. 5 Contents of the cysteine pool (CSH and CSS) (**a**), and cysteine redox ratio (CSH/CSS) (**b**) in roots of pea seedlings. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Values ± SE ($n = 3$) followed by a common letter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test

the number of modified spots in the presence of Cu is 25 spots, so in the presence of Ca, the number of modified spots decreases to only 15 spots and up to 12 spots with citrate. These results suggested either enhancement or inhibition of the expression and/or the abundance of proteins. Indeed, the protein content varied according to the physiological conditions and their regulation was achieved differently in response to the treatments.

3.5 Principal Component Analysis

Principal Component Analysis (PCA) was performed to illustrate the interplay between the studied parameters and the applied treatments (Fig. 8). The first (PC1) and second (PC2) components represent 84.57% and 8.98% of the variance, respectively. Cu content, oxidative stress biomarkers ($O_2^{\bullet -}$, $OH^{\bullet}$), pro-oxidant activity (LOX), –SH content, the antioxidative activities (SOD isoenzymes, GST, GR), and the thioredoxin system (Trx, NTR, Fdx, FNR) were loaded in the positive side of PC1. By contrast, GSH and CSH/CSS ratios were loaded in the opposite direction (Fig. 8a).

The scatter plot illustrating the applied treatments according to PC1 and PC2 showed a distinct clustering depending on treatment conditions. As expected, PC1 separated the seedling response to Cd stress and the other treatments. The treatments "Cu + Ca" and "Cu + citrate" exhibited a visible cluster, characterized by the lowest negative PC2 score values (− 0.763 and − 1.195, respectively; Fig. 8b) and almost the same score value as "Cu + H_2O" treatment on PC1 (− 0.297, − 0.252, and − 0.357 for Cu + Ca, Cu + citrate and Cu + H_2O, respectively; Fig. 8b).

Taken together, the two statistical analyses are consistent and confirmed of the effectiveness of Ca and citrate in improving pea seedling to Cu stress.

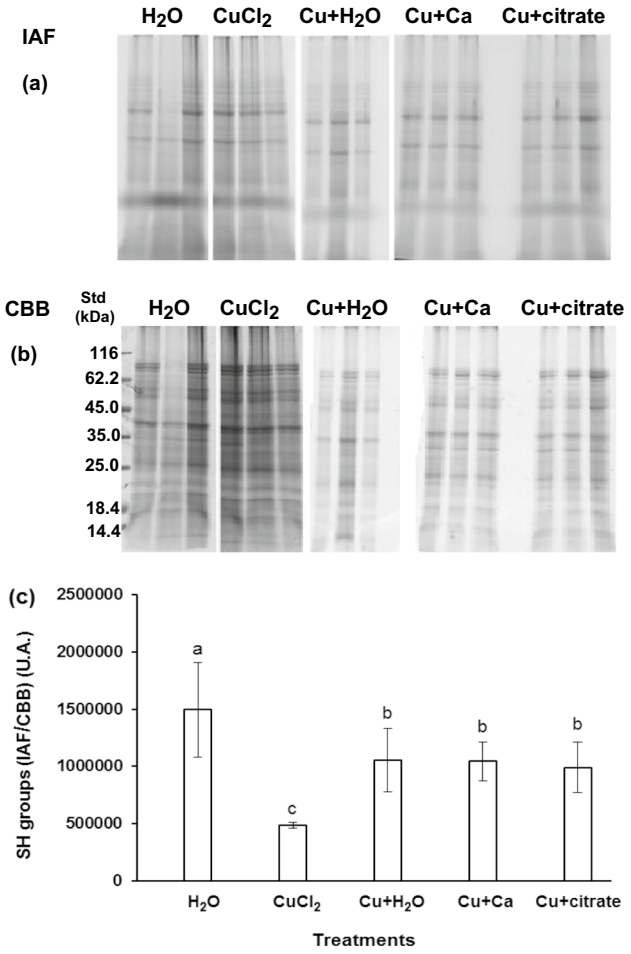

Fig. 6 Representative images of 1D gels (**a**, **b**) and total spot intensity (IAF/CBB) (**c**) of protein thiols in roots pea seedlings. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Proteins (80 µg) were stained with IAF labeling (**a**) (scanned with Typhoon 9400 scanner, 600 PMT) and with Coomassie CBB (**b**) (scanned with GS-800 calibrated densitometer)

