CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

| | |
|---|---|
| Title | Redox biology response in germinating Phaseolus vulgaris seeds exposed to copper: evidence for differential redox buffering in seedlings and cotyledon |
| Authors | Karmous, Ines;Trevisan, Rafael;El Ferjani, Ezzedine;Chaoui, Abdelilah;Sheehan, David |
| Publication date | 2017 |
| Original Citation | Karmous, I., Trevisan, R., El Ferjani, E., Chaoui, A. and Sheehan, D. (2017) 'Redox biology response in germinating Phaseolus vulgaris seeds exposed to copper: evidence for differential redox buffering in seedlings and cotyledon', PLOS ONE, 12(10), e0184396 (23pp). doi: 10.1371/journal.pone.0184396 |
| Type of publication | Article (peer-reviewed) |
| Link to publisher's version | http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0184396 - 10.1371/journal.pone.0184396 |
| Rights | © 2017, Karmous et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. - https://creativecommons.org/licenses/by/4.0/ |
| Download date | 2026-11-06 19:39:58 |
| Item downloaded from | https://hdl.handle.net/10468/6357 |

UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

PLOS ONE

RESEARCH ARTICLE

# Redox biology response in germinating *Phaseolus vulgaris* seeds exposed to copper: Evidence for differential redox buffering in seedlings and cotyledon

**Inès Karmous[1], Rafael Trevisan[2], Ezzeddine El Ferjani[1], Abdelilah Chaoui[1], David Sheehan[3,4]***

**1** Plant Toxicology and Molecular Biology of Microorganisms, Faculty of Sciences of Bizerta, Zarzouna, Tunisia, **2** Nicholas School of the Environment, Duke University, Durham, North Carolina, United States of America, **3** College of Science, Khalifa University of Science and Technology, Abu Dhabi, United Arab Emirates, **4** School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland

* david.sheehan@kustar.ac.ae

Check for updates

OPEN ACCESS

**Citation:** Karmous I, Trevisan R, El Ferjani E, Chaoui A, Sheehan D (2017) Redox biology response in germinating *Phaseolus vulgaris* seeds exposed to copper: Evidence for differential redox buffering in seedlings and cotyledon. PLoS ONE 12 (10): e0184396. https://doi.org/10.1371/journal.pone.0184396

**Editor:** Rodrigo Franco, University of Nebraska-Lincoln, UNITED STATES

**Received:** April 3, 2017

**Accepted:** August 23, 2017

**Published:** October 5, 2017

**Copyright:** © 2017 Karmous et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** The authors received no specific funding for this work.

**Competing interests:** The authors have declared that no competing interests exist.

**Abbreviations:** 6PGDH, 6-phosphogluconate dehydrogenase; AsA, ascorbate; Asc-GSH,

## Abstract

In agriculture, heavy metal contamination of soil interferes with processes associated with plant growth, development and productivity. Here, we describe oxidative and redox changes, and deleterious injury within cotyledons and seedlings caused by exposure of germinating (*Phaseolus vulgaris* L. var. soisson nain hâtif) seeds to copper (Cu). Cu induced a marked delay in seedling growth, and was associated with biochemical disturbances in terms of intracellular oxidative status, redox regulation and energy metabolism. In response to these alterations, modulation of activities of antioxidant proteins (thioredoxin and glutathione reductase, peroxiredoxin) occurred, thus preventing oxidative damage. In addition, oxidative modification of proteins was detected in both cotyledons and seedlings by one- and two-dimensional electrophoresis. These modified proteins may play roles in redox buffering. The changes in activities of redox proteins underline their fundamental roles in controlling redox homeostasis. However, observed differential redox responses in cotyledon and seedling tissues showed a major capacity of the seedlings' redox systems to protect the reduced status of protein thiols, thus suggesting quantitatively greater antioxidant protection of proteins in seedlings compared to cotyledon. To our knowledge, this is the first comprehensive redox biology investigation of the effect of Cu on seed germination.

## Introduction

Heavy metal contamination of agricultural soils can interfere with plant physiological processes associated with normal growth and development [1], and may induce alterations within plant cells [2, 3]. One of the underlying causes of tissue injury following exposure of plants to heavy metals is overproduction of reactive oxygen species (ROS) [3–5], which can react directly with biomolecules (lipids, DNA and proteins) leading to biochemical alterations [6].

ascorbate-glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; CO, carbonyls; FTSC, fluorescein-5-thiosemicarbazide; FNR, ferredoxin-NADP oxido-reductase; G6PDH, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione transferase; IAF, 5'-iodoacetamide fluorescein; IEF, isoelectric focusing; MDH, malate dehydrogenase; MDHAR, monodehydroascorbate reductase; NTR, NADPH-dependent thioredoxin reductase; POX, peroxidase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SH, thiols; SOD, superoxide dismutase; Trx, thioredoxin.

At the cellular level, plants have evolved both enzymatic and non-enzymatic defense mechanisms against deleterious effects of heavy-metal-induced oxidative stress [7]. Non-enzymatic antioxidant molecules include ascorbate (AsA), reduced glutathione (GSH), carotenoids, alkaloids, tocopherols, proline, and phenolic compounds (flavonoids, tannins, and lignin) which act as low $M_r$ free radical scavengers [8, 9]. Enzymatic antioxidant defenses include superoxide dismutase (SOD; EC 1.15.1.1) [10], glutathione peroxidases (GPX; EC 1.11.1.9), peroxidase (POX; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6), as well as the enzymes of the ascorbate-glutathione cycle (Asc-GSH) [11], ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Additionally, enzymatic functioning of the Asc-GSH, involving successive oxidation and re-reduction of Asc, GSH and NAD(P)H [12], depends on reducing power [13, 14] supplied directly at the expense of photosynthesis or via secondary NAD(P)H recycling dehydrogenase activities; glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.4), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), malate dehydrogenase (MDH; EC 1.1.1.37) and isocitrate dehydrogenase (threo-D-isocitrate: $NADP^+$ oxidoreductase, EC 1.1.1.42) [15].

In plants, modulation of cellular redox homeostasis involves three principal systems: glutaredoxin (Grx)/GSH/GR, thioredoxin/NADPH/NADPH-dependent thioredoxin reductase (EC 1.6.4.5) (Trx/NADPH/ NADPH-dependent thioredoxin reductase (NTR); EC 1.8.1.9) [16–18], and ferredoxin-NADP oxido-reductase (FNR, EC 1.18.1.2). Functioning of these redox system components is based on their redox activity [19, 20]. In germinating seeds, their roles have previously been investigated [21, 22]. Indeed, Trx has been shown to be involved in extensive changes in the redox state of major storage proteins, the thiols of which are converted from the oxidized (–S–S–) form to reduced (–SH) forms [21]. They also play a role in activation of proteases and inactivation of protease inhibitors [20–22]. Under normal conditions, the intracellular redox state is predominantly reducing, but processes such as oxidative stress, notably under abiotic- and biotic-induced stress, can shift the redox balance toward an oxidizing state [23–25]. Regulation of the redox status of proteins, including the sulfhydryl groups of cysteine, can act as a "switch" for the activity of proteins involved in specific signaling events and in cell cycle control [26].

Some of the recognized effects of Cu toxicity on bean seeds are: (1) alteration of germinative metabolism [27, 28]; and (2) disruption of the capacity of the ubiquitin-proteasome pathway to eliminate oxidatively-damaged proteins [29]. Besides, in cells, Cu exists in either a reduced ($Cu^+$) or an oxidized ($Cu^{2+}$) state, which makes Cu a redox-active metal by the induction of electron-transfer reactions. This redox-activity can also promote the generation of reactive oxygen radicals and affect every category of macromolecule. On these grounds, the present work aimed to shed more light on the mechanism of Cu-induced toxicity and on the cell defense response in bean cotyledons and seedlings. In particular, we are interested in elucidating changes in antioxidative enzymes (SOD, CAT, APX, POX and GPX) and enzymes of NAD(P)H-recycling dehydrogenases (G6PDH, 6PGDH and MDH) under Cu-induced stress. In addition, effects of Cu on the coenzyme pattern, NAD(P)H oxidase (EC 1.6.99.6; EC 1.6.99.3) activity and redox components (Grx, GR, Trx, NTR, Fd, FNR and peroxiredoxin (Prx; EC 1.11.1.15) were investigated.

## Materials and methods

### Germination and copper treatment conditions

Seeds of the bean (*Phaseolus vulgaris* L. var. soisson nain hâtif) were germinated at 25 ± 1.5˚C in the darkness in the presence of $H_2O$ or 200 μM $CuCl_2$, according to Karmous et al. [27]. Whole seedlings and cotyledons were collected, respectively, at days 3 and 9.

## Measurement of oxidative indicators

$H_2O_2$ levels were measured according to Sergiev et al. [30]. Carbonyl and thiol groups were determined according to the methods of Reznick and Packer [31] and Ellman [32], respectively. Measurements were performed using 0.5 mL of cotyledon or seedling extract in a total reaction volume of 2 mL.

## Enzyme assays

Cotyledons (6) or seedlings (12) were homogenized at 4˚C with 25 mM potassium phosphate buffer (pH 7.0), (1:5, w/v), containing 5 mM sodium ascorbate, followed by centrifugation for 30 min at 20,000 × g. The resulting supernatant was considered as soluble enzymatic fraction. Protein concentrations in the cotyledon or seedling extract were evaluated by the method of Bradford [33], using bovine serum albumin as standard protein. SOD activity was measured according to Mishra and Fridovich [34]. The enzyme assay mixture (2 mL) contained 1.88 U $mL^{-1}$ catalase, 62.5 mM sodium carbonate/bicarbonate (pH 10.4), 125 μM EDTA and 20 μL of the cotyledon or seedling extract. SOD activity was estimated at 490 nm, using epinephrine as standard. CAT activity was measured according to Aebi [35]. The enzyme assay mixture (2 mL) contained 10 mM $H_2O_2$ in 25 mM phosphate buffer (pH 7.0) and 100 μL of the cotyledon or seedling extract (diluted 10-fold). CAT activity was estimated by monitoring the decrease in absorbance of $H_2O_2$ reduction at 240 nm ($\varepsilon = 36 \times 10^{-6}$ $M^{-1}.cm^{-1}$). APX (EC 1.11.1.11) activity was measured according to Nakano and Asada [36]. The reaction mixture (2 mL) contained 0.5 mM ascorbate, 5 mM $H_2O_2$, 0.1 mM EDTA and 100 μL of the cotyledon or seedling extract. APX activity was determined by following the decrease in the absorbance of ascorbate at 290 nm ($\varepsilon = 2.8 \times 10^{-3}$ $M^{-1}.cm^{-1}$). POX activity was measured according to Fielding and Hall [37]. The reaction mixture (510 μL) contained 10 mM $H_2O_2$ in 25 mM potassium phosphate buffer (pH 7.0), 9 mM guaiacol and 10 μL of the cotyledon or seedling extract (diluted 5-fold). POX activity was estimated by the increase in absorbance of oxiguaiacol at 470 nm ($\varepsilon = 26.6$ $M^{-1}.cm^{-1}$). GPX activity was measured according to Nagalakshmi and Prasad [38]. The reaction mixture (520 μL) contained 50 mM phosphate buffer (pH 8.0), 100 mM NaCl, 1 mM GSH, 2.5 mM $H_2O_2$, 0.5 mM NADPH, 1 U GR and 20 μL of the cotyledon or seedling extract. Oxidation of NADPH was followed by measuring the decrease in absorbance at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}.cm^{-1}$, [17]). G6PDH and 6PGDH activities were measured in the medium (500 μL): 50 mM Tris-HCl buffer (pH 7.9), 5 mM $MgCl_2$ and 0.2 mM $NADP^+$, containing, respectively, 5 mM glucose-6-phosphate and 0.5 mM 6-phosphogluconate and 20 μL of the cotyledon or seedling extract. Production of NADPH was determined by measuring the increase in the absorbance at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}.cm^{-1}$, [20]). MDH activity was measured in the medium (500 μL): 400 mM hydrazine sulfate (pH 9.0), 500 mM glycine, 2.5 mM $NAD^+$, 50 mM malate sodium, 10 mM $MgCl_2$ and 10 μL of the cotyledon or seedling extract. The production of NADH was evaluated by measuring the increase in the absorbance at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}.$ $cm^{-1}$, Bergmeyer et al. [39]). Activities of Trx and NTR were measured in the reaction mixture (500 μL): 50 mM Tris-HCl, pH 8.1, 100 μM DTNB and 20 μL of the cotyledon or seedling extract, and containing 0.2 mM NADPH and 30 μg $mL^{-1}$ reduced Trx (NTR assay) or 15 mg $mL^{-1}$ NADPH and 0.1 μM NTR (Trx assay). The reduction of dithio-bis-nitrobenzoate (DTNB) was determined by measuring the increase in the absorbance at 412 nm ($\varepsilon = 13.6 \times 10^3$ $M^{-1}.cm^{-1}$,[40]). Grx activity was performed by HED reduction [41] in the medium (500 μL): 50 mM Tris-HCl (pH 8.0), containing 0.2 mM NADPH, 0.5 mM GSH, 0.5 U $mL^{-1}$ GR (Sigma), 0.5 mM hydroxyethyl disulfide HED and 20 μL of the cotyledon or seedling extract. The oxidation of NADPH was measured at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}.$ $cm^{-1}$). GR activity was determined according to Foyer and Halliwell [12], by following the rate of

