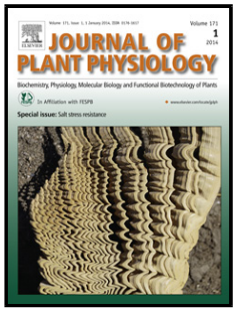
CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Protective role of exogenous phytohormones on redox status in pea seedlings under copper stress
Authors	Ben Massoud, Marouane;Sakouhi, Lamia;Karmous, Inès;Zhu, Yao;El Ferjani, Ezzedine;Sheehan, David;Chaoui, Abdelilah
Publication date	2017-12-09
Original Citation	Ben Massoud, M., Sakouhi, L., Karmous, I., Zhu, Y., El Ferjani, E., Sheehan, D. and Chaoui, A. (2018) 'Protective role of exogenous phytohormones on redox status in pea seedlings under copper stress'. Journal of Plant Physiology, 221, pp. 51-61. doi:10.1016/j.jplph.2017.11.014
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.jplph.2017.11.014
Rights	© 2017, Elsevier GmbH. All rights reserved. This manuscript version is made available under the CC-BY-NC-ND 4.0 license. - https://creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2026-11-02 16:34:19
Item downloaded from	https://hdl.handle.net/10468/5865

UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Accepted Manuscript



Title: Protective role of exogenous phytohormones on redox status in pea seedlings under copper stress

Authors: Marouane Ben Massoud, Lamia Sakouhi, Inès Karmous, Yao Zhu, Ezzedine El Ferjani, David Sheehan, Abdelilah Chaoui

PII: S0176-1617(17)30296-1
DOI: https://doi.org/10.1016/j.jplph.2017.11.014
Reference: JPLPH 52701

To appear in:

Received date: 20-8-2017
Revised date: 5-11-2017
Accepted date: 9-11-2017

Please cite this article as: Ben Massoud Marouane, Sakouhi Lamia, Karmous Inès, Zhu Yao, El Ferjani Ezzedine, Sheehan David, Chaoui Abdelilah.Protective role of exogenous phytohormones on redox status in pea seedlings under copper stress.*Journal of Plant Physiology* https://doi.org/10.1016/j.jplph.2017.11.014

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Title:

Protective role of exogenous phytohormones on redox status in pea seedlings under copper stress

Running title: Heavy metal stress alleviation

Marouane Ben Massoud[a,b], Lamia Sakouhi[a], Inès Karmous[a], Yao Zhu[c], Ezzedine El Ferjani[a], David Sheehan[b,d], Abdelilah Chaoui[a,*]

[a]*Plant Toxicology and Molecular Biology of Microorganisms, Faculty of Sciences of Bizerta, 7021 Zarzouna, Tunisia*

[b]*Proteomics Research Group, School of Biochemistry and Cell Biology & Environmental Research Institute, University College Cork, Ireland*

[c]*Teagasc Food Research Center, Department of Food Chemistry and Technology, Ashton, Dublin 15, Ireland*

[d]*College of Science, Khalifa University of Science and Technology, Abu Dhabi, United Arab Emirates*

M. Ben Massoud: benmassoudmarouane@yahoo.fr

L. Sakouhi:lamiasakouhi@hotmail.com

I. Karmous: karmous.ines@yahoo.fr

Y. Zhu:jnuspzy@gmail.com

E. El Ferjani: ezzferjani2002@yahoo.fr

D. Sheehan : d.Sheehan@ucc.ie

[*]A. Chaoui (Corresponding author): cabdelilah1@yahoo.fr

ACCEPTED MANUSCRIPT

ABSTRACT

The present work aims to provide insight on the role of phytohormone application in developing efficient practical defense strategies to improve plants tolerance under heavy metal contamination. For this purpose, pea (*Pisum sativum* L.) seeds were germinated in an aqueous solution of 200 µM $CuCl_2$ up to the 3rd day and then continued to germinate in the presence of distilled water (stress cessation) or were subjected to following combinations: Cu + 1 µM IAA and Cu + 1 µM GA_3 for 3 additional days. The results showed that copper excess induced oxidative stress in germinating seeds, which resulted in changes of the redox state of glutathione and cysteine, and proteomics revealed Cu-induced modifications of thiols (SH) and carbonyls (CO) (indicators of protein oxidation). However, application of IAA or GA_3 in the germination medium after 3 days of Cu exposure alleviated toxicity on seedlings, despite the persistence of Cu up to 6th day. This improving effect seems to be mediated by a cell Cu accumulation decrease and a protein reduced status recovery, since phytohormones modulate thioredoxin/ferredoxin systems in favor of protecting proteins against oxidation. In addition, an IAA and GA_3 protective effect was evidenced by a cellular homeostasis amelioration resulting from the balance conservation between the regeneration and consumption processes of glutathione and cysteine reduced forms. The exogenous effectors also induced modifications of profiles of SH and CO, suggesting changes in the regulation and expression of proteins that could be involved in defense mechanism against Cu stress.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abbreviations: CSH, reduced cysteine; CSS, oxidized cysteine; DTNB, 5,5'-dithio bis 2-nitrobenzoic acid; Fdx, ferredoxin; FNR, ferredoxin-NADP oxidoreductase; FTSC, fluorescein-5-thiosemicarbazide; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IAF, 5'-iodoacetamide fluorescein; NTR, NADPH-dependent thioredoxin reductase; Trx, thioredoxin

Keywords: Heavy metal Phytohormones Redox homeostasis Stress alleviation

1. Introduction

The functions of the components of redox status have been widely investigated in several plant systems, including seeds since they are considered as the sensor of the real physiological state in cells (Foyer and Noctor, 2005; Alkhalfioui et al., 2007). In addition, the regulation of the redox state of proteins, including the sulfhydryl groups of cysteine and methionine, is considered as a "switch" for the activity of several enzymes involved in specific signaling events(protein kinases, calcium signaling) and in cell cycle control (Alkhalfioui et al., 2007). However, under stress conditions, notably exposure to heavy metals, the cellular redox state can shift the redox balance toward an oxidizing state (Rouhier et al., 2010), which may result in altering many physiological processes associated with normal growth and development (Janas et al., 2010).

Copper is considered one of the toxic heavy metals that can affect one or more vital biochemical and physiological processes in plants and seedlings (Karmous et al., 2012; Chaoui et al., 2004; Chaoui and El Ferjani,2014). A delay in embryo growth has been often recorded after Cu exposure, and has been associated with many disorders of germinative metabolism (Karmous et al., 2012), as well as cellular oxidative state generation, mainly described by reactive oxygen species (ROS) over-production (Ahsan et al., 2007; Chaoui and El Ferjani,

ACCEPTED MANUSCRIPT

2014). Enzymatic and non-enzymatic defense mechanisms are activated to limit the induced oxidative stress damage (Wang et al., 2004). Indeed, several roles of glutathione have been reported, mainly in redox state homeostasis, regeneration of other antioxidants (Foyer and Noctor, 2005) and phytochelatins, as well as heavy metals sequestration in plants (Yadav, 2010). Likewise, GSH is implicated in the Asada-Halliwell cycle, exclusively existing in plants and essential for defense against oxidative stress (Foyer and Noctor, 2005). In this process, reduced glutathione (GSH) is converted to its oxidized form (GSSG; Foyer and Noctor, 2011), and in turn, the GSH is regenerated by glutathione reductase (GR) activity using NADPH as an electron donor, maintaining the cellular redox homeostasis by keeping optimum GSH/GSSG ratio (Gill et al., 2013). This parameter can also be changed by the activity of glutathione peroxidase (GPX). Plant glutathione peroxidases are functional peroxiredoxins distributed in several cell compartments and regulated during biotic and abiotic stresses (Navrot et al., 2006).