4 Discussion

Under normal physiological conditions, the continuous generation of oxygen-free radicals, known as reactive oxygen species (ROS), is a usual process in plants (Noctor et al. 2002). An overaccumulation of ROS, such as singlet oxygen ($1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), in plant cells has been considered as stress biomarkers under constraining environments, including heavy metals (Karmous et al. 2017; Sakouhi et al. 2021). As predicted, in the current study, the contents of $O_2^{\bullet -}$ and $OH^{\bullet}$ were found to increase in Cu-treated roots. The noted ROS rise could be a result of the Cu-induced interruption in the electron transfer processes in mitochondria and/or chloroplasts (Wang et al. 2004; Heyno et al. 2008; Bashri and Prasad 2015). Concomitantly, a significant increase of -SH pools occurred when seeds were Cu-challenged. Similar results were also reported in roots of Cd-stressed chickpea (Sakouhi et al. 2018). This response may constitute a protection way against Cu-induced oxidation resulting from the neosynthesis of thiol compounds with antioxidant activities and/or the upregulation of processes involved in the reduction of protein thiols. Remarkably, both Ca and citrate reduced Cu, ROS, and SH contents in pea roots. In agreement with our results, many effectors were reported to decrease heavy metal accumulation and oxidative stress particularly Ca (Borgmann et al. 2005) and citrate (Sebastian and Prasad 2018).

Superoxide dismutase is a pivotal antioxidant enzyme catalyzing the detoxification of $O_2^{\bullet -}$. Copper increased SOD and GST activities, indicating the activation of cellular antioxidant systems. Applications of Ca or citrate decreased the activities of these enzymes in Cu-treated roots. The ameliorating effects of Ca and citrate applications could also be correlated with decreased accumulation of stress biomarkers in Cu contaminated roots, thereby minimizing the risk of cell damage. Previous study, showed an increase of SOD in a concentration and exposure time-dependent manner in roots of *Brassica juncea* exposed to Cu, indicating the important role of this enzyme in combating heavy metal stress (Singh et al. 2010). Furthermore, application of effectors like auxin has also been shown to up-regulate the activity of SOD in *Lycopersicum esculentum* (Tyburski et al. 2009) and GST in *Zea mays* (Bocova et al. 2013).

The activity of LOX was considered as an indicator of oxidative stress, which catalyzed the oxygenation of polyunsaturated fatty acids into lipid hydroperoxides during responses to various environmental constraints (Chaoui and Ferjani, 2014). It appears that Cu can operate by enhancing LOX activity in pea seedlings (Fig. 2c). Under Cu-stress, Ca or citrate application counteracted the increase in LOX activity, indicating that down-regulation of LOX might be involved in Ca and citrate-induced antioxidant effects against Cu-stress. This result is correlated with a concomitant stimulation of the regenerative and consumption activities of GSH, namely GR and GPX (twice at the roots compared to control), but which seems to favor the latter.

Our results suggest that in roots, the Trx/NTR and Fdx/FNR systems improve the redox status of thiol proteins. Concomitantly, differential redox responses in roots suggest a major capacity of redox systems to prevent oxidation of protein thiols (Karmous et al. 2017). However, Ca and citrate addition restores these parameters to a level significantly close to control. To understand the redox status of glutathione and cysteine in Cu exposed roots, a proteomic approach was adopted. A particular attention was paid to the study of the impact of Cu combined with exogenous effectors on levels of glutathione and cysteine, which represents a