NADPH oxidation as a decrease in absorbance at 340 nm. The assay mixture (500 μL) contained 0.2 mM NADPH, 0.5 mM oxidized glutathione (GSSG) in 50 mM phosphate buffer (pH 7.0) and 20 μL of the cotyledon or seedling extract. Fd and FNR activities were assayed according to Green et al. [42]. The reaction mixture (500 μL) contained 20 μL of the cotyledon or seedling extract, 50 mM Tris-HCl (pH 7.8), 40 μM cytochrome C, 250 μM NADPH, 2 μM spinach leaf ferredoxin (FNR assay) or 0.1 μM FNR (Fd assay). The reduction of cytochrome C was monitored by the increase in absorbance at 550 nm ($\varepsilon = 19.1 \times 10^{-3}$ $M^{-1}.cm^{-1}$). Prx activity was measured in the medium (500 μL): 50 mM potassium phosphate (pH 7.0), containing 150 μM NADPH, 1 mM GSH, 0.5 U GR, 50 μM Grx, 1 mM $H_2O_2$ and 20 μL of the cotyledon or seedling extract. Oxidation of NADPH was monitored as a decrease in absorbance at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}.cm^{-1}$) [41]. Activities of NADPH oxidase and NADH oxidase were measured using 20 μL of the cotyledon or seedling extract in a total reaction mixture of 500 μL, containing 100 mM sodium acetate (pH 6.5), 1 mM $MnCl_2$, and 0.5 mM *p*-coumaric acid, containing 0.2 mM NADPH (NADPH oxidase assay) or 0.2 mM NADH (NADH oxidase assay). Oxidation of NAD(P)H was measured as a decrease in absorbance at 340 nm ($\varepsilon = 6.22$ $M^{-1}.cm^{-1}$[43]).

## Coenzyme extraction and measurement

Reduced (NADPH and NADH) and oxidized ($NADP^+$ and $NAD^+$) forms of coenzyme were extracted according to the method of Zhao et al.[44], respectively, in 0.1 M NaOH and 0.1 M HCl, followed by centrifugation at 20,000 × g at 10 min at 4˚C. Twenty μL of cotyledon or seedling extract in 500 μL total reaction volume were used for quantification according to the procedures described by Matsumura and Miyachi [45].

## Protein carbonyls and thiols

Proteins were extracted by homogenization (1:5, w/v) of cotyledons or seedlings in 10 mM Tris-HCl, pH 7.2, 500 mM saccharose, 1 mM EDTA, 150 mM KCl and 1 mM PMSF. After centrifugation at 20,000 ×g for 1 h at 4˚C, supernatant was collected.

Protein carbonyls (CO) and thiols (SH) were labelled, respectively, at a final concentration of l mM with fluorescein-5-thiosemicarbazide (FTSC) and 0.2 mM 5'-iodoacetamide fluorescein (IAF). After 2 h incubation at 37˚C for 150 min in the dark, proteins were precipitated with an equal volume of 20% TCA and centrifuged at 20,000 ×g for 3 min at 4˚C. The pellets were then resuspended and washed three times with 100% ethanol/ethyl acetate (1:1) and 96% acetone, respectively, for CO and SH groups. Pellets obtained were resuspended in Tris-HCl 0.5 M pH 6.8, glycerol 10%, SDS 0.5% and bromophenol blue, then applied to 1D SDS-PAGE gels (12%, 120 V) [46] (Mini-PROTEAN system, Bio-Rad). Gels were scanned in a Typhoon Trio Scanner 9400 (Control v5.0 + variable Mode Imager-RA 501: PRT<I/06/004, GE Healthcare, UK; excitation, 490–495 nm; emission, 515–520 nm). Protein-associated fluorescence intensity (arbitrary units, AU) was analyzed using Quantity One image analysis software (BioRad, Hercules, CA, USA). Gels were then stained with Colloidal Coomassie Brilliant Blue G250 [47], scanned with a calibrated densitometer GS-800 (BioRad, Hercules, CA, USA) and the total OD was measured and normalized by the previously-used software.

For 2D gels, proteins were separated according to their pI (first dimension: isoelectric focusing IEF), then according to their molecular weight (second dimension: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SDS-PAGE). Proteins were first rehydrated in 5 M urea, 2 M thiourea, 2% CHAPS, 4% ampholyte (Pharmalyte 3–10, Amersham-Pharmacia Biotech, Little Chalfont, Bucks, UK), 1% Destreak reagent (Amersham-Pharmacia Biotech), and trace amounts of 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 Immobiline™ Dry Strip IPG, Bio-Sciences AB, Bio-Rad, Hercules, CA, USA), for the separation of a final volume of 125 μl. Proteins were focused on a Protean IEF Cell (Bio-Rad) for at least 15 h at room temperature, according to the following steps: (1) a linear voltage increase until 250 V for 15 min, (2) 10,000 V for 2 h (50 μA/ strip), (3) focusing at 20,000 V, and (4) hold at 500 V. Following IEF, strips were equilibrated for 20 min in equilibration buffer; 6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, containing 2% DTT and then for 20 min in equilibration buffer containing 2.5% iodoacetamide. IPG strips were then loaded onto 12% SDS-PAGE gels (PROTEAN Plus Dodeca Cell Bio-Rad). After protein separation, gels were scanned for fluorescence as described above and then stained with Colloidal Coomassie Brilliant Blue R-250 followed by densitometry scanning. Normalization of FTSC- and IAF-labeled protein spots and Coomassie-staining intensity was performed using Progenesis SameSpots Software (Ref: S/No.62605/3787; Nonlinear USA Inc/2530 Meridian Parkway/ 3rd Floor Durham/ NC 27713/ USA) as per the manufacturer's instructions. Fluorescence spots were normalized to protein intensity for the same gel revealing increased fluorescence.

### Statistical analysis

All experiments were performed at least in triplicate. Values are means ± standard error SE, of three technical and five biological replicates. These were compared for significance of differences at $p < 0.05$ using the ANOVA test followed by Student's t test analysis. Images of 2D gels were subjected to landmarking alignment so that corresponding spots were matched with each other. This models protein spots mathematically as a 3D Gaussian distribution and determines maximum absorption after raw image correction and background subtraction. Spot intensities were normalized to make the total density in each gel image equal, and quantitative and qualitative analyses were performed. The protein spots were detected automatically and then edited manually to remove streaks, speckles, and artifacts. Two D gels were replicated at least three times and the results reported as means ± SD. Analyses of variance (one-way ANOVA) followed by Tukey's *post hoc* multiple comparison tests were performed using the software package Statistica 8.0 to compare Cu-treated tissues with controls. Statistically significant differences between all spots in 2D gel image were established at $p<0.05$ and assessed using Student's t test.

## Results

### Effects of copper on seed germination

Cu strongly inhibited germination of bean seeds, as evidenced by decreased growth of the Cu-treated seedlings over 9 days (Fig 1). A two-day delay in germination was evident in Cu-treated seeds (Fig 2A and 2B). However, the seedling length showed drastic decrease with increasing Cu concentration (Fig 2C–2F). Nevertheless, for the present study, we chose 200 μM Cu as our working concentration and days 3 and 9 for, respectively, seedlings and cotyledons.

### Response of antioxidant enzyme systems to copper-induced stress

A significant increase of $H_2O_2$ content was observed in Cu-treated cotyledon extracts although no significant increase was evident in seedling extracts after 3 days' treatment (Table 1). In S1 Appendix, we also recorded an increase in MDA levels in both tissues after exposure to Cu. Hence, we were interested to ascertain the mechanisms by which bean seeds respond to Cu-induced stress. Indeed, marked enhancement of the antioxidant enzymatic activities; SOD, CAT and peroxidases (APX, GPX and POX) in seedlings (Table 1) and cotyledons (Table 1)

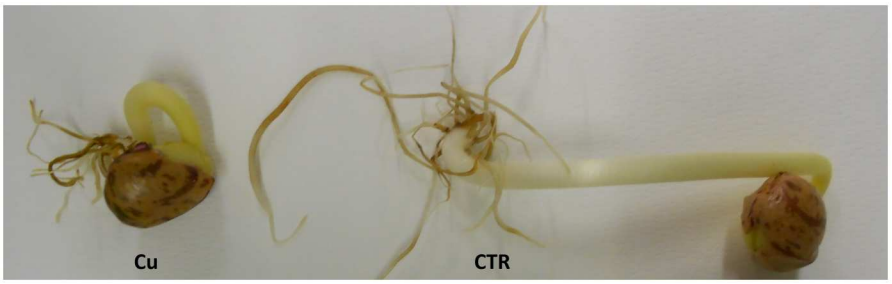



**Fig 1. Growth of bean (*Phaseolus vulgaris* L. var. soisson nain hâtif) seedlings germinated in the presence of distilled water or 200 μM $CuCl_2$.**

https://doi.org/10.1371/journal.pone.0184396.g001

were evident after Cu treatment. This increase was significant for all antioxidant enzymes (except SOD and APX in cotyledons), as compared to controls. In addition, time courses of enzyme activities suggested that, in seedlings, SOD and CAT activities increased after only 4 hours of germination while POX, APX and GPX increased after 24 hours (Fig 3). In cotyledons, SOD, CAT and APX activities increased from the first day of germination, with more significant activation at days 3, 6 and 9 (Fig 3). However, GPX and POX showed increased activities after day 3.

Upon Cu treatment, activities of NAD(P)H-independent dehydrogenases, notably, G6PDH, 6PGDH and MDH were significantly enhanced in both cotyledons and seedlings (Table 1). These biochemical observations led us to examine changes in protein redox status in response to Cu exposure, as well as possible relationships between protein thiol management and thiol-dependent enzymatic redox systems.

## Redox changes under copper-induced stress

Levels of both CO and -SH groups were higher in Cu-treated seedlings whilst, in cotyledons, an increase in CO level versus a net decline in level of protein -SH was observed (Table 2). This suggested that protein thiol status was affected by oxidation due to Cu in both organs. In addition, when compared to respective controls, cotyledons of Cu-treated seeds showed a significant decrease in Trx activity, but no significant variation in Grx activity and a marked increase in GR and NTR activities (Table 3). However, in seedlings, a significant increase in the activities of NTR and Trx was evident with no significant increase in GR and Grx activities in the presence of Cu (Table 3). These data suggest that, as a consequence of Cu-induced stress, there is a more protective effect of Grx/GR and Trx/NTR systems on protein thiols in seedlings than in cotyledons. Curiously, the Fd/FNR system was highly stimulated in seedlings whilst it was inhibited in cotyledons (Table 3). Prx activity also increased in both seedlings and cotyledons, as compared with controls, which may implicate this enzyme in Cu defense.