Scientists have been more interested in developing alternative ways and potential strategies to counteract the adverse effects of environmental stressors via the exogenous application of chemicals to improve plant tolerance, such as polyamines, brassinosteroids (Choudhary et al., 2012), nitric oxide (Hu et al., 2007), sulfur (Anjum et al., 2008), β-estradiol (Chaoui and El Ferjani,2014), progesterone (Genisel et al., 2013), salicylic acid (Belkhadi et al., 2010), calcium (Sakouhi et al., 2016) and organic acids (malate, oxalate, citrate and benzoate) (Gao et al., 2012).

On these grounds, the aim of this investigation was to shed more light on the mechanism by which IAA and GA_3 interact with the redox components in germinating pea seeds subjected to 200 µM $CuCl_2$ treatment. The cysteine and glutathione contents were analyzed by HPLC, concomitant with the possible changes occurring within the accumulation profile of Cu in the two parts of the seedling and some enzyme activities involved in modulating the cellular redox homeostasis (Rouhier et al., 2008): thioredoxin (Trx), NADPH-dependent thioredoxin

ACCEPTED MANUSCRIPT

reductase (NTR), ferredoxin (Fdx) and ferredoxin-NADP oxidoreductase (FNR). The activities of GPX and GR were also measured to estimate the recycling of glutathione (Mittler, 2002). The oxidation state of the proteins was studied by quantifying their contents in thiol and carbonyl indicators. Proteins CO and SH were labeled with specific substrates, and analyzed by one- and two-dimensional electrophoresis.

2. Material and methods

2.1. Germination and treatment conditions

Pea (*Pisum sativum* L. var. douce province) seeds were disinfected with 2% sodium hypochlorite for 10 min, then rinsed three times with distilled water, and germinated at 25°C in the presence of distilled water (control) or 200 µM $CuCl_2$. At day 3, Cu was replaced by the following treatments; 1µM IAA and 1 µM GA_3were applied in combinations with Cu: "Cu+IAA" and "Cu+GA_3", while"Cu+H_2O" consisted in discarding completely Cu and carrying on germination with H_2O (stress cessation). At day 6, the seedlings were harvested and separated into roots and shoots, weighed, then stored at -80°C until use.

2.2.Copper content determination

After wet digestion of the oven-dried seedlings (10 ml per 0.1 g dry weight) with an acid mixture (HNO_3:$HClO_4$, 4:1), Cu concentrations were determined by an atomic absorption spectrophotometer (Perkin Elmer). Sigma Diagnostic Standards (Copper Atomic Absorption Solutions, Sigma-Aldrich) were diluted in appropriate ranges with 0.1 N HNO_3 (65% HNO_3, Sigma-Aldrich, Analytical grade) and used for calibration.

2.3.Glutathione and cysteine quantification

ACCEPTED MANUSCRIPT

Lyophilized samples were homogenized at 4°C in the presence of 0.2 M potassium-phosphate buffer, pH 7.5 (Chwatko et al., 2014), and obtained homogenates were immediately subjected to derivation or reduction experiments. The derivation reaction was performed using 1 mM DTNB (5,5'-dithio bis 2-nitrobenzoic acid prepared in 0.5 M K-phosphate buffer, pH 8; Katrusiak et al., 2001). After incubation at 4°C for 5 min, 7 M phosphoric acid was added to stop the reaction, and the homogenate was centrifuged at 12,000*g* for 10 min at 4°C. The obtained supernatant was filtered using a 0.45 µm filter and stored at -20°C in amber glass vials for HPLC measurements. For total glutathione and cysteine determination, a reduction of oxidized forms (GSSG and CSS, respectively) was performed with 10 mM DTT prior to the derivation reaction. GSSG and CSS concentrations were calculated as the difference in the contents of total and reduced forms (GSH and CSH, respectively).

The chromatographic separation was achieved using a reverse-phase column Agilent (USA), 1100 Series, connected to a UV-visible detector set at 330 nm (Katrusiak et al., 2001). A volume of 20 µL of extract was injected into Zorbax Eclipse Plus C18 column (5 µm, 4.6 x 250 mm^2). The mobile phase consisted of acetonitrile as mobile phase A and acidified water, adjusted to pH 3.5 with acetic acid, as mobile phase B at a flow rate of 1.2 $mLmin^{-1}$. The proteins were eluted according to the following profile: 0-15 min, 10% A; 15-16 min, 100% A; 16-20 min, 10% A. The identification of peaks was based on the comparison of retention times with those obtained with corresponding standards. The total and reduced forms of thiol compounds were quantified using calibration curves made from DTNB-derived glutathione and cysteine (Sigma) solutions.

2.4. Enzyme assays

Protein extracts were obtained as the supernatants of the centrifugation (20,000*g* for 15 min at 4°C) of fresh tissue homogenates prepared in 25 mM potassium-phosphate buffer pH 7.0, containing 5 mM sodium ascorbate.

ACCEPTED MANUSCRIPT

GR (EC 1.6.4.2) activity was determined according to Foyer and Halliwel (1976), by measuring the rate of NADPH oxidation-decrease in absorbance at 340 nm($\varepsilon = 6.22 \times 10^3$ $M^{-1}.cm^{-1}$). The assay mixture contained 0.2 mM NADPH, 0.5 mM oxidized glutathione (GSSG) in 50 mM phosphate buffer (pH 7.0) and enzyme extract.

GPX (EC 1.11.1.9) activity was measured according to Nagalashmi and Prasad (2001). The reaction mixture 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 enzyme extract. Oxidation of NADPH was followed by measuring the decrease in absorbance at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}.cm^{-1}$).

Activities of Trx and NTR (EC 1.8.1.9) were measured in the reaction mixture: 50 mM Tris-HCl (pH 8.1), 100 µM DTNB and enzyme extract, containing 0.2 mM NADPH and 30 µg/mL reduced Trx (NTR assay) or 15 mg mL^{-1} NADPH and 0.1 µM NTR (Trx assay) (Jacquot et al., 1994). The reduction of DTNB was determined by measuring the increase in the absorbance at 412 nm ($\varepsilon = 13.6 \times 10^3$ $M^{-1}.cm^{-1}$).

Activities of Fdx and FNR (EC 1.18.1.2) were assayed according to Green et al. (1991). The reaction mixture contained 50 mM Tris-HCl (pH 7.8), 40 µM cytochrome C, 250 µM NADPH, 2 µM spinach leaf Fdx (FNR assay) or 0.1 µM FNR (Fdx assay) and the enzyme extract. The reduction of cytochrome C was monitored by the increase in absorbance at 550 nm ($\varepsilon = 19.1 \times 10^{-3}$ $M^{-1}.cm^{-1}$).

Protein concentrations in extracts were evaluated by the method of Bradford (1976), using bovine serum albumin as standard protein.

2.5. Proteomic analysis of thiols and carbonyls

Extraction of proteins was performed by homogenization of samples in 10 mM Tris-HCl, pH 7.2, containing 500 mM saccharose, 1 mM EDTA, 150 mM KCl and 1 mM PMSF.

ACCEPTED MANUSCRIPT

After centrifugation at 20,000*g*for 1 h at 4°C, supernatants were collected. Protein CO and SH were labeled with the respective substrates l mM fluorescein-5-thiosemicarbazide (FTSC) and 0.2 mM 5'-iodoacetamide fluorescein (IAF). After 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 obtained pellets were re-suspended and washed three times with 100% ethanol/ethyl acetate (1:1) and 96% acetone, respectively, for CO and SH assays, and were then re-suspended in 0.5 M Tris-HCl pH 6.8, 10% glycerol , 0.5% SDS and bromophenol blue, then applied to 1D SDS-PAGE (12%, 120 V) (Laemmli, 1970) (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). Quantity One Image analysis software (BioRad, Hercules, CA, USA) was used to analyze protein-associated fluorescence intensity. Colloidal Coomassie Brilliant Blue (CBB)G-250 (Dyballa and Metzger, 2009) was used to stain the gels, and a calibrated densitometer GS-800 (BioRad, Hercules, CA, USA) allowed to scan them, then measure and normalize the total OD.