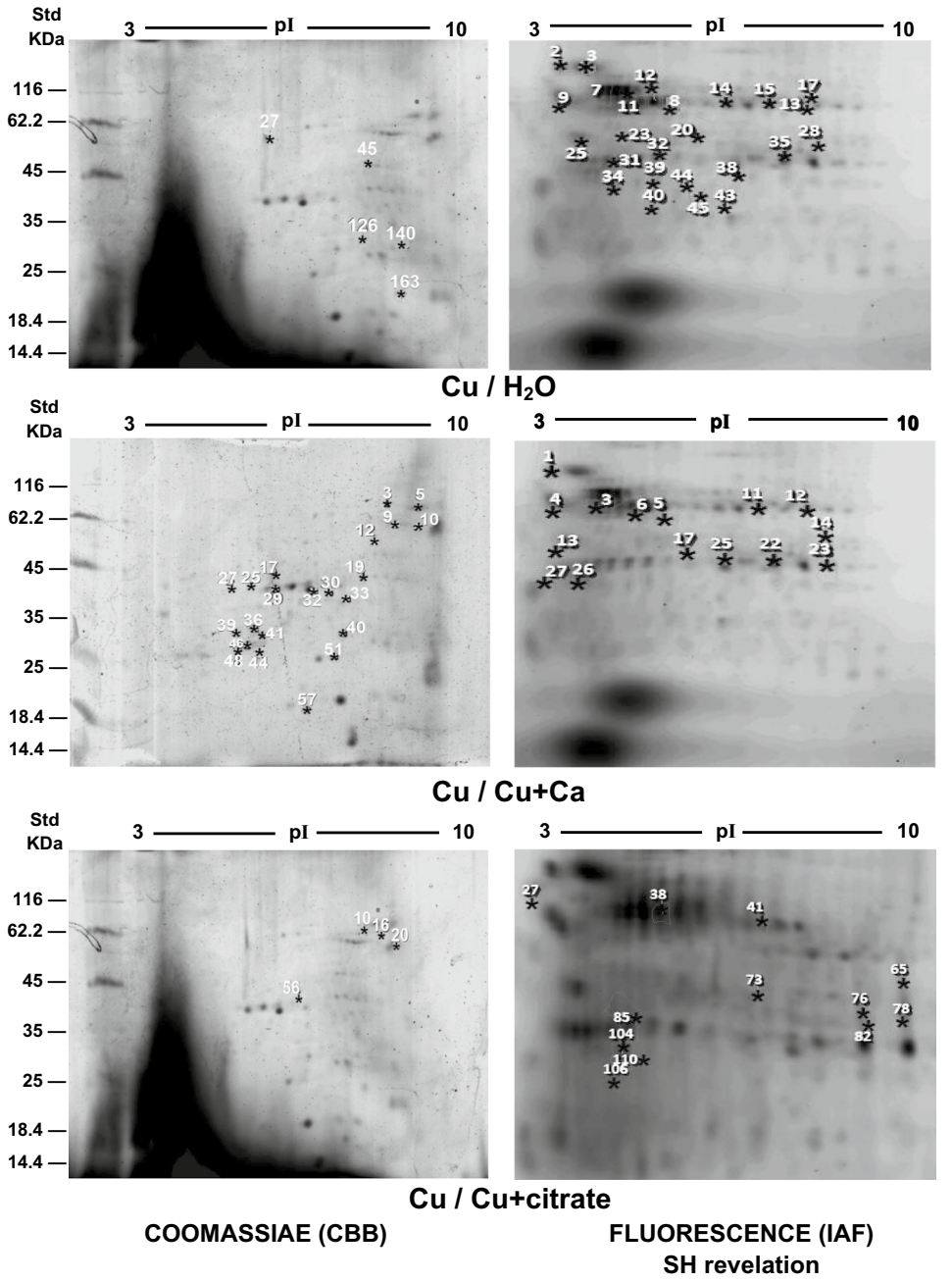

Fig. 7 Expression profile of protein thiol groups in roots of pea seedlings. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate". Proteins (150 µg) were stained with colloidal Coomassie Brilliant (CBB) and separated by 2-D SDS-PAGE. Spots of interest in the representative gels from CBB staining and IAF labeling were scanned with GS-800 calibrated densitometer and Typhoon 9400 scanner (600 PMT and 500 PMT), respectively

Table 1 Summary of quantitative changes of proteins containing thiol groups (revelation by IAF labeling) in pea roots. The seeds germinated for 3 days in H_2O or 200 µM $CuCl_2$ and then transferred up to 6 days in H_2O or $CuCl_2$ combined with 10 mM calcium "Cu + Ca" or 100 µM citric acid "Cu + citrate" (data are from Fig. 7)