To assess potential contributions of coenzyme forms in response to Cu-induced stress, possible changes in total quantities of nicotinamide coenzymes (oxidized form + reduced form) were examined. The enzymatic activities responsible for oxidation of the reduced forms of coenzyme were also measured. A net increase in total coenzyme levels was found in both cotyledons and seedlings (Table 4). The redox ratio of coenzymes ($NADP^+$ /NADPH and $NAD^+$ /NADH), as well as NADPH oxidase activity increased significantly in seedlings, whilst only the $NAD^+$ /NADH ratio and NADH oxidase activity increased significantly in cotyledons. These findings suggested elevated levels of oxidized coenzyme forms ($NADP^+$ and $NAD^+$) in response to Cu treatment, as compared with controls (Table 4).

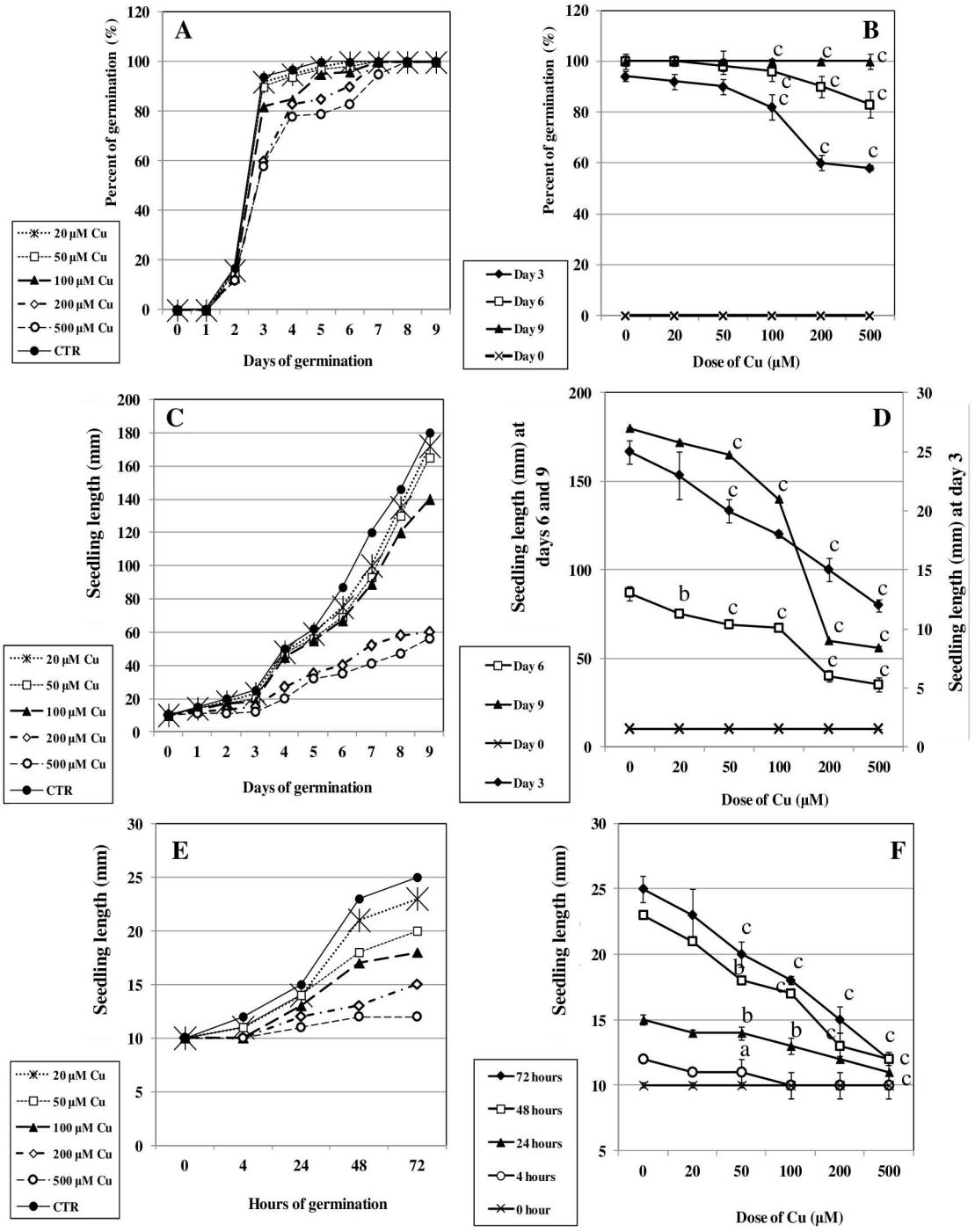



**Fig 2. Time courses of (A, B) germination rate and (C, D, E, F) seedling length in bean seeds in the absence (CTR) and in the presence of $CuCl_2$ (concentrations 20, 50, 100, 200, 500 µM).** Letters indicate significant differences compared with the respective control sample (a: $p < 0.05$, b: $p < 0.01$ and c: $p < 0.001$).

https://doi.org/10.1371/journal.pone.0184396.g002

## Changes in protein thiol and carbonyl groups

Analysis of 1D SDS PAGE gels revealed a significant decrease in total protein thiols in seedlings (Fig 4), but an increase in cotyledons (Fig 5). In addition, representative 2D gel images of

**Table 1. Level of $H_2O_2$ and activities of antioxidant enzymes in the seedlings after 3 days (a) and cotyledons after 9 days (b) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**

| Parameters | Unit | CTR | Cu |
|---|---|---|---|
| $H_2O_2$ | (a) μmol. $g^{-1}$ fresh weight | 0.90±0.13 | 1.27±0.02 |
| | (b) μmol. $g^{-1}$ fresh weight | 1.53±0.24 | 3.10±0.32 * |
| SOD | (a) U. $mg^{-1}$ proteins | 27.47±4.15 | 33.56±1.93 * |
| | (b) U. $mg^{-1}$ proteins | 13.64±2.58 | 23.42±1.34 * |
| CAT | (a) mU. $mg^{-1}$ proteins | 85.32±12.26 | 204.36±11.08 *** |
| | (b) U. $mg^{-1}$ proteins | 72.83±21.02 | 229.20±83.49 * |
| APX | (a) U. $mg^{-1}$ proteins | 1.52±0.48 | 4.02±0.23 *** |
| | (b) U. $mg^{-1}$ proteins | 2.23 ± 0.05 | 4.68 ± 2.20 |
| POX | (a) mU. $mg^{-1}$ proteins | 360.69±51.04 | 561.65±27.58 *** |
| | (b) mU. $mg^{-1}$ proteins | 1069.68 ± 227.44 | 1622.57± 295.70 * |
| GPX | (a) mU. $mg^{-1}$ proteins | 91.35±25.76 | 240.18±31.33 *** |
| | (b) mU. $mg^{-1}$ proteins | 28.66 ± 6.93 | 50.96 ± 5.64 ** |
| G6PDH | (a) mU. $mg^{-1}$ proteins | 28.46±5.29 | 104.22±27.99 ** |
| | (b) mU. $mg^{-1}$ proteins | 6.71 ± 2.54 | 13.63 ± 1.18 ** |
| 6PGDH | (a) mU. $mg^{-1}$ proteins | 137.35±25.87 | 289.67±39.81 *** |
| | (b) mU. $mg^{-1}$ proteins | 33.53 ± 7.48 | 67.84 ± 5.67 *** |
| MDH | (a) mU. $mg^{-1}$ proteins | 138.48±38.13 | 364.29±42.81 *** |
| | (b) U. $mg^{-1}$ proteins | 1.05 ± 0.02 | 1.97 ± 0.01 |

Values are means ± SE (n = 5). Asterisks indicate significant differences compared with the respective control sample

* $p < 0.05$,

** $p < 0.01$

*** $p < 0.001$

https://doi.org/10.1371/journal.pone.0184396.t001

total proteins showed 1,174 and 599 spots, respectively, in seedlings and cotyledons (Fig 6; Table 5). Amongst these, 77 and 34, respectively, were significantly modified (±1.5-fold), compared to controls ($p<0.05$). It was noted in these preparations that the majority of proteins focused in the pI range 5–10 and in the $M_r$ range 15–75 kDa. Comparison of spot patterns between Cu-treated and control samples revealed more increase than decrease of proteins, in the presence of Cu in both tissues, suggesting activation of biosynthesis upon heavy metal exposure. Fluorescence intensity measurements after IAF labeling showed 4 and 27 spots of interest, respectively, containing -SH groups ($p< 0.05$ and $> = 1.5$-fold). In cotyledons, all the proteins corresponding to 4 spots seemed to be increased in abundance whilst, in the seedlings, no significant variation was detected between replicates in the presence of Cu (13 increases *vs* 14 decreases, Fig 6). Figs 7 and 8 showed an increase in the total CO, respectively, in the seedlings and the cotyledons after Cu exposure. Carbonylation is known to be a general indicator of protein oxidation [6, 32]. These findings were corroborated by 2D gel analysis using FTSC-specific fluorescence. The representative 2D gels of CO groups of proteins showed 610 and 356 total protein spots, respectively, in cotyledons and seedlings. Among these, 234 and 159 corresponded with spots detected by fluorescence after FTSC labeling (Table 6). The interesting spots (significant at $p<0.05$ and $> = 1.5$ fold), 29 and 3 spots respectively, in cotyledons and seedlings (Fig 9) also showed more increase than decrease by Cu, suggesting enhanced protein biosynthesis under Cu-induced stress.

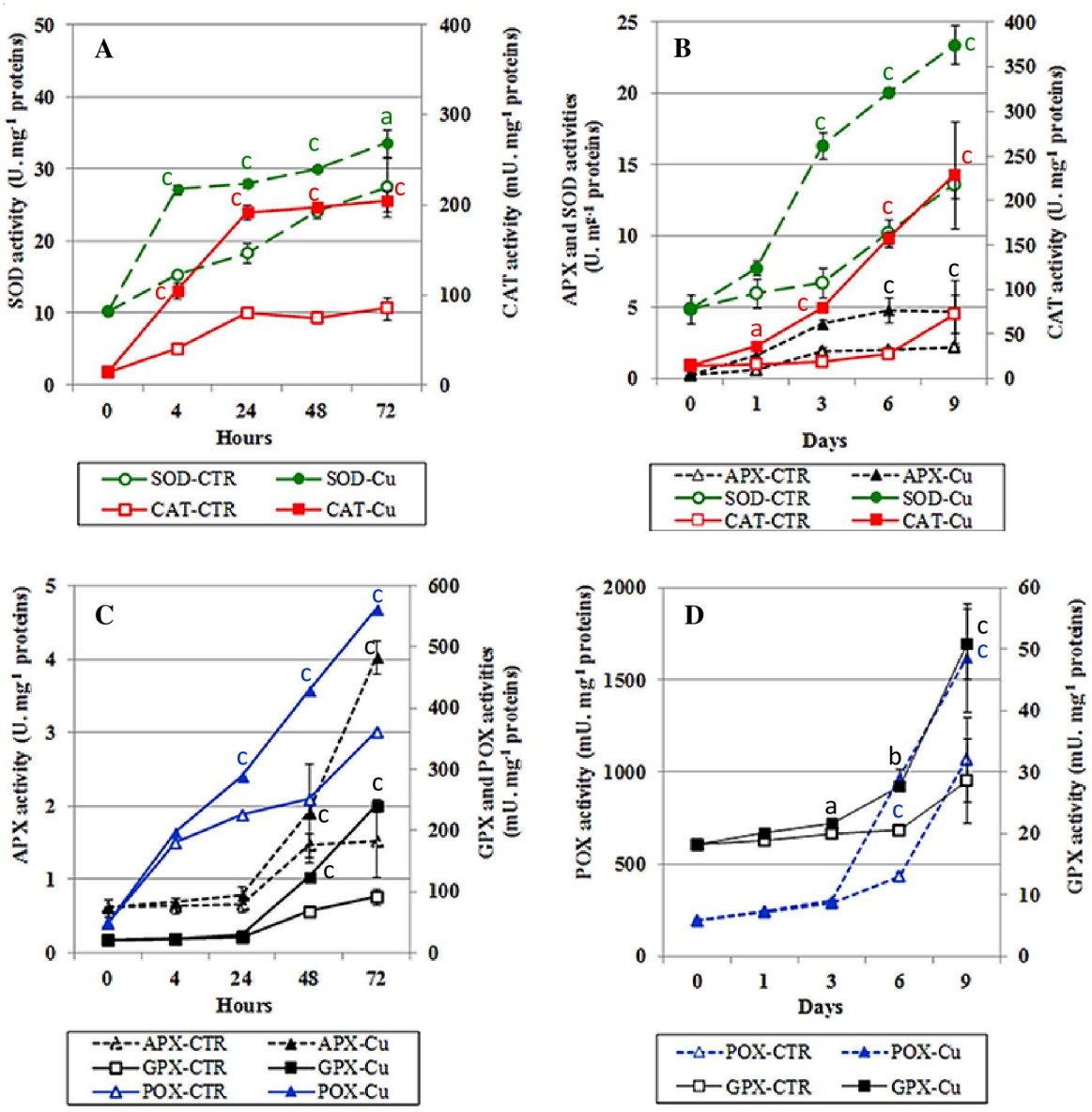



**Fig 3. Enzymatic activities of SOD, CAT and peroxidases (APX, GPX and POX) in (A, C) seedlings and (B, D) cotyledons of bean seeds during germination in the presence of distilled water (CTR) or 200 μM Cu.** Values are means ± SE (n = 5). Letters indicate significant differences compared with the respective control sample (a: $p < 0.05$, b: $p < 0.01$ and c: $p < 0.001$).