For 2D gels, proteins were separated according to their pI (first dimension: isoelectric focusing), then according to their molecular weight (second dimension: 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 bromophenol blue, and then immobilized in IPG strips (70×3×0.5 mm),pH 3-10 and linear gradient (GE Healthcare Immobiline ^{TM}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, starting with 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 subsequently equilibrated for 20 min in equilibration buffer ; 6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol,

ACCEPTED MANUSCRIPT

2% DTT, pH 8.8, and then for 20 min in equilibration buffer containing 2.5% iodoacetamide. IPG strips were then loaded onto 12% SDS-PAGE (PROTEAN Plus Dodeca Cell Bio-Rad) for protein separation. Gels were scanned afterwards for fluorescence and then stained with CBB G-250 followed by densitometry scanning. Normalization of FTSC- and IAF-labeled protein spots and CBB-staining intensity was performed using Progenesis Same Spots Software (Ref: S/No.62605/3787; Nonlinear USA Inc/2530 Meridian Parkway/3rd Floor Durham/NC27713/USA) as per the manufacturer's instructions. Fluorescence spots were normalized to protein intensity for the same gel revealing increased fluorescence.

2.6. Statistical analysis

All experiments were performed at least in triplicate. Values are means ± SE of three technical and five biological replicates. Significance of differences was tested at $p<0.05$ using ANOVA test followed by Tukey's test $HSD_{p=0.05}$. Images of protein spots of 2D gels were subjected to Landmarking alignment (3D Gaussian distribution), and their intensities were corrected by background subtraction and normalized, then subjected to quantitative and qualitative analyses. One-way ANOVA followed by Tukey's *post hoc* multiple comparison tests were performed using the software package Statistica 8.0. Statistically significant differences between all spots in 2D gel image were established at $p<0.05$ and assessed using the Student's *t*-test.

3. Results and discussion

One of the underlying causes of tissue injury following exposure of plants to heavy metals is the generation of oxidative stress, resulting in alterations within almost all cellular components, including proteins (Davie, 2001). Plant cells contain numerous proteins with disulfide reductase activity. Notably, Trx and Fdx can play a crucial role in plant responses to

ACCEPTED MANUSCRIPT

stress due to their thiol redox property. Thus, in the present study, we investigated the possible changes occurring within the different components of the redox systems after the subsequent application of the phytohormones IAA and GA_3 to the Cu-contaminated germinating medium of pea seeds in both root and shoot tissues. Our results showed a significant increase in the activities of Trx, NTR, Fdx and FNR in the presence of Cu (Fig. 1), which suggests (1) their contribution to maintain the reduced form of protein SH, but at the same time (2) a potential depletion of the cellular stock of reducing power (NADPH), notably by NTR and FNR activities (Green et al., 1991; Jacquot et al., 1994). It has been assumed that the tolerance to heavy metal toxicity is more dependent on the availability of reduced cell metabolites, such as NAD(P)H, than on antioxidant enzymes capacity of plant tissues (Cuypers et al., 2000; Léon et al., 2002; Sakouhi et al., 2016). Such enhancement in activities of redox proteins agrees with their fundamental role in the protection of plants under abiotic stress (Rouhier et al., 2010), and notably the repair of damaged proteins (Arner and Holmgren, 2000). However, IAA or GA_3 were able to recover the activities of redox enzymes to nearly control values (Fig. 1). This ameliorating effect seems to occur as though we completely omit Cu and replace it with H_2O (Cu+H_2O experiment), which suggests that exogenous effectors were able to completely alleviate the negative effect of Cu, despite its persistence in the germination medium. This finding suggests the involvement of phytohormones in modulating the thiols status of proteins and reducing the intracellular oxidative stress damages, which might confer tolerance to pea seeds against Cu contamination.

Copper treatment drastically reduced glutathione levels, especially the reduced form (by 71 and 82% as compared to respective controls in root and shoot; Fig.2A, C), leading to a substantial glutathione redox state disruption (Fig. 2B, D). GSH depletion could be associated with increased GPX activity (Fig. 3A) despite an enhancement in the reduced-form recycling capacity by GR activity (Fig. 3B). The imbalance in the GSH/GSSG ratio has been reported in

ACCEPTED MANUSCRIPT

plants exposed to various environmental stresses, notably cold (Radyuk et al., 2009), Cd (Anjum et al., 2011) and Cu (Russo et al., 2008).The GSH depletion (Fig. 2) could be also attributed to (a) the biosynthesis of phytochelatins involved in Cu ions sequestration, (b) potential GSH conjugation to copper ions through the glutathione-S-transferase activity (Delalande et al., 2010) and (c) to the consumption of GSH by the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Nevertheless, the introduction of IAA and GA_3 into the germination medium restored glutathione contents (Fig. 2A, C) and GSH/GSSG balance (Fig. 2B, D) to levels similar to those of the control roots and shoots. The glutathione redox state protection, despite a slowing-down of the reduced form recycling via the GR activity (Fig. 3B), appears to be the result of the significant decrease in GPX activity when Cu was applied in combination with phytohormones (Fig. 3A). A similar IAA correction of GSH and GSSG contents has also been reported in response to cadmium stress in *Trigonella foenum-graecum* (Bashri and Prasad, 2016), and the sensitivity of *Arabidopsis thaliana* seedlings to copper can even be regulated by auxin (Song et al., 2017).

Cysteine is an important amino acid for its role as a precursor of many molecules involved in antioxidant defense, such as vitamins, cofactors and antioxidants, namely glucosinolates, thioninesand glutathione, and the implication of its redox status in cellular homeostasis is increasingly studied in the plant cell (Álvarez et al.,2012). The behavior of the oxidoreduction state of cysteine (Fig. 4) is similar to that of glutathione (Fig. 2). Late treatment with IAA or GA_3 of Cu-stressed germinating seeds reversed the disruptive effect of heavy metal on cysteine levels and CSH/CSS ratio in the two parts of the seedlings (Fig. 4). The phytohormone's protective effect on cysteine redox state significantly exceeded that of Cu suppression (Cu+H_2O experiment *versus* Cu+IAA and Cu+GA_3; Fig. 4B, D). Several lines of argument suggest the involvement of cysteine in the response of plants to abiotic stresses, including heavy metals: (i) Cd tolerance of transgenic tobacco plants is correlated to an over-

ACCEPTED MANUSCRIPT

expression of cysteine synthase (Kawashima et al., 2004), (ii) increased thiol biosynthesis of transgenic poplar expressing a wheat *O*-cetylserine (thiol) lyase enhances resistance to hydrogen sulfide and sulfur dioxide toxicity, and (iii) the exogenous application of cysteine to basil seeds and seedlings mitigated even the detrimental effect of cobalt stress (Azarakhsh et al., 2015).

To investigate the redox changes with respect to proteins, thiol (SH; Fig.5) and carbonyl (CO; Fig. 6) groups were analyzed by proteomics. Heavy metal-induced oxidative stress may result in damage to proteins, mainly oxidation of thiols and formation of carbonyls (Suzuki et al., 2010). Indeed, oxidative injury can trigger conformational changes, making protein thiols more reactive towards cationic groups and modifying their susceptibility to alkylation, either by increasing their exposure to the matrix or by decreasing side-chain *pKa* values (Lin et al., 2002). Here, the comparison of dynamic responses towards Cu by 1Dproteomic analysis revealed significant changes in abundance of SH and CO groups of protein species (Figs. 5, 6). Interestingly, the protein SH profile showed a significant decrease under Cu stress (Fig. 5), suggesting the oxidation of protein thiols in both roots and shoots and the enhancement of CO groups formation (Fig. 6), which can induce many reactions including alkylation (Lin et al., 2002) and glutathionylation (Hansen et al., 2009). Nonetheless, application of IAA and GA_3 showed a significant recovery in both parameters (Figs. 5, 6).