Treatments	H_2O/Cu	Cu/Cu + Ca	Cu/Cu + citrate
Number of spots modified > 1.5-fold with $p < 0.05$	25	15	12

limiting factor for GSH biosynthesis, as well as the response of Trx/NTR and Fdx/FNR systems, main intracellular redox actors that controls the redox state of protein thiol groups by catalyzing the formation of intramolecular disulfide bridges. Several studies suggested that reductive enzymatic activities, in particular those linked to the Trx/NTR and Fdx/FNR systems, are crucial for cellular redox regulation. These systems have been shown to play a fundamental role in the control of redox status in plants subjected to abiotic stress (Rouhier et al. 2008, 2010; Karmous et al. 2017).

Non-enzymatic antioxidants (GSH and cysteine) play a vital role in maintaining cellular redox potential for abiotic

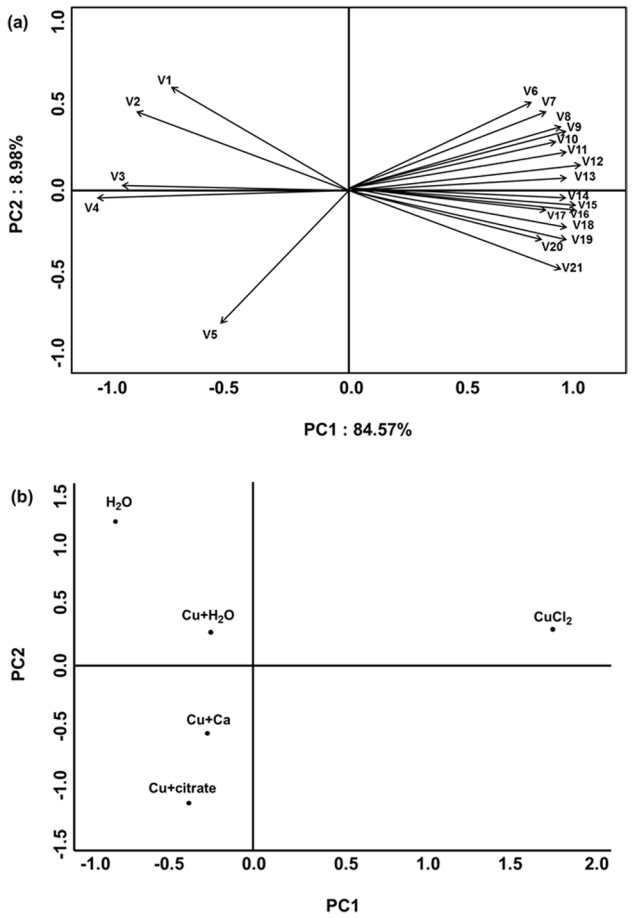

Fig. 8 Principal Component Analysis (PCA) of various parameters (**a**) and treatments (**b**) applied to germinating pea seeds. The first component (PC1) represents 84.57% of the inertia and the second component (PC2) explains 8.98% of the inertia. **V1**, CSH; **V2**, GSH/GSSG; **V3**, CSH/CSS; **V4**, GSH; **V5**, GSSG; **V6**, CSS; **V7**, Cu/Zn-SOD; **V8**, SH; **V9**, NTR; **V10**, Cu; **V11**, LOX; **V12**, GST; **V13**, Fe-SOD; **V14**, GPX; **V15**, GR; **V16**, $O_2^{\bullet-}$; **V17**, FNR; **V18**, Fdx; **V19**, $OH^{\bullet}$; **V20**, Trx; **V21**, Mn-SOD

stress tolerance by scavenging overproduced ROS. The perturbation of the redox state of glutathione has been reported in plants exposed to various environmental stresses, such as salinity (Hefny and Abdel-Kader 2009), cold (Radyuk et al. 2009), Cd (Romero-Puertas et al. 2006; Rehman and Anjum 2010; Anjum et al. 2011), Zn (Cuypers et al. 2001), Pb (Kumar et al. 2012), and Cu (Russo et al. 2008). The decline of GSH under Cu stress (Fig. 4a) could be attributed to high: (i) use of GSH by the ascorbate–glutathione cycle (Noctor and Foyer 1998), (ii) conjugation of GSH to Cu through the glutathione-*S*-transferase activity (Delalande et al. 2010), and (iii) biosynthesis of phytochelatins involved in metal sequestration and compartmentalization. The addition of Ca or citrate in the germination medium restored almost the GSH contents and improved the GSH/GSSG balance in the roots.