https://doi.org/10.1371/journal.pone.0184396.g003

## Discussion

Because of their sessile character, plants confront various environmental stresses during their life cycles. Heavy metals, particularly Cu, represent a serious problem in agricultural production [48]. Research into germination and seedling growth (post-germination phase) are considered fundamental to evaluating the toxic effects of heavy metals on important agronomic plants such as beans [49]. It has been suggested that vulnerability of bean seeds towards copper stress can be partly explained by disruption of metabolic pathways affecting seedling growth [27, 28]. In the present work, a significant delay in seedling growth (Figs 1 and 2) was shown

**Table 2. Levels of protein carbonyl and thiol groups in the seedlings (3 days-old) and the cotyledons (9 days-old) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**

| Parameters | Tissues | Unit | CTR | Cu |
|---|---|---|---|---|
| -CO | Seedlings | μmol $mg^{-1}$ proteins | 7.37±0.15 | 9.24±0.09 *** |
| | Cotyledons | nmol $mg^{-1}$ proteins | 7.96±1.89 | 15.12±0.30 ** |
| -SH | Seedlings | nmol $mg^{-1}$ proteins | 90.90±18.40 | 292.83±19.98 *** |
| | Cotyledons | nmol $mg^{-1}$ proteins | 65.11±4.52 | 35.74±3.14 * |

Values are means ± SE (n = 4). Asterisks indicate significant differences compared with the respective control sample

* p < 0.05,

** p < 0.01

*** p < 0.001

https://doi.org/10.1371/journal.pone.0184396.t002

to be associated with metabolic disturbances possibly occurring in both seedlings and cotyledons. In fact, investigation of the changes in antioxidant metabolism and cellular redox status confirmed that Cu induced intrinsic production of ROS, notably $H_2O_2$ (Table 1). Many heavy-metal-stressed species have been reported to defend against ROS overproduction [50–52]. In the present work, the formation of $H_2O_2$ seems to be mediated by the redox-active Cu. Therefore, metal ions-catalyzed reactive oxygen radicals might be potent mediators of the cellular oxidative injury, which can damage proteins, nucleic acids, and lipids. Indeed, in addition to lipid peroxidation (see increased malondialdehyde levels in S1 Appendix), we aimed to investigate mainly changes affecting proteins. Cu exhibits an affinity for the sulfur in cysteine or methionine, and $Cu^{2+}$ binds to oxygen or imidazole nitrogen groups of aspartic and glutamic acid, or histidine. In addition, Cu can displace other metals, such as zinc, from their cognate

**Table 3. Activities of redox enzymes in the seedlings (3 days-old) and the cotyledons (9 days-old) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**

| Parameters | Tissues | CTR | Cu |
|---|---|---|---|
| NTR | Seedlings | 0.68±0.23 | 1.36±0.29 * |
| | Cotyledons | 2.07±0.89 | 4.00±0.56 * |
| Trx | Seedlings | 2.10±0.37 | 6.51±1.79** |
| | Cotyledons | 1.56±0.29 | 1.33±0.31 |
| GR | Seedlings | 12.09±2.28 | 18.40±1.26** |
| | Cotyledons | 14.89±2.26 | 15.30±6.07 |
| Grx | Seedlings | 18.34±3.62 | 26.02±6.1* |
| | Cotyledons | 5.10±1.56 | 4.81±2.01 |
| FNR | Seedlings | 16.34±3.20 | 42.28±7.78 *** |
| | Cotyledons | 9.37±3.62 | 4.12±1.26 * |
| Fd | Seedlings | 25.91±8.09 | 50.53±5.37 ** |
| | Cotyledons | 5.55±1.94 | 2.36±2.78 |
| Prx | Seedlings | 67.18±19.2 | 282.02±41.72 *** |
| | Cotyledons | 79.57±4.38 | 100.00±12.56 * |

Values are means ± SE (n = 5). NTR activity was expressed in U/mg proteins in cotyledons and mU/mg proteins inseedlings. Activities of Trx, GR, Grx, FNR, Fd and Prx were expressed in mU/mg proteins in both cotyledons and seedlings. Asterisks indicate significant differences compared with the respective control sample

* p < 0.05,

** p < 0.01

*** p < 0.001

https://doi.org/10.1371/journal.pone.0184396.t003

**Table 4. Activities of NAD(P)H oxidases and redox ratios of coenzymes in the seedlings (3 days-old) and the cotyledons (9 days-old) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**

| Parameters | Tissues | CTR | Cu |
|---|---|---|---|
| Total coenzymes | Seedlings | 91.62±1.38 | 107.14±1.43 *** |
| | Cotyledons | 1870.61±22.27 | 2953.85±36.85 ** |
| $NADP^+$ /NADPH | Seedlings | 0.68±0.04 | 2.02±0.19 *** |
| | Cotyledons | 1.57±0.24 | 1.91±0.35 |
| $NAD^+$ /NADH | Seedlings | 6.04±0.64 | 15.20±0.10 *** |
| | Cotyledons | 4.54±0.60 | 6.35±0.75 *** |
| NADPH oxidase | Seedlings | 694.57±51.74 | 841.67±31.45 * |
| | Cotyledons | 368.89±100.95 | 429.59±43.85 |
| NADH oxidase | Seedlings | 397.52±127.33 | 540.23±128.32 |
| | Cotyledons | 384.14±103.19 | 579.81±72.24 * |

Values are means ± SE (n = 5). Total coenzymes (oxidized and reduced forms) were expressed in nmol/g fresh weight, and activities of NAD(P)H oxidases were expressed in mU/mg proteins. Asterisks indicate significant differences compared with the respective control sample

* $p < 0.05$,

** $p < 0.01$

*** $p < 0.001$

https://doi.org/10.1371/journal.pone.0184396.t004

ligands in metalloproteins, which can result in inappropriate protein structures or inhibition of activity of many important cellular enzymes.

Additionally, generation of oxidative stress has been reported in germinating legume seeds after heavy metal exposure [53, 54]. Here, endogenous $H_2O_2$ accumulation, triggers stimulation of antioxidant enzymes SOD, CAT and peroxidases (APX, GPX and POX), thus allowing enhanced elimination of $H_2O_2$ in seedlings and cotyledon tissues after Cu exposure (Table 1; Fig 3). Enzymatic antioxidative response differs between seedlings and cotyledons, however, with respect to the order of activation of the antioxidative enzymes during germination (Figs 3 and 4). Changes in antioxidant concentrations and activities of ROS-processing enzymes have been associated with seed germination under heavy metals [55]. POXs are considered to be heavy-metal stress-related enzymes and are sometimes used as stress markers in metal poisoning scenarios [13]. Effects of heavy metals on antioxidant enzyme activities and their involvement in defense mechanisms against oxidative damage have been widely reported in the literature, but remain controversial and vary amongst plant species, different tissues and varying exposure regimes [3, 13, 53, 56]. Cu also inhibits some enzymes such as acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2), G6PDH, isocitrate dehydrogenase, CAT, GPX and glutathione transferases. Antioxidant systems are likely to be involved in defense against heavy metal-imposed oxidative stress, but might also be direct biochemical targets for metallic ion-induced toxicity.

In addition to their enzymatic antioxidant capacity, plant tolerance to heavy metal-induced toxicity depends crucially on the availability of reduced cofactors, such as NAD(P)H [13, 14]. The key antioxidant and redox systems such as Trx, Grx and the Asc-GSH cycle depend heavily on NADPH rather than NADH for reducing equivalents. Hence, in response to oxidative stress, cells may need to shift from pathways producing NADH to others producing NADPH, such as the pentose phosphate pathway [15]. In this regard, increased activity of NAD(P)H-independent dehydrogenases, notably G6PDH, 6PGDH and MDH, in both seedlings and cotyledons (Table 1) after Cu treatment, most likely enable increased availability of NADPH to stressed cells [14, 54, 55].

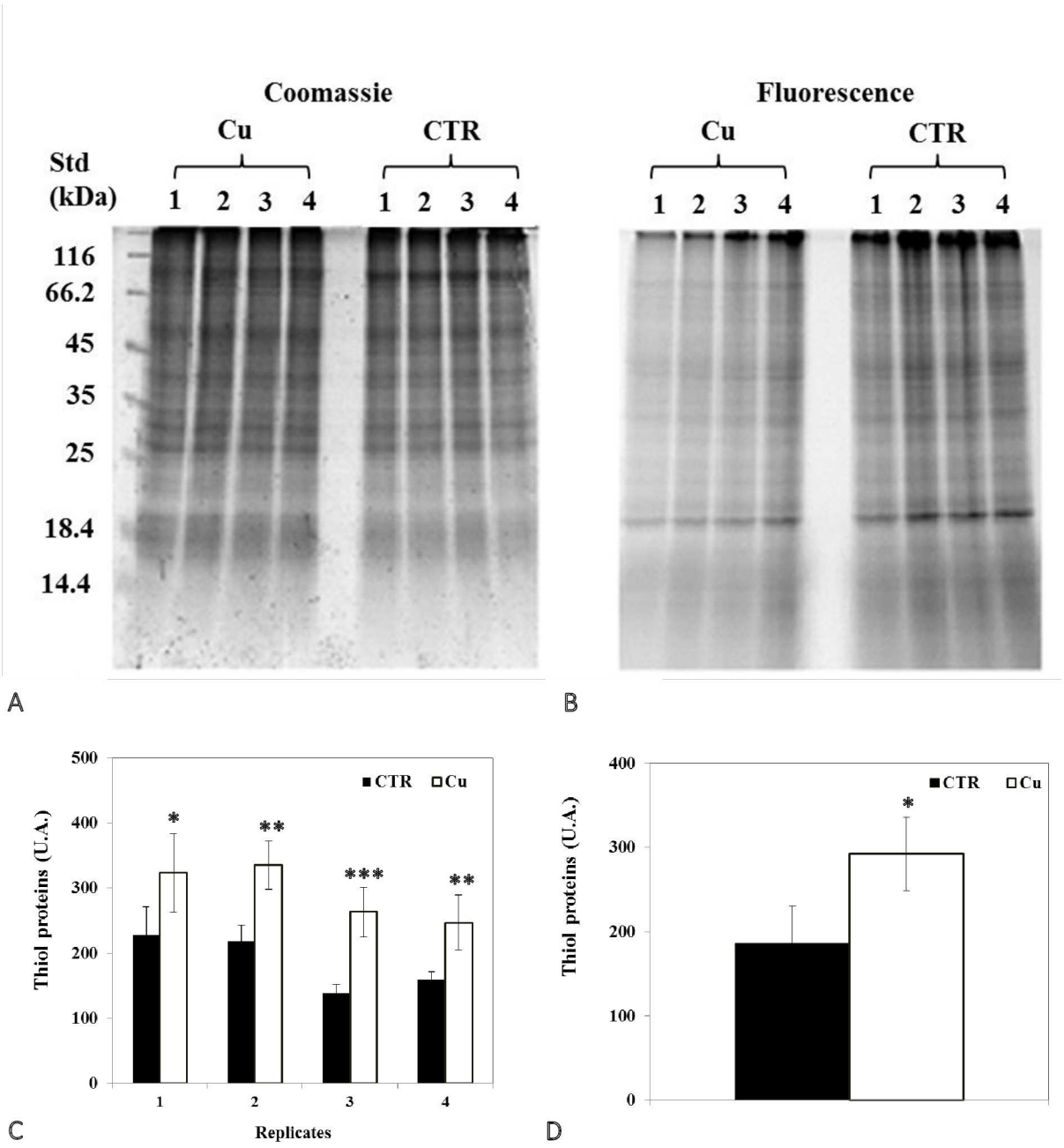



**Fig 4. Representative images of 1DE gels of proteins (100 μg) in seedlings of bean seeds germinated for 3 days. (A) In the presence of distilled water (CTR) or (B) 200 μM Cu. Gels were stained with Coomassie G-250 (scanned with GS-800 calibrated densitometer) and with IAF labeling (scanned with Typhoon 9400 scanner).** Total optical densities for each lane obtained from IAF staining were normalized with those from Coomassie G-250 staining of the same gel. (C, D) Levels of proteins containing thiol groups. Values shown are (C) means of 4 biological replicates (±SD) numbered from 1 to 4, and (D) means of 4 technical replicates (±SD). Each measurement was performed in an extract obtained from several seedlings. Analyses were performed using ANOVA, student's T test; *p<0.05, **p<0.01, ***p<0.001.