The 2D-separation of proteins showed different spots after IAF or FTSC labeling and CBB staining (Fig. 7). In fact, Table1recorded the significant modifications occurring within SH and CO groups of proteins in roots and shoots of control (H_2O) *versus* Cu treatment (H_2O/Cu) and of Cu *versus* Cu+IAA and Cu+GA_3 treated samples (Cu/Cu+IAA and Cu/Cu+GA_3, respectively). The data showed that Cu toxicity induced many alterations within proteins. For example, in roots, 37 spots were significantly modified when revealed by FTSC (CO groups). However, the application of IAA and GA_3 also exhibited important quantitative

ACCEPTED MANUSCRIPT

changes as compared to treatment with Cu alone - notably, 36 spots (SH)and 32 spots (CO) in roots and shoots of Cu+GA_3- and Cu+IAA-treated seedlings, respectively. These changes suggest that the application of phytohormones can modulate the Cu-induced alteration in proteins of redox status, which may result in protection against the toxicity of heavy metals. The proteomics approach is increasingly used to detect the modifications of protein thiols in response to oxidative stress (Lin et al., 2002). Indeed, Ahsan et al. (2009) and Printz et al. (2013) reported that heavy metals disrupt the availability of several proteins, including those that regulate the redox status. Modifications mainly concern the expression, abundance and regulation of proteins. For example, excess copper induced proteomic changes in germinating rice seeds (Ahsan et al., 2007), and proteomics also revealed alleviating effects of GA_3 on salt-stressed rice (Wen et al.,2010) and of IAA under manganese toxicity (Gangwar et al., 2011). The quantitative findings reported in this paper should be completed by qualitative analyses. Further work is needed to identify modified proteins by mass spectrometry.

The appreciable positive effects of phytohormones on the Cu-stressed seedling redox state could be correlated with the reduction of heavy metal accumulation by almost 32% and 45% in roots and shoots, respectively (Table 2). In agreement with our results, GA_3 and IAA were reported to decrease heavy metal accumulation in *Arabidopsis thaliana* (Zhu et al., 2012), broad bean and lupin (Sharaf et al., 2009) and eggplant plants (Singh and Prasad 2015). In the green alga *Chlorella vulgaris*, auxins and gibberellin alleviated stress symptoms by inhibiting heavy metal accumulation (Piotrowska-Niczyporuk et al., 2012) and a greater accumulation of proline subsequent to IAA treatment in wheat seedlings exposed to Cd-stress was reported by Agami and Mohamed (2013). This suggests proline implication in the stabilization of macromolecules structures and functions under stress conditions. The mechanism by which phytohormones reduce metal accumulation is still unclear. Wang et al. (2013) suggested that IAA reduced Al accumulation in wheat root apex by increasing efflux of malic acid, with high

ACCEPTED MANUSCRIPT

binding capacity with rhizosphere active Al, and by increasing rhizosphere pH via the regulation of plant cell plasma membrane H^+-ATPase activity. Moreover, Zhu et al. (2012; 2013) showed that auxins enhanced the content of hemicellulose1, which contributed to the fixation of Cd^{2+} in the cell wall, thus reducing its translocation from roots to shoots, while GA's protective effect against Cd injury resulted from the down-regulated cadmium transporter gene IRT1 expression. Likewise, He et al. (2015) reported that GA_3 caused Pb sequestration in cell walls, modifying its subcellular distribution.

4. Conclusion

The data reported here show evidence that the protective effects of IAA and GA_3 against Cu-induced alterations can be mediated by lowering heavy metal accumulation and by providing a thiols redox state correction, thereby, a suitable reducing environment to prevent, counteract or repair potential injuries from oxidation of sensitive cell sites, which may, ultimately, restore a normal growth of pea seedlings. Our findings also suggest that the phytohormones, although added after 3 days of copper exposure, induced almost total recovery in redox homeostasis by alleviating the damage of proteins. Proteomics may represent an innovative practical way of better understanding the improvement of plant tolerance to heavy metal stress through the supply of exogenous chemicals favorable for optimal growth.

Acknowledgments

Financial support for this work was received from the Tunisian Ministry of Higher Education, Scientific Research and Technology and School of Biochemistry and Cell Biology & Environmental Research Institute, University College Cork, Ireland (Grant to Marouane Ben

ACCEPTED MANUSCRIPT

Massoud). The authors wish to thank Mrs. Sihem Ben Hassine for technical assistance in HPLC analyzes.

References

Agami, R.A., Mohamed, G.F., 2013. Exogenous treatment with indole-3-acetic acid and salicylic acid alleviates cadmium toxicity in wheat seedlings. Ecotoxicol. Environ. Saf. 94, 164–171.

Ahsan, N., Lee, D.G., Lee, S.H., Kang, K.Y., Lee, J.J., Kim, P.J., Yoon, H.S., Kim, J.S., Lee, B.H., 2007. Excess copper induced physiological and proteomic changes in germinating rice seeds. Chemosphere 67, 1182-1193.

Ahsan, N., Renaut, J., Komatsu S., 2009. Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. Proteomics 9, 2602-2621.

Alkhalfioui, F., Renard, M., Vensel, W.H., Wong, J., Tanaka, C.K., Hurkman, W.J., Buchanan, B.B., Montrichard, F., 2007. Thioredoxin-linked proteins are reduced during germination of *Medicago truncatula* seeds. Plant Physiol. 144, 1559-1579.

Álvarez, C., ÁngelesBermúdez, M., Romero, L.C., Gotor, C., García, I., 2012. Cysteine homeostasis plays an essential role in plant immunity. New Phytol. 193, 165-177.

Anjum, N.A., Umar, S., Ahmad, A., Iqbaland, M., Khan, N.A., 2008. Sulphur protects mustard (*Brassica campestris* L.) from cadmium toxicity by improving leaf ascorbate and glutathione. Plant Growth Regul. 54, 271-279.

Anjum, S.A., Wang, L.C., Farooq, M., Hussain, M., Xue, L.L., Zou, C.M., 2011. Brassinolide application improves the drought tolerance in maize through modulation of enzymatic antioxidants and leaf gas exchange. J. Agro. Crop Sci. 197, 177-185.

ACCEPTED MANUSCRIPT

Arner, E.S., Holmgren, A., 2000. Physiological functions of thioredoxin and thioredoxin reductase. Eur. J. Biochem. 267, 6102-6109.

Azarakhsh, M.R., Asrar, Z., Mansouri, H., 2015. Effects of seed and vegetative stage cysteine treatments on oxidative stress response molecules and enzymes in *Ocimum basilicum* L. under cobalt stress. J. Soil Sci. Plant Nutr. 15, 651-662.

Bashri, G., Prasad, S.M., 2016. Exogenous IAA differentially affects growth, oxidative stress and antioxidants system in Cd stressed *Trigonella foenum-graecum* L. seedlings: Toxicity alleviation by up-regulation of ascorbate-glutathione cycle. Ecotoxicol. Environ. Saf. 132, 329-338.

Belkhadi, A., Hediji, H., Abbes, Z., Nouairi, I., Barhoumi, Z., Zarrouk, M., Chaïbi, W., Djebali, W., 2010. Effects of exogenous salicylic acid pre-treatment on cadmium toxicity and leaf lipid content in *Linumus itatissimum* L. Ecotoxicol. Environ. Saf. 73, 1004-1011.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Chaoui, A., Jarrar, B., El Ferjani, E., 2004. Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. J. Plant Physiol. 161, 1225-1234.

Chaoui, A., El Ferjani, E., 2014. Heavy metal-induced oxidative damage is reduced by β-estradiol application in lentil seedlings. Plant Growth Regul. 74, 1-9.

Choudhary, S.P., Oral, H.V., Bhardwaj, R., Yu, J.Q., Tran, L.S.P., 2012. Interaction of brassinosteroids and polyamines enhances copper stress tolerance in *Raphanus sativus*. J. Exp. Bot. 63, 5659-5675.