Glutathione plays an important role in scavenging ROS or toxic compounds with the help of the antioxidant enzymes GPX and GST (Szalai et al. 2009). Treatment of pea seedlings with Cu decreased GSH content with increased GR and GST activities. The level of GSSG also increased due to the oxidation of GSH during the scavenging of ROS. However, exogenous Ca and citrate applied to the Cu-treated pea seedlings further stimulated GSH content along with decreased GR and GST activities. In agreement with our results, an increase in GSH levels was reported in orange and *Sedum alfredii* exposed to Ca-Cd co-treatment (López-Climent et al. 2014). Also, Bashri and Prasad (2016) showed that Cd leads to a decrease in GSH and an increase in GSSG contents in *Trigonella foenum-graecum*, whereas the addition of auxin corrects both alterations. However, several studies have shown the important role of the application of some ionic competitors such as calcium and organic acid citrate and others in maintaining the redox cell balance (Mishra et al. 2013; Rahman et al. 2016; Jin Feng et al. 2018). Contrarily to GSH, the cell cysteine levels have been studied by other researchers and an increase in accumulation of this amino acid has been reported in tobacco exposed to Cd and poplar to H_2S (Kawashima et al. 2004; Nakamura et al. 2009). Nakamura et al. (2009) attributed this over-accumulation to *neo* synthesis through the overexpression of cysteine synthase. According to Azarakhsh et al. (2015), exogenous cysteine addition to basil seeds and seedlings would be able to mitigate the detrimental effects of cobalt.

The antioxidant enzymes studied here are a part of vital defense mechanisms developed by roots of *Pisum sativum* treated with Cu in order to protect against ROS and could be expected to lead to oxidative modification of proteins. This study probed whether the presence of Cu alone or combined with citrate or Ca induce multiple oxidative changes to proteins, including quantitative and qualitative changes in the thiol groups in the roots of pea seedlings. Owing to their abundance, proteins are the major cellular target to a range of reactive radicals (Davies 2005). Our experiments in 1-D SDS-PAGE revealed many differences between treatments in the detection of thiols proteins. Interestingly, the protein SH profile showed a significant decrease under Cu stress, on the contrary of the spectrophotometric measurement, probably overestimated due to the presence of phytochelatins of small molecular weights, and which proteomic analysis might not take into account. Hence, 1-D result suggested the oxidation of protein thiols in roots under Cu-stress. These redox modifications could induce many reactions like alkylation (Lin et al. 2002) and glutathionylation (Hansen et al. 2009). Nonetheless, the exogenous application of Ca or citrate showed a significant recovery in thiol proteins of Cu-treated roots.

To further investigate the redox changes in thiol proteins, an analysis for the separation of the proteins with

2D electrophoresis was used. The results obtained for free thiols spot density proteins confirm those obtained in the enzymatic assay. It seems therefore that the presence of Cu modified to some extent the effects of Ca and citrate separately on protein status in pea seedlings. Ahsan et al. (2009) demonstrated that heavy metals cause disruption of most cellular mechanisms and differential accumulation of proteins involved in the regulation of cellular redox status. Similarly, Printz et al. (2013) observed a decreased abundance of major proteins involved in cellular processes, such as those that scavenged ROS or involved in redox regulation. Also, other proteomic approaches have demonstrated the establishment of an oxidative stress state under environmental stress conditions attested by (1) downregulation of ubiquitinated proteins and thioredoxin during germination (Zhang et al. 2009), (2) up-regulation of proteins associated with antioxidant defense (He and Yang 2013) and abundance of those involved in ROS sequestration (Nanjo et al. 2012, 2013), (3) alteration of the proteins involved in redox homeostasis (Wang and Liu 2008) and (4) induction of heat shock proteins (Kottapalli et al. 2013) and metallothioneins (Zhang et al. 2009).