https://doi.org/10.1371/journal.pone.0184396.g004

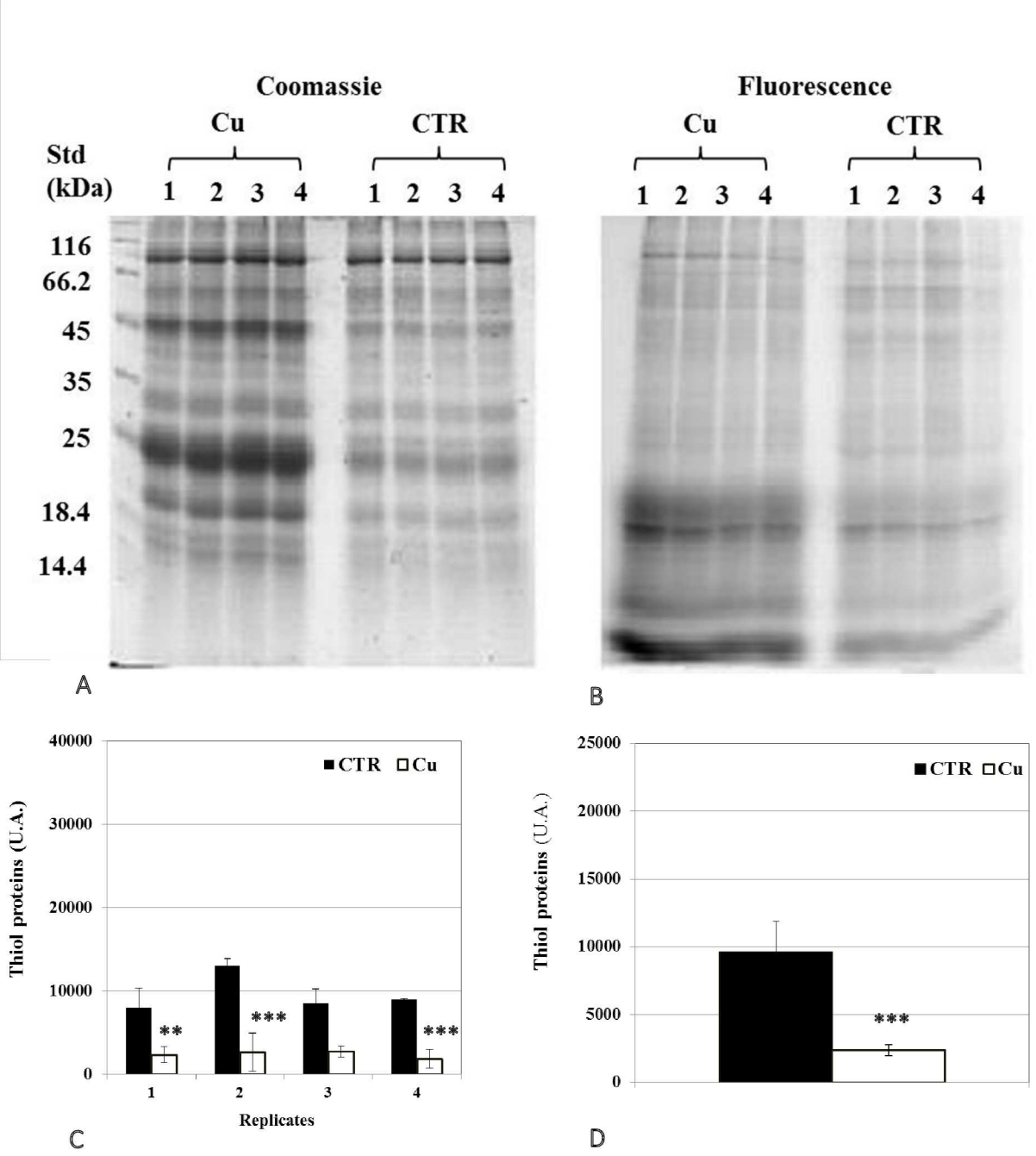



**Fig 5. Representative images of 1DE gels of proteins (150 μg) in cotyledons of bean seeds germinated for 9 days in the presence of (A) distilled water (CTR) or (B) 200 μM Cu. Gels were stained with Coomassie G-250 (scanned with GS-800 calibrated densitometer) and with IAF labeling (scanned with Typhoon 9400 scanner).** Total optical densities for each lane obtained from IAF staining were normalized with those from Coomassie G-250 staining of the same gel. (C, D) Levels of proteins containing thiol groups. Values shown are (C) means of 4 biological replicates (±SD) numbered from 1 to 4, and (D) means of 4 technical replicates (±SD). Each measurement was performed in an extract obtained from several cotyledons. Analyses were performed using ANOVA, student's T test; **p<0.01, ***p<0.001.

https://doi.org/10.1371/journal.pone.0184396.g005

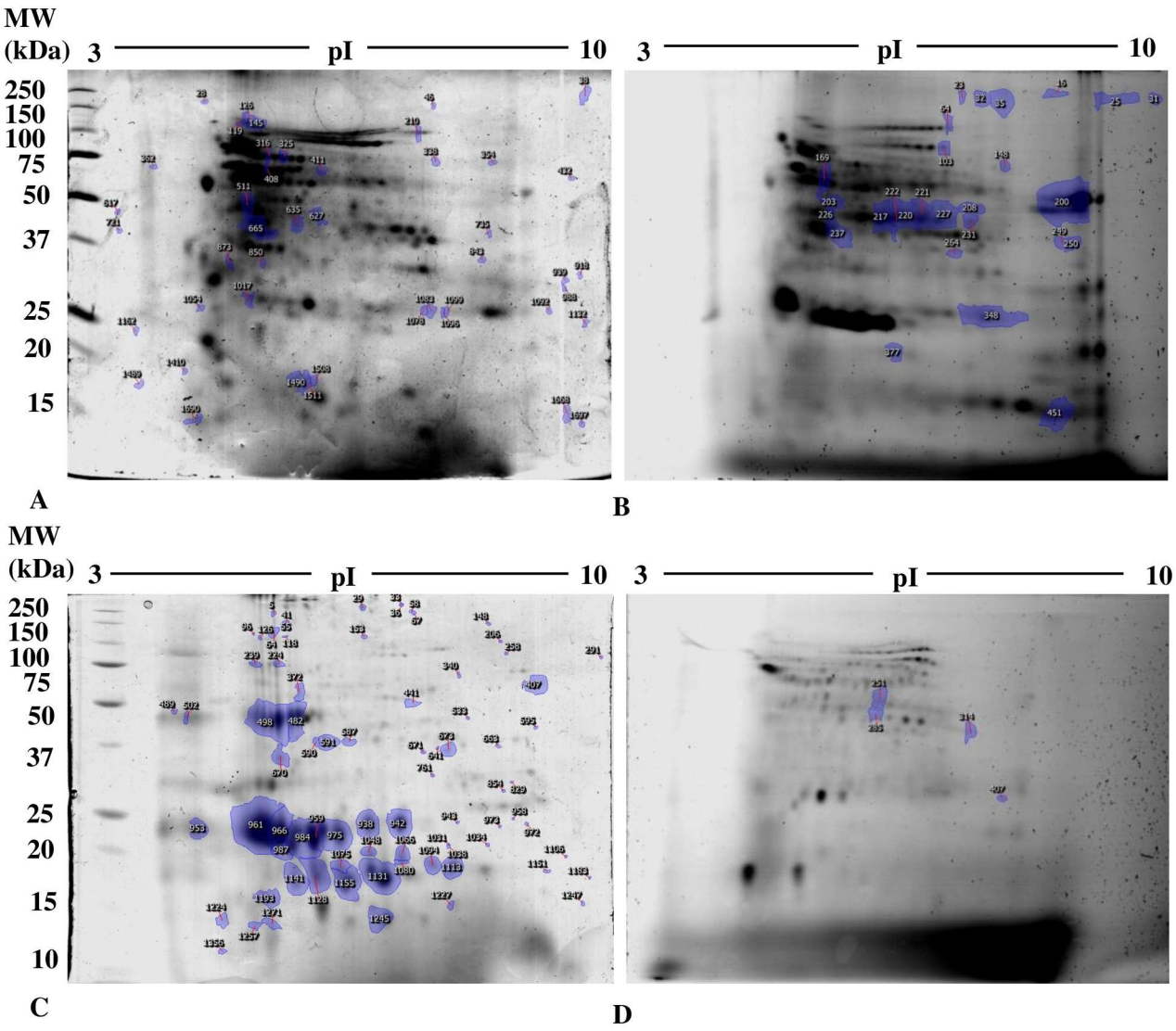



**Fig 6. Profiles of the expression of proteins containing thiol groups in (A, B) seedlings and (C, D) cotyledons of bean seeds germinated for 9 days in the presence of distilled water (CTR) or 200 μM Cu.** Proteins (800 μg) were labeled with IAF and separated by 2-D SDS-PAGE. Figures show spots of interest in representative gels from (A, C) colloidal Coomassie Brilliant G-250 staining (scanned with GS-800 calibrated densitometer) and (B, D) IAF labeling (scanned with Typhoon 9400 scanner; 800 PMT). Numbers correspond to spots of $p<0.05$ and Fold induction >1.5 (spots identified).

https://doi.org/10.1371/journal.pone.0184396.g006

In the current study, protein redox status and the major intracellular redox actors that control formation/reduction of intra- and/or inter-molecular disulfide bridges were also studied. Analysis of the components of different redox systems suggests that, in cotyledons, neither the Trx/NTR nor Grx/GR systems were involved in improving the protection of protein thiols to oxidation, possibly due to direct inhibition by Cu ions of the redox enzymes. Cu also seems to induce differential redox responses in cotyledons and seedlings. In fact, it seems that both Trx and Grx enzymes had not improved the redox status of thiols in cotyledons. However, in seedlings the levels of all components of the redox systems were elevated, thus suggesting a contribution of Trx/NTR/NADPH, despite the vulnerability of the coenzymes to enzymatic oxidation. A decreased level of reduced protein thiols was found coupled with increased

**Table 5. Proteins containing thiol groups (IAF labeling) in the seedlings (3 days-old) (a) and the cotyledons (9 days-old) (b) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**

| Number of spots | Coomassie | Fluorescence |
|---|---|---|
| Total | (a) 599 | 260 |
| | (b) 1174 | 500 |
| Significantly modified by Cu (p< = 0.05) | (a) 33 | 33 |
| | (b) 92 | 7 |
| Fold increase > = 1.5 | (a) 33 | 27 |
| | (b) 77 | 4 |
| p< = 0.05, Fold increase > = 1.5 | (a) 34 | 27 |
| | (b) 77 | 4 |
| Increased by Cu | (a) 20 | 13 |
| | (b) 47 | 4 |
| Decreased by Cu | (a) 14 | 14 |
| | (b) 30 | 0 |

https://doi.org/10.1371/journal.pone.0184396.t005

carbonyl content in cotyledons (Table 2), indicating extensive protein oxidation [29]. But in seedlings, despite an increase in protein carbonyl content, enhanced protein thiol levels (Table 2) suggest that thiol status is protected via Trx and Grx activities (Table 3). Redox systems are thought to play fundamental roles in controlling plant redox and defense status when subjected to abiotic stress [18, 24, 25]. Indeed, Trx are involved in protection against oxidative damage by regeneration of Prxs and methionine sulphoxide reductases, allowing detoxification of various peroxides and protein repair [57]. Trx h can act as a hydrogen donor for the peroxiredoxin-1 Cys, which protects macromolecules in seedlings against oxidation during the early phases of imbibition [58]. In cotyledons, loss of Fd and FNR activities may generate intracellular oxidative stress whilst, in seedlings, the Fd/FNR system appears to be involved in modulation of redox status (Table 3). On the other hand, increased Prx activity in both seedlings and cotyledons after Cu exposure suggest an antioxidant role, most probably *via* POX activity using $H_2O_2$, peroxynitrite and hydroperoxides as substrates [59].