ACCEPTED MANUSCRIPT

Chwatko, G., Kuźniak, E., Kubalczyk, P., Borowczyk, K., Wyszczelska-Rokiel, M., Głowacki, R., 2014. Determination of cysteine and glutathione in cucumber leaves by HPLC with UV detection. Anal. Methods 6, 8039-8044.

Cuypers, A., Vangronsveld, J., Clijsters, H., 2000. Biphasic effect ofcopper on the ascorbate-glutathione pathway in primary leaves of *Phaseolus vulgaris* L. seedlings during the early stages of metal assimilation. Physiol. Plant. 110, 512-517.

Davie, K.J., 2001. Degradation of oxidized proteins by the 20S proteasome. Biochimie 83, 301-310.

Delalande, O., Desvaux, H., Godat, E., Valleix, A., Junot, C., Labarre, J., Boulard, Y., 2010. Cadmium–glutathione solution structures provide new insights into heavy metal detoxification. FEBS J 277, 5086–5096.

Dyballa, N., Metzger, S., 2009. Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. J. Vis. Exp. 30, e1431, doi:10.3791/1431.

Foyer, C.H., Halliwell, B., 1976. The presence of gluthathione and gluthathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133, 21-25.

Foyer, C., Noctor, G., 2005. Oxidant and antioxidant signaling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28, 1056-1071.

Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155, 2-18.

Gangwar, S., Singh, V.P., Prasad, S.M., Maurya, J.N., 2011. Differential responses of pea seedlings to indole acetic acid under manganese toxicity. Acta Physiol. Plant. 33, 451-462.

ACCEPTED MANUSCRIPT

Gao, Y., Miao, C., Xia, J., Luo, C., Mao, L., Zhou, P., Shi, W., 2012. Effect of citric acid on phytoextraction and antioxidative defense in *Solanum nigrum* L. as a hyperaccumulator under Cd and Pb combined pollution. Environ. Earth Sci. 65, 1923-1932.

Genisel, M., Turk, H., Erdal, S. 2013. Exogenous progesterone application protects chickpea seedlings against chilling-induced oxidative stress. Acta Physiol. Plant. 35, 241-251.

Gill, S.S., Anjum, N.A., Hasanuzzaman, M., Gill, R., Trivedi, D.K., Ahmad, I., Pereira, E., Tuteja, N., 2013. Glutathione and glutathione reductase: A boon in disguise for plant abiotic stress defense operations. Plant Physiol. Biochem. 70, 204-212.

Green, L.S., Yee, B.C., Buchanan, B.B., Kamide, K., Sanada, Y., Wada, K.,1991. Ferredoxin and ferredoxin-NADP reductase from photosynthetic and non photosynthetic tissues of tomato. Plant Physiol. 96, 1207-1213.

He, S., He, Z., Wu, Q., Wang, L., Zhang, X., 2015. Effects of GA_3 on plant physiological properties, extraction, subcellular distribution and chemical forms of Pb in *Lolium perenne*. Int. J. Phytoremediation 17, 1153–1159.

Hu, K.D., Hu, L.Y., Li, Y.H., Zhang, F.Q., Zhang, H., 2007. Protective roles of nitric oxide on germination and antioxidant metabolism in wheat seeds under copper stress. J. Plant Growth Regul. 53, 173-183.

Jacquot, J.P., Rivera-Madrid, R., Marinho, P., Kollarova, M., Le Maréchal, P., Miginiac-Maslow, M., Meyer Y., 1994. *Arabidopsis thaliana* NADPH thioredoxin reductase. J. Mol. Biol. 235, 1357-1363.

Janas, K.M., Zielińska-Tomaszewska, J., Rybaczek, D., Maszewski, J., Posmyk, M.M., Amarowicz, R., Kosińska, A., 2010. The impact of copper ions on growth, lipid peroxidation, and phenolic compound accumulation and localization in lentil (*Lens culinaris Medic*.). J. Plant Physiol. 167, 270-276.

ACCEPTED MANUSCRIPT

Karmous, I., Jaouani, K., Chaoui, A., El Ferjani, E., 2012. Proteolytic activities in *Phaseolus vulgaris* cotyledons under copper stress. Physiol. Mol. Biol. Plants 18, 337-343.

Katrusiak, A.E., Paterson, P.G., Kamencic, H., Shoker, A., Lyon, A.W., 2001. Pre-column derivatization high-performance liquid chromatographic method for determination of cysteine, cysteinyl–glycine, homocysteine and glutathione in plasma and cell extracts. J. Chromatogr. B. Biomed. Sci. Appl. 758, 207-212.

Kawashima, C.G., Noji, M., Nakamura, M., Ogra, Y., Suzuki, K.T., Saito, K., 2004. Heavy metal tolerance of transgenic tobacco plants over-expressing cysteine synthase. Biotechnol. Lett. 26, 153-157.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680-685.

Léon, A.M., Palma, J.M., Corpas, F.J., Comez, M., Romero-Puertas, M.C., Chatterjee, D., Mateos, R.M., Del Río, L.A., Sandalio, L.M., 2002.Antioxidative enzymes in cultivars of pepper plants with different sensitivity of cadmium. Plant Physiol. Biochem. 40, 813–820.

Lin, T.K., Hughes, G., Muratovska, A., Blaikie, F.H., Brookes, P.S., Darley-Usmar, V. Smith R.A., Murphy M.P., 2002. Specific modification of mitochondrial protein thiols in response to oxidative stress. A proteomics approach. J. Biol. Chem. 277, 17048-17056.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410.

Nagalakshmi, N., Prasad, M.N.V. 2001. Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci. 160, 291-299.

ACCEPTED MANUSCRIPT

Navrot, N., Collin, V., Gualberto, J., Gelhaye, E., Hirasawa, M., Rey, P., Knaff, D.B., Issakidis, E, Jacquot, J.P., Rouhier, N., 2006. Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol. 142, 1364-1379.

Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49, 249-279.

Piotrowska-Niczyporuk, A., Bajguz, A., Zambrzycka, E., Godlewska-Żyłkiewicz, B., 2012. Phytohormones as regulators of heavy metal biosorption and toxicity in green alga *Chlorella vulgaris* (Chlorophyceae). Plant Physiol. Biochem.52, 52–65.

Printz, B., Sergeant, K., Lutts, S., Guignard, C., Renaut, J., Hausman, J.F., 2013. 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. 12, 5160-5179.

Radyuk, M.S., Domanskaya, I.N., Shcherbakov, R.A., Shalygo, N.V., 2009. Effect of low above-zero temperature on the content of low-molecular antioxidants and activities of antioxidant enzymes in green barley leaves. Russ. J. Plant Physiol. 56, 175-180.

Rouhier, N., Stéphane, D.L., Jacquot, J.P., 2008. The role of glutathione in photosynthetic organisms, emerging functions for glutaredoxins and glutathionylation. Ann. Rev. Plant Biol. 59, 143-166.

Rouhier, N., Couturier, J., Johnson, M.K., Jacquot, J.P., 2010. Glutaredoxins: roles in iron homeostasis. Trends Biochem. Sci. 35, 43-52.

Russo, M., Sgherri, C., Izzo, R., Navari-Izzo, F., 2008. *Brassica napus* subjected to copper excess: Phospholipases C and D and glutathione system in signaling. Environ. Exp. Bot. 62, 238-246.

ACCEPTED MANUSCRIPT

Sakouhi, L., Rahoui, S., Ben Massoud, M., Munemasa, S., El Ferjani, E., Murata, Y., Chaoui, A., 2016. Calcium and EGTA alleviate cadmium toxicity in germinating chickpea seeds. J. Plant Growth Regul. 35(4), 1064-1073.

Sharaf, A.M., farghal, I.I., Sofy, M.R., 2009. Role of gibberellic acid in abolishing the detrimental effects of Cd and Pb on broad bean and lupin plants. Res. J. Agric. Biol. Sci. 5, 668-673.

Singh, S., Prasad, S.M., 2015. IAA alleviates Cd toxicity on growth, photosynthesis and oxidative damages in eggplant seedlings. Plant Growth Regul. 77, 87-98.