5 Conclusions

In conclusion, the results of this study demonstrated that exogenous Ca and citrate alleviated Cu-induced oxidative stress by ameliorating the cellular redox status. This effect was achieved by limiting the Cu-induced increase of antioxidant enzymes and the maintaining of the redox status of glutathione and cysteine. Furthermore, the separation of proteins by 1D and 2D showed that Ca or citrate could protect protein thiol groups against oxidation in roots of Cu-treated pea seedlings, by reducing metal accumulation in tissues through Cu entry restriction. Figure 9 exhibits the protective effects of Ca and citrate supply against Cu toxicity in pea roots.

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Author contribution MBM performed all laboratory experiments, statistically analyzed the data, and wrote the manuscript; OK helped in manuscript drafting; LS performed PCA Analysis; YZ performed Proteomic analysis; SBH performed HPLC analysis; DS supervised the proteomic experiments and helped in the proteomic analyses; AC and WD 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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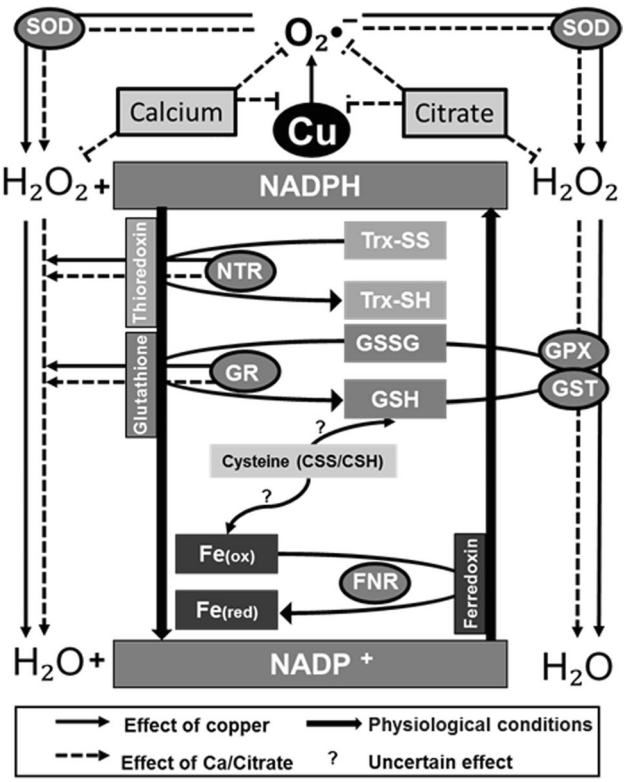

Fig. 9 Diagram representing the protective events mediated by calcium and citrate supplementations which could attenuate Cu toxicity in pea roots through: (i) thiol, glutathione and cysteine homeostasis, and (ii) thioredoxin and ferredoxin regeneration systems. Exposure to Cu stress boosted superoxide anion ($O_2^{\bullet-}$) accumulation which synergistically increased cell damages and disruption of redox homeostasis. However, exogenous calcium and citrate prevented the over-accumulation of $O_2^{\bullet-}$ by modulating superoxide dismutase (SOD) isoenzymes activities. Superoxide anion is recognized as a precursor for hydrogen peroxide (H_2O_2), which is quenched by antioxidants systems such as glutathione reductase (GR), glutathione-*S*-transferase (GST), and glutathione peroxidase enzymes (GPX). Calcium and citrate application decreased the three enzymes activities in Cu-treated pea roots, which could also be correlated with a down accumulation in H_2O_2 levels. The enzymes mentioned above contribute also to regulate redox status of glutathione (GSH) using NADPH as cofactor which contributes to H_2O_2 detoxification. NADPH-recycling metabolisms involve cysteine, thioredoxin (Trx)/NADPH-thioredoxin reductase (NTR) and ferredoxin (Fdx)/ferredoxin-NADP reductase (FNR) systems. Both calcium and citrate restored these systems (Trx/NTR and Fdx/FNR) to a level significantly close to control, which was associated to an improvement of redox status of thiol (SH) proteins. Although $NADP^+$ is necessary to reduced ferredoxin, NADPH is also a key factor for the regeneration of reduced forms of GSH and Trx-SH by GR and NTR activities, respectively. Trx/Fdx regeneration systems strengthen thiol levels and consequently enhance protein redox homeostasis

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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