In response to Cu stress, high levels of oxidized coenzymes compared to reduced ones accumulated in seedling and cotyledon tissues (Table 4), despite increased NAD(P)H-independent dehydrogenase activities. This observation is most likely due to enhanced consumption of NADPH following the induction of NTR activity in cotyledons and both NTR and GR activity in seedlings. Another explanation could be stimulation of enzymes oxidizing reduced coenzymes. For example, enhanced NAD(P)H oxidase activity could result in elevation of oxidized forms of coenzymes at the expense of reduced ones, thus increasing $NAD(P)^+$/NAD(P)H ratios [53].

Cu-induced biochemical disturbances in germinating bean seeds, including modulation of activities of antioxidant enzymes, could prevent oxidative damage. However, differential redox responses in cotyledon and seedling tissues suggest a major capacity of redox systems to prevent oxidation of protein thiols in seedlings in particular. Protein thiol status of seedlings was not affected by Cu with an apparent increase in the reduced SH pool (Tables 3 and 4). This could be explained by higher Trx and Grx activities on protein thiols, underlining these proteins' fundamental rules in controlling seedling redox homeostasis (despite other antioxidative alterations also described here). Recycling of protein thiols also appears to occur mainly *via* redox systems (Trx/NTR), (Grx/GR), (Fd/FNR) and Prx, rather than *via* NADPH. These results are corroborated by the study of proteomic changes occurring to SH and CO groups of proteins in both cotyledon and seedling.

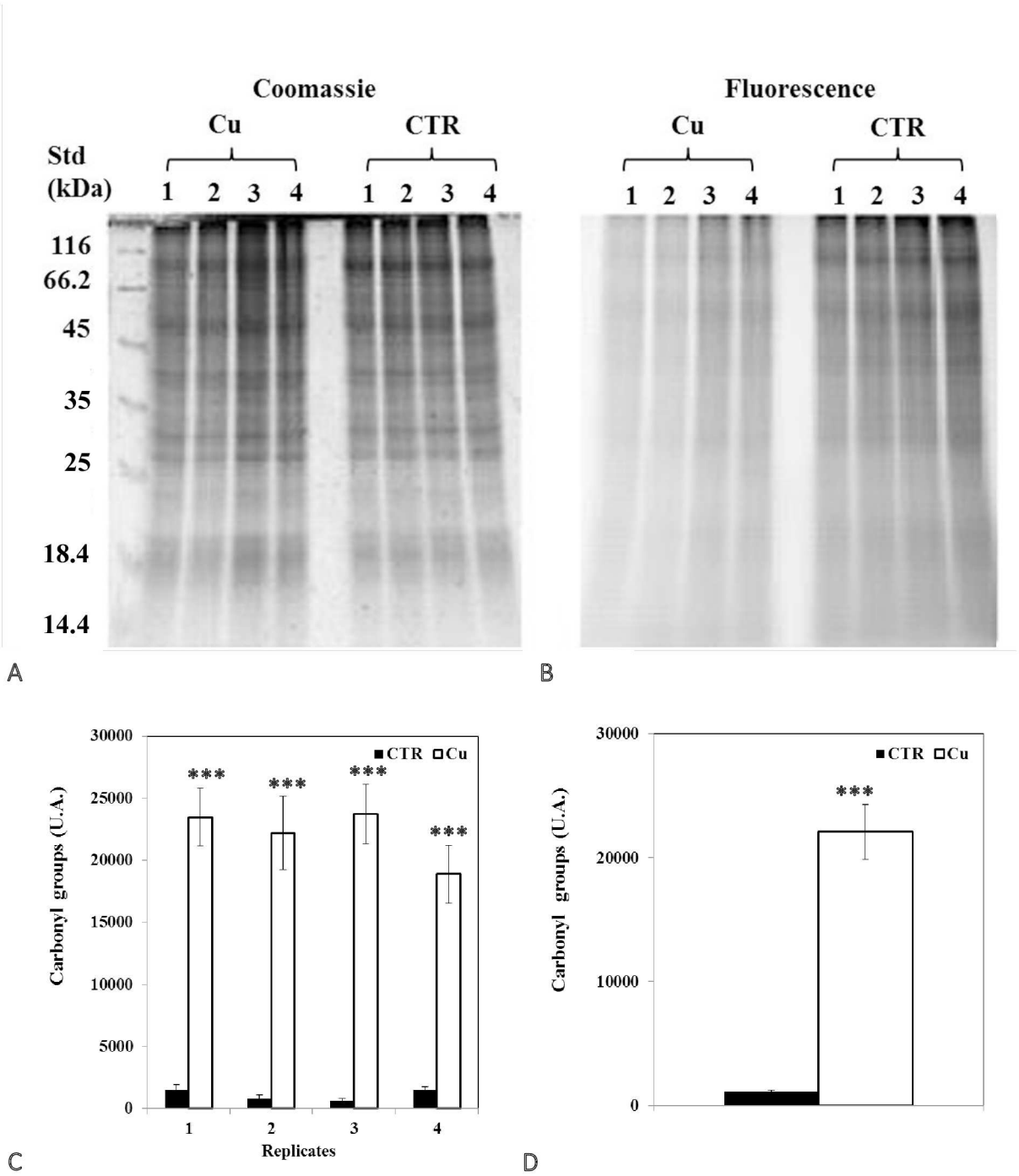



**Fig 7. Representative images of 1DE gels of proteins (150 μg) in seedlings of bean seeds germinated for 9 days in (A) the presence of distilled water (CTR) or (B) 200 μM Cu.** Gels were stained with Coomassie G-250 (scanned with GS-800 calibrated densitometer) and with FTSC labeling (scanned with Typhoon 9400 scanner). Total optical densities for each lane obtained from FTSC staining were normalized with those from Coomassie G-250 staining of the same gel. (C, D) Levels of proteins containing carbonyl groups. Values shown are means of (C) 4 biological replicates (±SD) numbered from 1 to 4, and means of (D) 4 technical replicates (±SD). Each measurement was performed in an extract obtained from several cotyledons. Analyses were performed using ANOVA, student's T test; *p<0.05, ***p<0.001.

https://doi.org/10.1371/journal.pone.0184396.g007

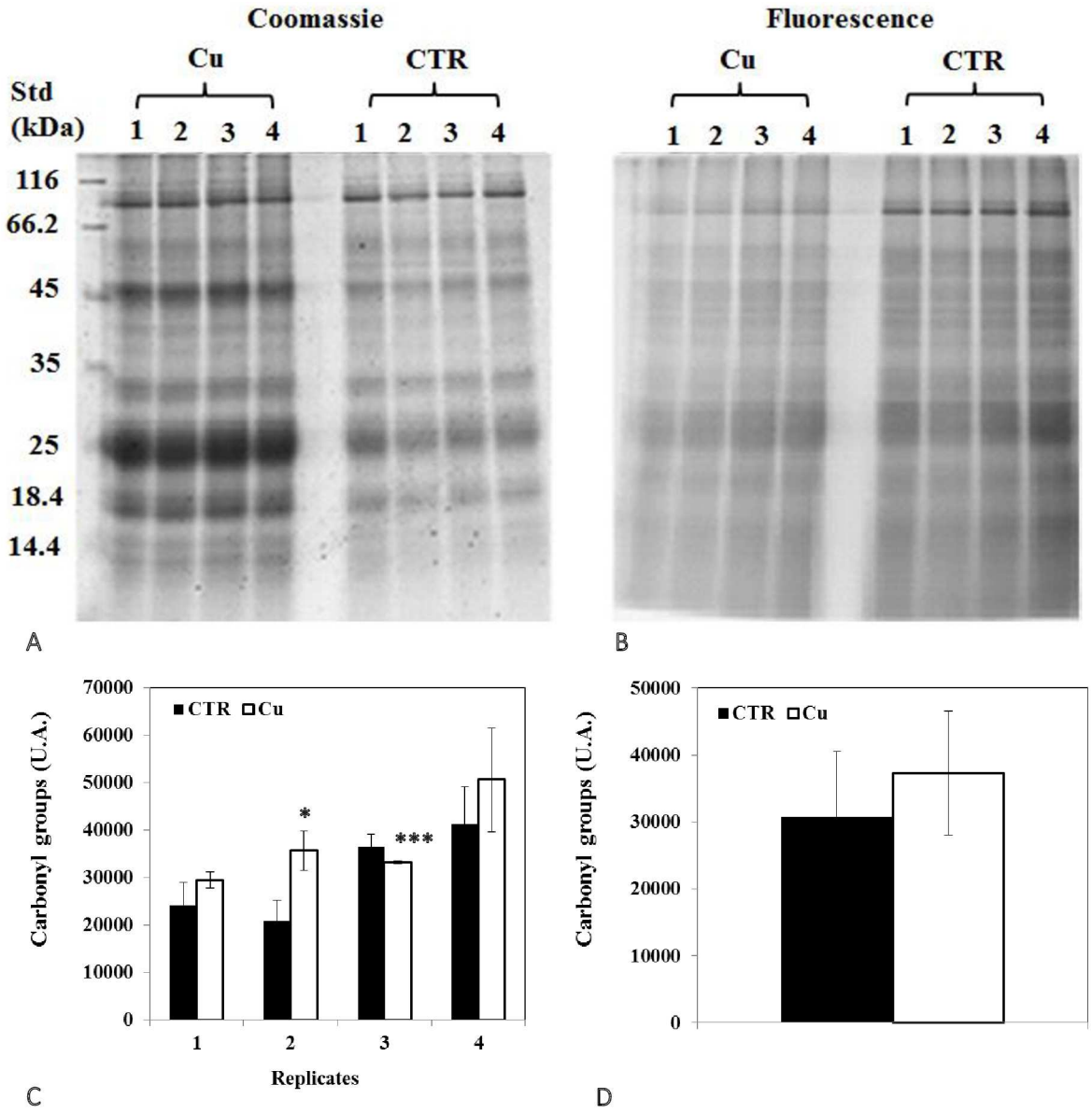



**Fig 8. Representative images of 1DE gels of proteins (100 μg) in cotyledons of bean seeds germinated for 3 days in the presence of (A) distilled water (CTR) or (B) 200 μM Cu.** Gels were stained with Coomassie G-250 (scanned with GS-800 calibrated densitometer) and with FTSC labeling (scanned with Typhoon 9400 scanner). Total optical densities for each lane obtained from FTSC staining were normalized with those from Coomassie G-250 staining of the same gel. (C, D) Levels of proteins containing carbonyl groups. Values shown are (C) means of 4 biological replicates (±SD) numbered from 1 to 4, and (D) means of 4 technical replicates (±SD). Each measurement was performed in an extract obtained from several cotyledons. Analyses were performed using ANOVA, student's T test; ***p<0.001.