Song, Y., Zhou, L., Yang, S., Wang, C., Zhang, T., Wang, J., 2017. Dose-dependent sensitivity of *Arabidopsis thaliana* seedling root to copper is regulated by auxin homeostasis. Environ. Exp. Bot.139, 23-30.

Suzuki, Y.J., Carini, M., Butterfield, D.A., 2010. Protein carbonylation, Antioxid. Redox Signal. 12(3), 323-325.

Wang, Q., Nian, F., Zhao, L., Li, F., Yang, H., Yang, Y., 2013. Exogenous indole-3-acetic acid could reduce the accumulation of aluminum in root apex of wheat (*Triticum aestivum* L.) under Al stress. J. Soil Sci. Plant Nutr. 13, 534–543.

Wang, S.H., Yang, Z.M., Yang, H., Lu, B., Li, S.Q., Lu, Y.P., 2004. Copper-induced stress and antioxidative responses in roots of *Brasica juncea* L. Bot. Bull. Acad. Sin. 45, 203-212.

Wen, F., Zhang, Z.H., Bai, T., Xuand, Q., Pan, Y.H., 2010. Proteomics reveals the effects of gibberellic acid (GA_3) on salt-stressed rice (*Oryza sativa* L.) shoots. Plant Sci. 178, 170-175.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Yadav, S.K., 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S. Afr. J. Bot. 76, 167-179.

Zhu, X.F., Jiang, T., Wang, Z.W., Lei, G.J., Shi, Y.Z., Li, G.X., Zheng, S.J., 2012. Gibberellic acid alleviates cadmium toxicity by reducing nitric oxide accumulation and expression of IRT1 in *Arabidopsis thaliana*. J. Hazard. Mater. 239-240, 302-307.

Zhu, X.F., Wang, Z.W., Dong, F., Lei, G.J., Shi, Y.Z., Li, G.X., Zheng, S.J., 2013. Exogenous auxin alleviates cadmium toxicity in *Arabidopsis thaliana* by stimulating synthesis of hemicellulose 1 and increasing the cadmium fixation capacity of root cell walls. J. Hazard. Mater. 263(2), 398-403.

ACCEPTED MANUSCRIPT

Figures list

ACCEPTED MANUSCRIPT

Fig. 1. Activities of Trx (**A**), Fdx (**B**), NTR (**C**) and FNR (**D**) in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3". Values ± SE (n=5) followed by a common letter for the same organ are not different at 0.05 level of significance, using ANOVA followed by Tukey's test.

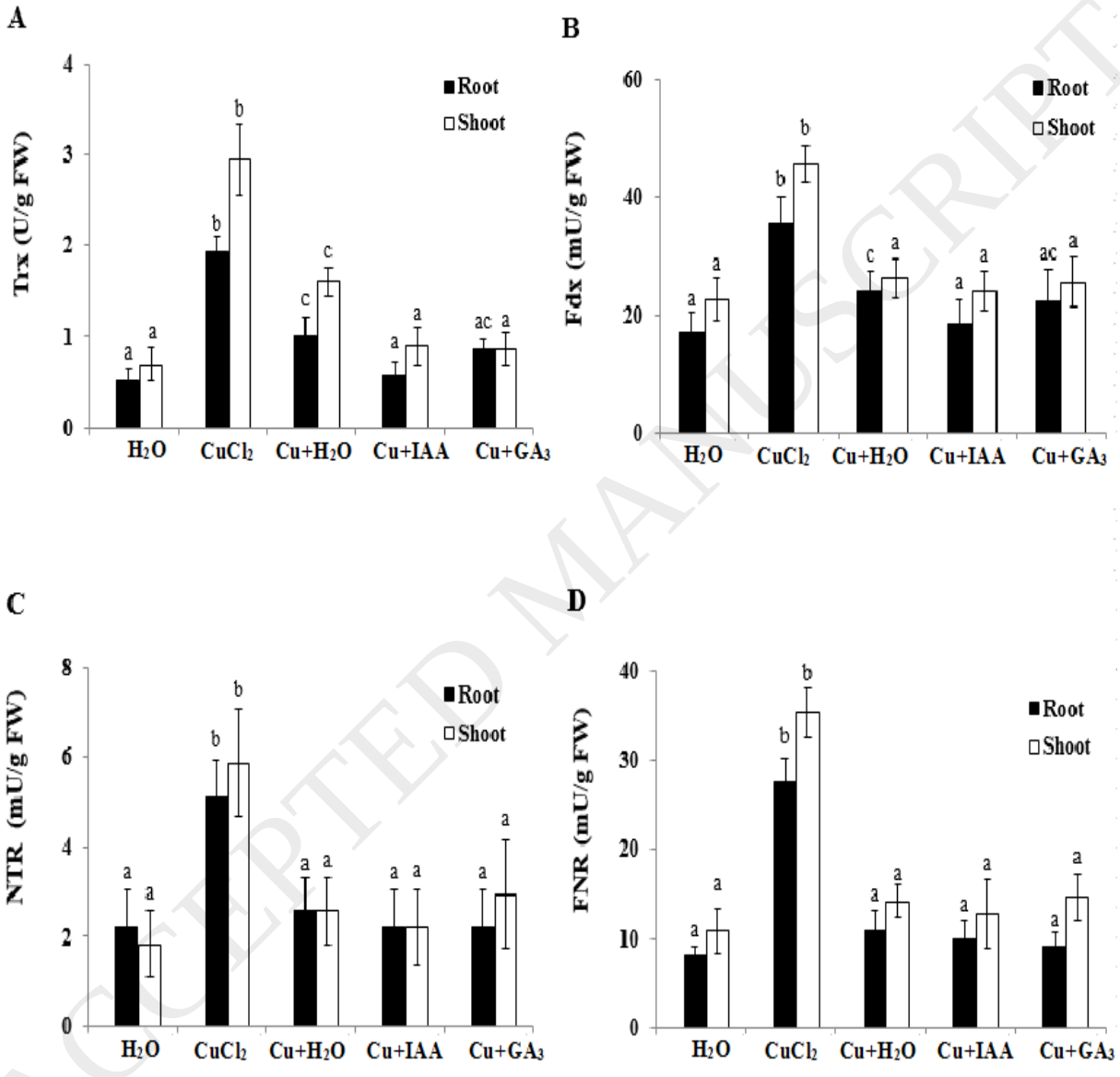
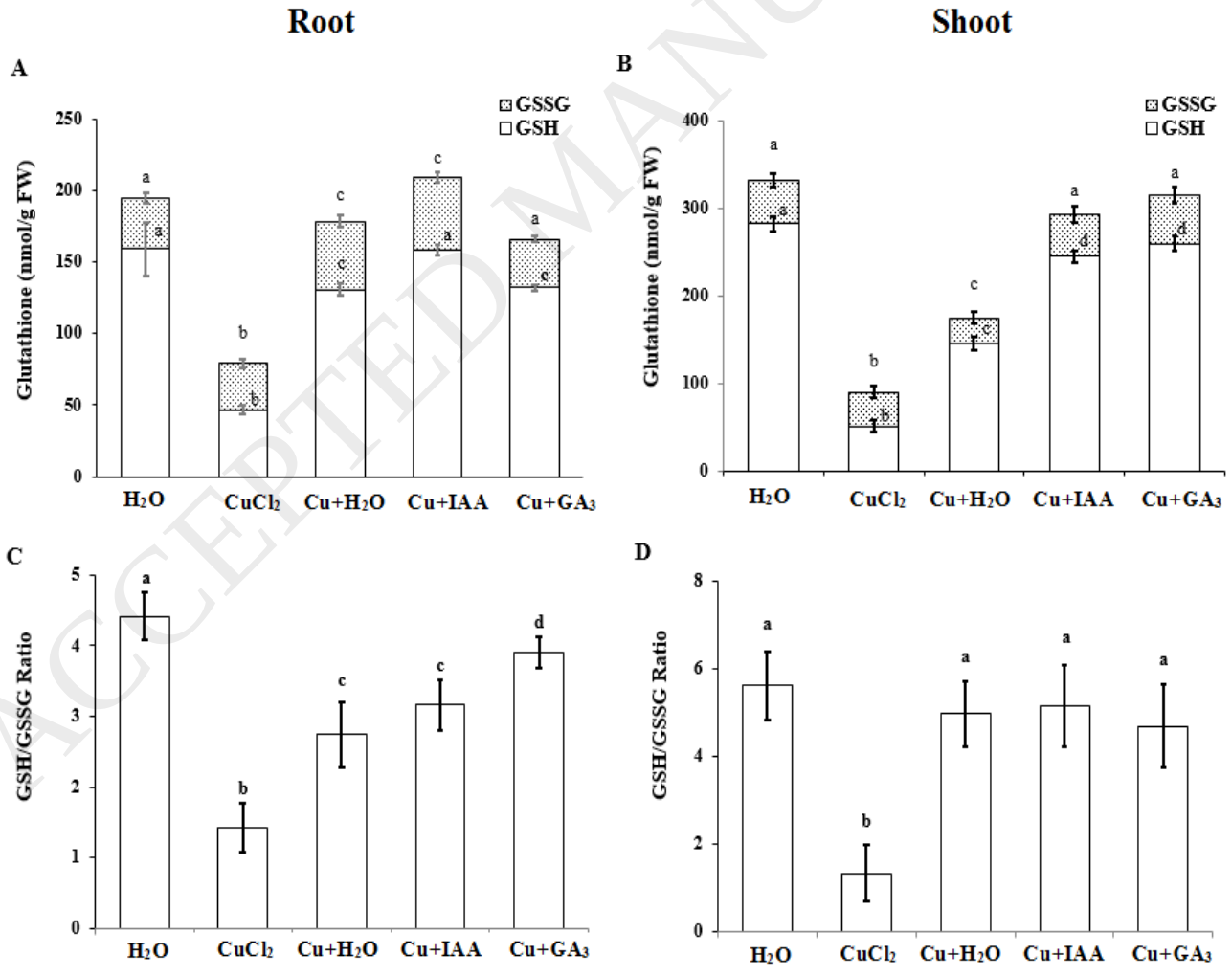


Figure 1

ACCEPTED MANUSCRIPT

Fig. 2. Contents of reduced (GSH) and oxidized (GSSG) glutathione (**A, B**) and redox ratio (GSH/GSSG) (**C**,**D**) in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3". Values ± SE (n=5) followed by a common letter for the same parameter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test.



ACCEPTED MANUSCRIPT

Figure 2

Fig. 3. Activities of GPX (**A)** and GR (**B)** in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3". Values ± SE (n=5) followed by a common letter for the same organ are not different at 0.05 level of significance, using ANOVA followed by Tukey's test.

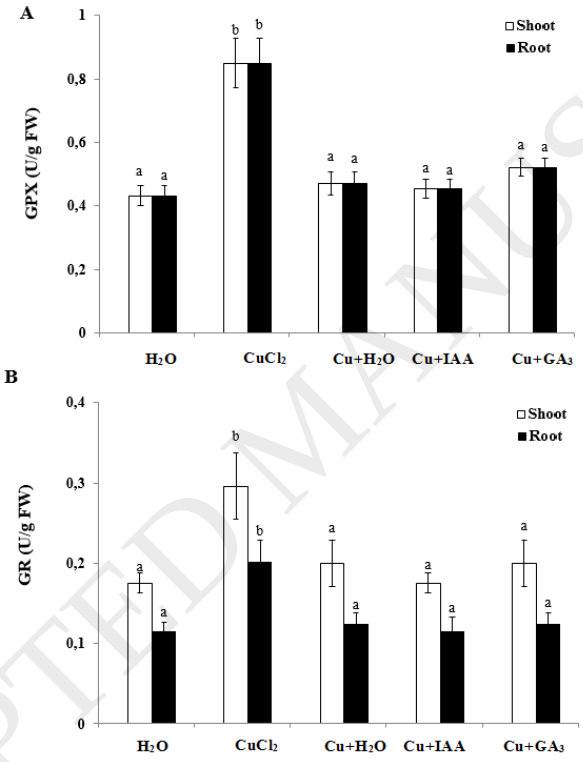


Figure 3

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Fig. 4. Contents of reduced (CSH) and oxidized (CSS) cysteine (**A, B**) and redox ratio (CSH/CSS) (**C**,**D**) in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3".Values ± SE (n=5) followed by a common letter for the same parameter are not different at 0.05 level of significance, using ANOVA followed by Tukey's test.

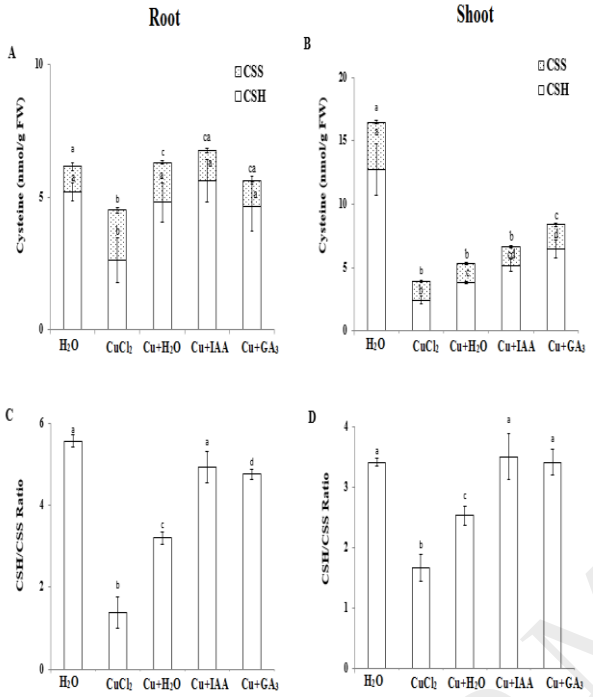


Figure 4

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Fig. 5. Representative images of 1DE gels **(A, B, D, E)** of proteins (80 µg) in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$, and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3". Gels were stained with IAF labeling (scanned with Typhoon 9400 scanner) **(A, D)** and with Coomassie G-250 (CBB) (scanned with GS-800 calibrated densitometer) **(B, E)**. Levels of protein thiols in root (**C)** and shoot **(F**). Quantity one image analysis software was used to analyze protein-associated fluorescence intensity (arbitrary units, AU) by determining IAF/CBB ratio. Values shown are means of 3 individual measurements (±SE). Values ± SE followed by a common letter are not different at 0.05 level of significance using ANOVA test followed by the Student's *t*-test.

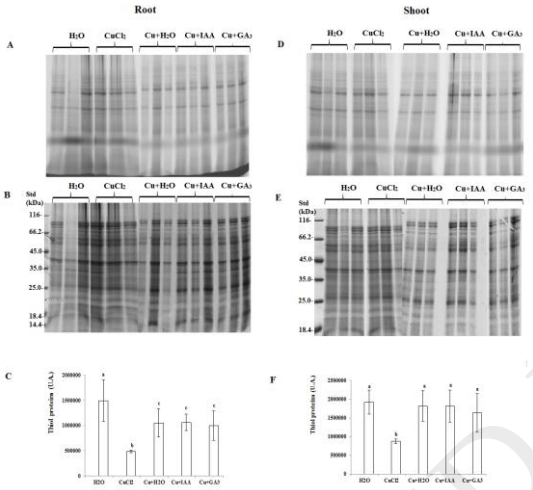


Figure 5

ACCEPTED MANUSCRIPT

Fig. 6. Representative images of 1DE gels **(A, B, D, E)** of proteins (80 µg) in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$, and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3". Gels were stained with FTSC labeling (scanned with Typhoon 9400 scanner) **(A, D)** and with Coomassie G-250 (CBB) (scanned with GS-800 calibrated densitometer) **(B, E)**. Levels of protein carbonyls in root (**C)** and shoot **(F**). Quantity one image analysis software was used to analyze protein-associated fluorescence intensity (arbitrary units, AU) by determining (FTSC/CBB). Values shown are means of 3 individual measurements (±SE). Values ± SE followed by a common letter are not different at 0.05 level of significance, using ANOVA test followed by Student's t test.