https://doi.org/10.1371/journal.pone.0184396.g008

Simultaneous profiling of many proteins represents a novel way to compare dynamic responses towards heavy metals [60]. Proteomics is increasingly used to detect effects of environmental contaminants in ecotoxicology [61, 62]. 2DE analysis revealed significant changes in abundance of SH and CO groups of protein species in cotyledons and the seedlings of Cu-stressed bean seeds (Figs 6–9). Oxidative stress can trigger conformational changes making protein thiols more reactive towards cationic groups and modifying their susceptibility to

**Table 6. Proteins containing carbonyl groups (FTSC labeling) in the seedlings (3 days-old) (a) and the cotyledons (9 days-old) (b) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**

| Number of spots | Coomassie | Fluorescence |
|---|---|---|
| Total | **(a)** 356 | 159 |
| | **(b)** 610 | 234 |
| Significantly modified by Cu (p< = 0.05) | **(a)** 32 | 5 |
| | **(b)** 130 | 31 |
| Fold increase > = 1.5 | **(a)** 27 | 3 |
| | **(b)** 122 | 29 |
| p< = 0.05, Fold increase > = 1.5 | **(a)** 27 | 3 |
| | **(b)** 122 | 29 |
| Increased by Cu | **(a)** 19 | 1 |
| | **(b)** 86 | 18 |
| Decreased by Cu | **(a)** 8 | 2 |
| | **(b)** 36 | 11 |

https://doi.org/10.1371/journal.pone.0184396.t006

oxidation, either by increasing their exposure to the matrix or by decreasing side-chain *pKa* values [63]. In addition, transitory oxidative stress may increase protection of thiols, e.g. by glutathionylation or formation of sulphenic acid [64]. Heavy metals disrupt the majority of cellular mechanisms as well as causing differential accumulation of proteins involved in the regulation of cellular redox status [65]. Decreased availability of the main proteins involved in important cellular processes has also been reported [66].

In the present study, we have profiled the role of a network of ROS-detoxifying enzymes in protecting bean seeds from Cu-induced stress. Whilst antioxidant protection mechanisms have an important role in Cu stress tolerance in both cotyledons and seedlings, we have discovered subtle differences in the two organs. Notably, we found a greater capacity for protein protection in seedlings compared to cotyledons.

One of the likely ways for ROS to interact with proteins is through thiol modification of cysteine residues, which can be oxidised to varying degrees triggering changes in protein conformation and activity [67]. Redox signalling in higher plants may include the activation of the mitogen activated protein kinase cascade, inhibition of phosphatases and activation of $Ca^{2+}$ channels and $Ca^{2+}$ -binding proteins [68], as well as effects of plant hormones on signalling networks [69]. Kranner and Seal proposed [70] a triphasic stress model of seed redox control under salt stress, whereby the 'alarm' phase involves stress perception and transduction through the ROS-RNS-hormone signalling network, post-translational modification of macromolecules and an altered transcriptome so that the protection and repair machinery become activated and upregulated, respectively, in response to the perception of a stress and / or the initial damage caused. Under continuing stress (time or severity), the 'resistance' phase is reached when sufficient gene products required for protection and repair are produced to maintain viability. The resistance phase includes inducible protection (e.g. upregulated antioxidants that protect macromolecules from further damage), repair mechanisms (e.g. synthesis of DNA repair enzymes), and the elimination of redundant cells that are damaged beyond repair.

On the other hand, our understanding of seed tolerance or resistance to heavy metal exposure is far from complete and future research should be directed to achieve a better understanding of the mechanisms by which they act or interact with biomolecules and metabolic pathways during early and post-germination phases. The present work suggests that differential responses within different organs of the same seed may be due to differential mechanisms

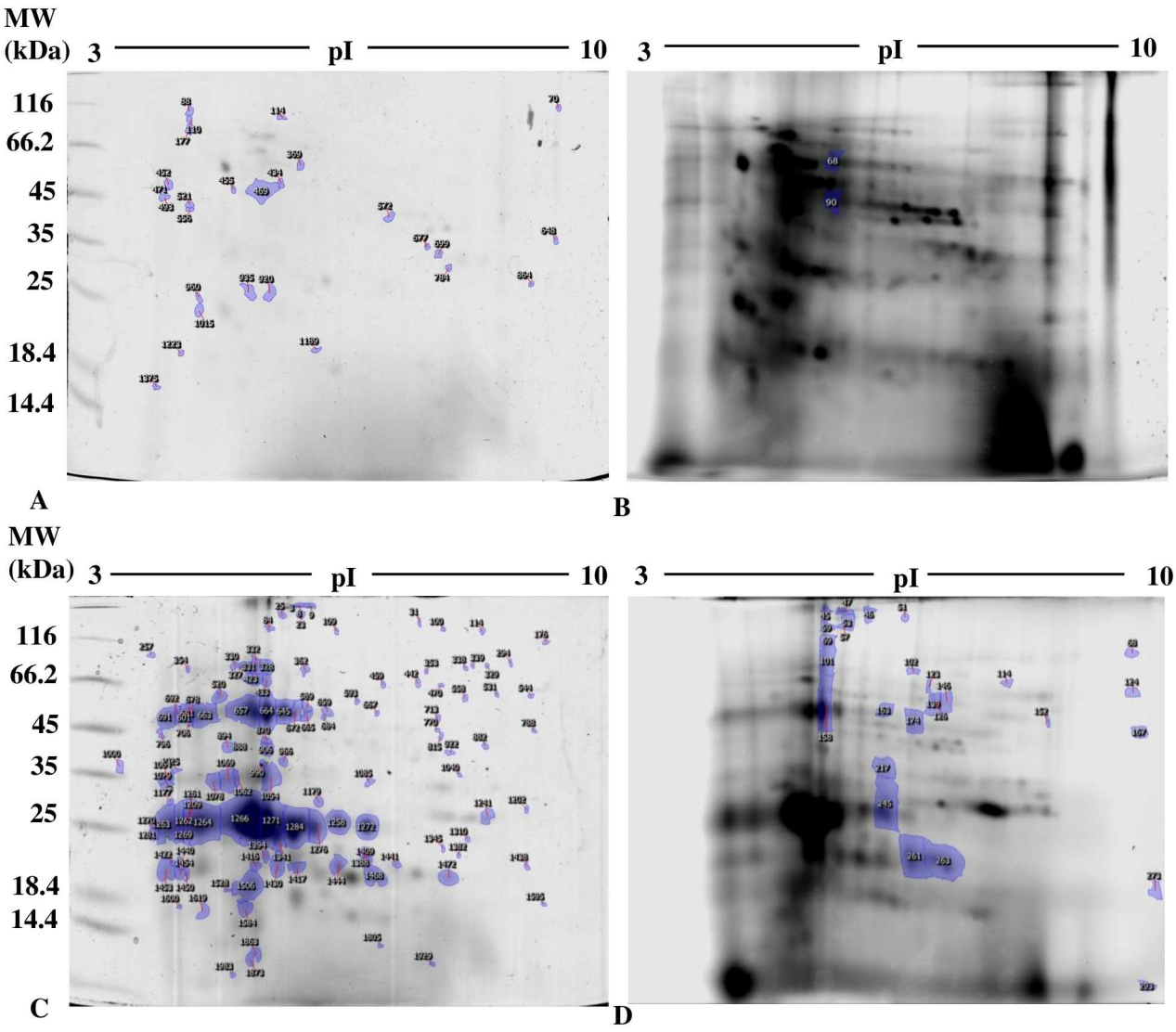



**Fig 9. Profiles of the expression of proteins containing carbonyl groups in (A, B) seedlings and (C, D) cotyledons of bean seeds germinated for 9 days in the presence of distilled water (CTR) or 200 μM Cu.** Proteins (1200 μg) were labeled with FTSC and separated by 2-D SDS-PAGE. Figures show spots of interest in representative gels from (A) colloidal Coomassie Brilliant G-250 staining (scanned with GS-800 calibrated densitometer) and (B) FTSC labeling (scanned with Typhoon 9400 scanner; 600 PMT). Numbers correspond to spots of $p<0.05$ and Fold induction >1.5 (spots identified).

https://doi.org/10.1371/journal.pone.0184396.g009

that confer stress resistance. There might also be much interaction, with feedback loops between gene expression, transcription and translation as well as interconnections between the various biochemical pathways responsible for metal tolerance, such as those that define the redox hub comprising ROS, antioxidants and plant hormones. Once we have achieved a more complete understanding of the pathways that confer tolerance to salinity and drought, it may be possible to up- or down-regulate sets of genes until those required for salt tolerance, or more generally, stress tolerance, have been identified. Our approach has profiled profound biochemical changes associated with development of oxidative stress under environmental stress conditions. The data reported here provide novel insights that may lead to a broader understanding of molecular responses to Cu-induced stress in higher plants, and the resulting consequences for growth, development and enhanced agricultural productivity.

## Supporting information

**S1 Appendix. Levels of MDA in the seedlings (3 days-old) and the cotyledons (9 days-old) of germinated bean seeds in the presence of $H_2O$ (CTR) or 200 μM Cu.**
(DOCX)

## Author Contributions

**Conceptualization:** Inès Karmous, Ezzeddine El Ferjani, Abdelilah Chaoui.

**Data curation:** David Sheehan.

**Investigation:** Inès Karmous, Rafael Trevisan.

**Methodology:** Rafael Trevisan, Abdelilah Chaoui, David Sheehan.

**Resources:** David Sheehan.

**Supervision:** Ezzeddine El Ferjani, Abdelilah Chaoui, David Sheehan.

**Writing – original draft:** Inès Karmous, Ezzeddine El Ferjani, Abdelilah Chaoui, David Sheehan.

**Writing – review & editing:** Abdelilah Chaoui, David Sheehan.

## References


1. Maksymiec W, Krupa Z. Jasmonic acid and heavy metals in Arabidopsis plants—a similar physiological response to both stressors? J Plant Physiol 2002; 159 (5): 509–15.
2. Janas K, Zielińska-Tomaszewska J, Rybaczek D, Maszewski J, Posmyk MM, Amarowicz R, et al. The impact of copper ions on growth, lipid peroxidation, and phenolic compound accumulation and localization in lentil (*Lens culinaris Medic.*) seedlings. J Plant Physiol 2010; 167(4): 270–76. https://doi.org/10.1016/j.jplph.2009.09.016 PMID: 19900731
3. Schutzendubel A, Polle A. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 2002; 53(372): 1351–65. PMID: 11997381
4. Monferrán MV, Sanchez Agudo JA, Pignata ML, Wunderlin DA. Copper-induced response of physiological parameters and antioxidant enzymes in the aquatic macrophyte *Potamogeton pusillus*. Environ Pollut 2009; 157(8): 2570–76.
5. Sharma SS, Dietz K-J. The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci 2009; 14(1): 43–50. https://doi.org/10.1016/j.tplants.2008.10.007 PMID: 19070530
6. Yruela I. Copper in plants. Brazil J Plant Physiol 2005; 17(1): 145–56.
7. Wang SH, Zhang Q, Jin GM, Jiang SJ, Jiang D, He QY, et al. Copper-Induced Oxidative Stress and Responses of the Antioxidant System in Roots of *Medicago sativa*. J Agron Crop Sci 2011; 197(6): 418–29.
8. Brown EJ, Khodr H, Hider RC, Rice-Evans CA. Structural dependence of flavonoid interactions with Cu2+ ions: implications for their antioxidant properties. Biochem J 1998; 330(3): 1173–8.
9. Lavid N, Schwartz A, Yarden O, Tel-Or E. The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the waterlily (Nymphaeaceae). Planta 2001; 212(3): 323–31. https://doi.org/10.1007/s004250000400 PMID: 11289596
10. Kumar M, Sugatha P, Hebbar K. Superoxide dismutase isozymes and their heat stability in coconut (*Cocos nucifera L.*) leaves. Ann Biol 2014; 30(4): 593–7.
11. Jimenez A, Hernández JA, Del Río LA, Sevilla F. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiol 1997; 114(1): 275–84. PMID: 12223704
12. Foyer CH, Halliwell B. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 1976; 133(1): 21–5. https://doi.org/10.1007/BF00386001 PMID: 24425174
13. Cuypers A, Vangronsveld J, Clijsters H. Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of *Phaseolus vulgaris* seedlings during the early stages of metal assimilation. Physiol Plantarum 2000; 110(4): 512–7.

14. León AM, Palma JM, Corpas FJ, Gómez M, Romero-Puertas MC, Chatterjee D, et al. Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. Plant Physiol Biochem 2002; 40(10): 813–20.
15. Mattioni C, Gabbrielli R, Vangronsveld J, Clijsters H. Nickel and cadmium toxicity and enzymatic activity in nitrolerant and non-tolerant populations of *Silene italica Pers*. J Plant Physiol 1997; 150(1–2): 173–7.
16. Jacquot JP, Eklund H, Rouhier N, Schürmann P. Structural and evolutionary aspects of thioredoxin reductases in photosynthetic organisms. Trends Plant Sci 2009; 14(6): 336–43. https://doi.org/10.1016/j.tplants.2009.03.005 PMID: 19446492
17. Navrot N, Collin V, Gualberto J, Gelhaye E, Hirasawa M, Rey P, et al. Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol 2006; 142(4): p. 1364–79. https://doi.org/10.1104/pp.106.089458 PMID: 17071643
18. Rouhier N, Lemaire SD, Jacquot J-P. The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu Rev Plant Biol 2008; 59: 143–66. https://doi.org/10.1146/annurev.arplant.59.032607.092811 PMID: 18444899
19. Ishiwatari Y, Nemoto K, Fujiwara T, Chino M, Hayashi H. *In situ* hybridization study of the rice phloem thioredoxin h mRNA accumulation—possible involvement in the differentiation of vascular tissues. Physiol Plantarum 2000; 109(1): 90–6.
20. Lozano RM, Wong JH, Yee BC, Peters A, Kobrehel K, Buchanan BB. New evidence for a role for thioredoxin h in germination and seedling development. Planta 1996; 200(1): 100–6.
21. Alkhalfioui F, Renard M, Vensel WH, Wong J, Tanaka CK, Hurkman WJ, et al. Thioredoxin-linked proteins are reduced during germination of *Medicago truncatula* seeds. Plant Physiol 2007; 144(3): 1559–79. https://doi.org/10.1104/pp.107.098103 PMID: 17513483
22. Montrichard F, Renard M, Alkhalfioui F, Duval FD, Macherel D. Identification and differential expression of two thioredoxin h isoforms in germinating seeds from pea. Plant Physiol 2003; 132(3): 1707–15. https://doi.org/10.1104/pp.102.019562 PMID: 12857849
23. Lemaire SD. The glutaredoxin family in oxygenic photosynthetic organisms. Photosynth Res 2004; 79 (3): 305–18. https://doi.org/10.1023/B:PRES.0000017174.60951.74 PMID: 16328797
24. Rouhier N, Couturier J, Johnson MK, Jacquot JP. Glutaredoxins: roles in iron homeostasis. Trends Biochem Sci 2010; 35(1): 43–52. https://doi.org/10.1016/j.tibs.2009.08.005 PMID: 19811920
25. Traverso JA, Vignols F, Cazalis R, Pulido A, Sahrawy M, Cejudo FJ, et al. PsTRXh1 and PsTRXh2 are both pea h-type thioredoxins with antagonistic behavior in redox imbalances. Plant Physiol 2007; 143 (1): 300–11. https://doi.org/10.1104/pp.106.089524 PMID: 17098852
26. Foyer CH, Noctor G. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ 2005; 28(8): 1056–71.
27. Karmous I, El Ferjani E, Chaoui A. Copper excess impairs mobilization of storage proteins in bean cotyledons. Biol Trace Elem Res 2011; 144(1–3): 1251–9. https://doi.org/10.1007/s12011-011-9115-9 PMID: 21681466
28. Karmous I, Jaouani K, Chaoui A, El Ferjani E. Proteolytic activities in *Phaseolus vulgaris* cotyledons under copper stress. Physiol Mol Biol Plants 2012. 18(4): 337–43. https://doi.org/10.1007/s12298-012-0128-4 PMID: 24082496
29. Karmous I, Chaoui A, Jaouani K, Sheehan D, El Ferjani E, Scoccianti V. et al. Role of the ubiquitin-proteasome pathway and some peptidases during seed germination and copper stress in bean cotyledons. Plant Physiol Biochem 2014; 76: 77–85. https://doi.org/10.1016/j.plaphy.2013.12.025 PMID: 24486582
30. Sergiev I, Alexieva V, Karanov E. Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. C R Acad Bulg Sci 1997; 51(3): 121–4.
31. Reznick AZ, Packer L. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. Method Enzymol 1994. 233: 357–63.
32. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82(1): 70–7. PMID: 13650640
33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72(1–2): 248–54.
34. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; 247(10): 3170–5. PMID: 4623845
35. Aebi H. Catalase *in vitro*. Method Enzymol 1984; 105: 121–6.
36. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 1981; 22(5): 867–80.

37. Fielding J, Hall J. A biochemical and cytochemical study of peroxidase activity in roots of *Pisum sativum I*. A comparison of dab-peroxidase and guaiacol-peroxidase with particular emphasis on the properties of cell wall activity. J Exp Bot 1978; 29(4): 969–81.

38. Nagalakshmi N, Prasad M. Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci 2001; 160(2): 291–9. PMID: 11164601

39. Bergmeyer H, Enzymes as biochemical reagents. Hexokinase. Method Enzymat Anal 1974; 1: 425–522.

40. Jacquot JP, Rivera-Madrid R, Marinho P, Kollarova M, Le Maréchal P, Miginiac-Maslow M, et al. *Arabidopsis thaliana* NAPHP thioredoxin reductase: cDNA characterization and expression of the recombinant protein in *Escherichia coli*. J Mol Biol 1994; 235(4): 1357–63. https://doi.org/10.1006/jmbi.1994.1091 PMID: 8308900

41. Rouhier N, Gelhaye E, Jacquot JP. Exploring the active site of plant glutaredoxin by site-directed mutagenesis. FEBS Lett 2002; 511(1–3): 145–9. PMID: 11821065

42. Green LS, Yee BC, Buchanan BB, Kamide K, Sanada Y, Wada K. Ferredoxin and ferredoxin-NADP reductase from photosynthetic and nonphotosynthetic tissues of tomato. Plant Physiol 1991; 96(4): 1207–13.

43. Ishida A, Ookubo K, Ono K. Formation of hydrogen peroxide by NAD (P) H oxidation with isolated cell wall-associated peroxidase from cultured liverwort cells, *Marchantia polymorpha L*. Plant Cell Physiol 1987; 28(4): 723–6.

44. Zhao Z, Hu X, Ross CW. Comparison of tissue preparation methods for assay of nicotinamide coenzymes. Plant Physiol 1987; 84(4): 987–8. PMID: 16665633

45. Matsumura H, Miyachi S. Cycling assay for nicotinamide adenine dinucleotides. Method Enzymol 1980; 69: 465–70.

46. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227(5259): 680–5. PMID: 5432063

47. Dyballa N, Metzger S. Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. J Viz Exp 2009; (30): e1431–e1431.

48. Flemming C, Trevors J. Copper toxicity and chemistry in the environment: a review. Water Air Soil Pollut 1989; 44(1–2): 143–58.

49. Ahsan N, Lee DG, Lee SH, Kang KY, Lee JJ, Kim PJ, et al. Excess copper induced physiological and proteomic changes in germinating rice seeds. Chemosphere 2007; 67(6): 1182–93. https://doi.org/10.1016/j.chemosphere.2006.10.075 PMID: 17182080

50. Bouazizi H, Jouili H, Geitmann A, El Ferjani E. Effect of copper excess on H 2 O 2 accumulation and peroxidase activities in bean roots. Acta Biolog Hungar, 2008; 59(2): 233–45.

51. Chaoui A, Ghorbal MH, El Ferjani E. Effects of cadmium-zinc interactions on hydroponically grown bean (*Phaseolus vulgaris L.*). Plant Sci 1997; 126(1): 21–8.

52. Chaoui A, Mazhoudi S, Ghorbal MH, El Ferjani E. Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris L.*). Plant Sci 1997; 127(2): 139–47.

53. Chaoui A, El Ferjani E. Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum L.*) seedlings. C R Biol 2005; 328(1): 23–31. PMID: 15714877

54. Zhang H, Zhang FQ, Xia Y, Wang GP, Shen ZG. Excess copper induces production of hydrogen peroxide in the leaf of *Elsholtzia haichowensis* through apoplastic and symplastic CuZn-superoxide dismutase. J Hazard Mater 2010; 178(1): 834–43.

55. Garnczarska M, Wojtyla Ł. Differential response of antioxidative enzymes in embryonic axes and cotyledons of germinating lupine seeds. Acta Physiol Plant 2008; 30(4): 427–32.

56. Posmyk M, Konte R, Janas K. Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicol Environ Safe 2009; 72(2): 596–602.

57. Arnér ES, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem, 2000; 267(20): 6102–9. PMID: 11012661

58. Aalen R., Peroxiredoxin antioxidants in seed physiology. Seed Sci Res 1999; 9(4): 285–295.

59. Wood Z.A., Schroder E, Robin Harris J, Poole LB. Structure, mechanism and regulation of peroxiredoxins. Trend Biochem Sci 2003; 28(1): 32–40. PMID: 12517450

60. Kültz D, Fiol D, Valkova N, Gomez-Jimenez S, Chan SY, Lee J. Functional genomics and proteomics of the cellular osmotic stress response in non-model organisms. J Exp Biol 2007; 210(9): 1593–601.

61. Chora S, McDonagh B, Sheehan D, Starita-Geribaldi M, Roméo M, Bebianno MJ. Ubiquitination and carbonylation of proteins in the clam *Ruditapes decussatus*, exposed to nonylphenol using redox

proteomics. Chemosphere 2010; 81(10): 1212–7. https://doi.org/10.1016/j.chemosphere.2010.09.038 PMID: 20943247

62. Tedesco S, Doyle H, Blasco J, Redmond G, Sheehan D. Exposure of the blue mussel, *Mytilus edulis*, to gold nanoparticles and the pro-oxidant menadione. Comp Biochem Physiol C: Toxicol Pharmacol 2010; 151(2): 167–74.
63. Lin T-K, Hughes G, Muratovska A, Blaiki FH, Brookes PS, Darley-Usmar V, et al. Specific Modification of Mitochondrial Protein Thiols in Response to Oxidative Stress: A proteomics approach. J Biol Chem 2002; 277(19): 17048–56. https://doi.org/10.1074/jbc.M110797200 PMID: 11861642
64. Hansen RE, Roth D, Winther JR. Quantifying the global cellular thiol—disulfide status. P Natl Acad Sci USA 2009; 106(2): p. 422–7.
65. Ahsan N, Renaut J, Komatsu S. Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. Proteomics 2009; 9(10): 2602–21. https://doi.org/10.1002/pmic.200800935 PMID: 19405030
66. Printz B, Sergeant K, Lutts S, Guignard C, Renaut J, Hausman JF. From tolerance to acute metabolic deregulation: contribution of proteomics to dig into the molecular response of alder species under a polymetallic exposure. J Proteome Res 2013; 12(11): 5160–79. https://doi.org/10.1021/pr400590d PMID: 24015726
67. Colville L, Kranner I. Desiccation tolerant plants as model systems to study redox regulation of protein thiols. Plant Growth Regul 2010; 62(3): 241–55.
68. Møller IM, Jensen PE, Hansson A. Oxidative modifications to cellular components in plants. Annu Rev Plant Biol 2007; 58: 459–81. https://doi.org/10.1146/annurev.arplant.58.032806.103946 PMID: 17288534
69. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al., ROS signaling: the new wave? Trends Plant Sci 2011; 16(6): 300–9. https://doi.org/10.1016/j.tplants.2011.03.007 PMID: 21482172
70. Kranner I, Seal CE. Salt stress, signalling and redox control in seeds. Funct Plant Biol 2013; 40(9): 848–59.