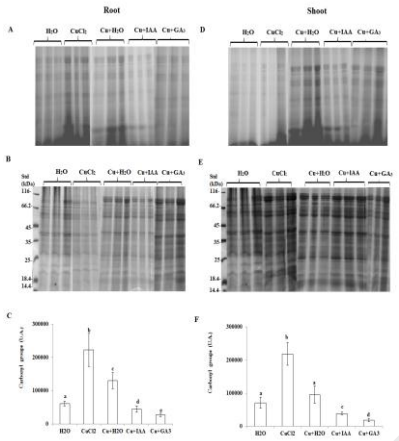


Figure 6

ACCEPTED MANUSCRIPT

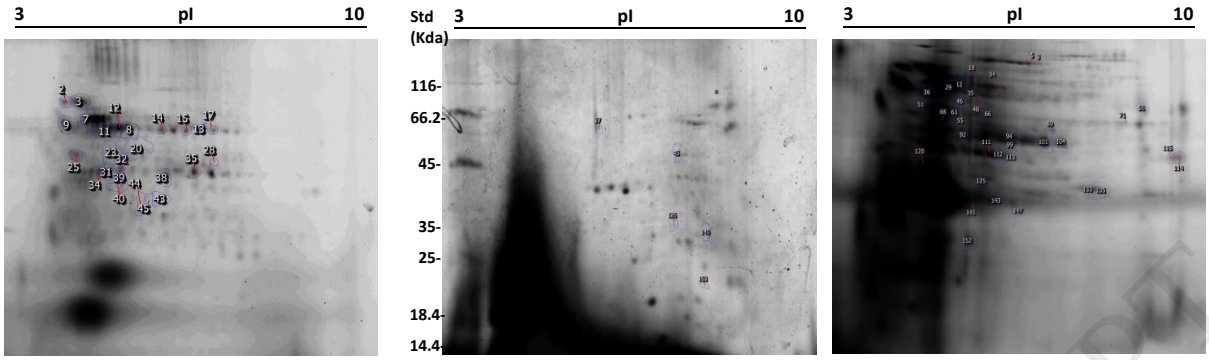
ACCEPTED MANUSCRIPT

Fig. 7. Profiles of proteins containing thiol and carbonyl groups in pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3". Proteins (150 µg) were labeled with IAF or FTSC and separated by 2D SDS-PAGE. Images show spots of interest in representative gels from colloidal Coomassie Brilliant G-250 (CBB) staining (scanned with GS-800 calibrated densitometer) and IAF or FTSC labeling (scanned with Typhoon 9400 scanner; 800 PMT). Numbers correspond to identified spots whose change>1.5 fold with $p<0.05$ (see summary of significant quantitative changes in Table 1).

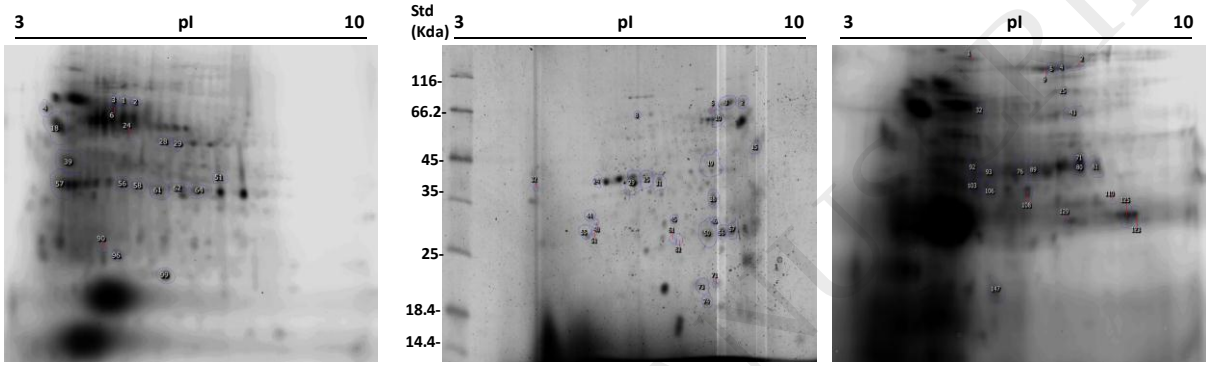
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

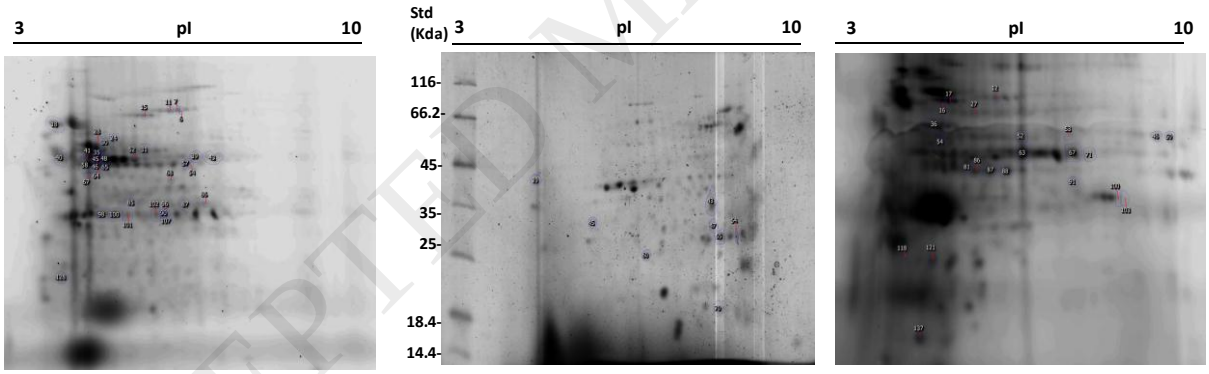
Root



H_2O / Cu



Cu / Cu+ IAA



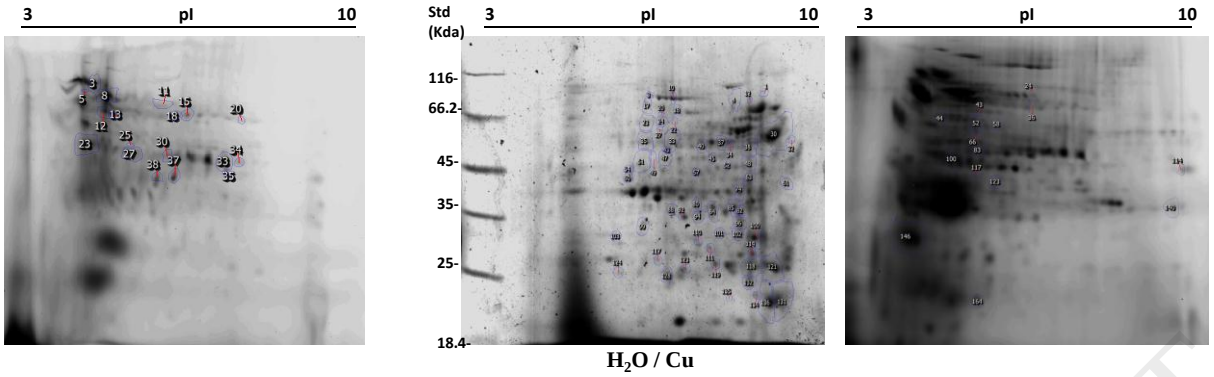
Cu / Cu+GA_3

FLUORESCENCE (IAF) **COOMASSIE (CBB)** **FLUORESCENCE (FTSC)**

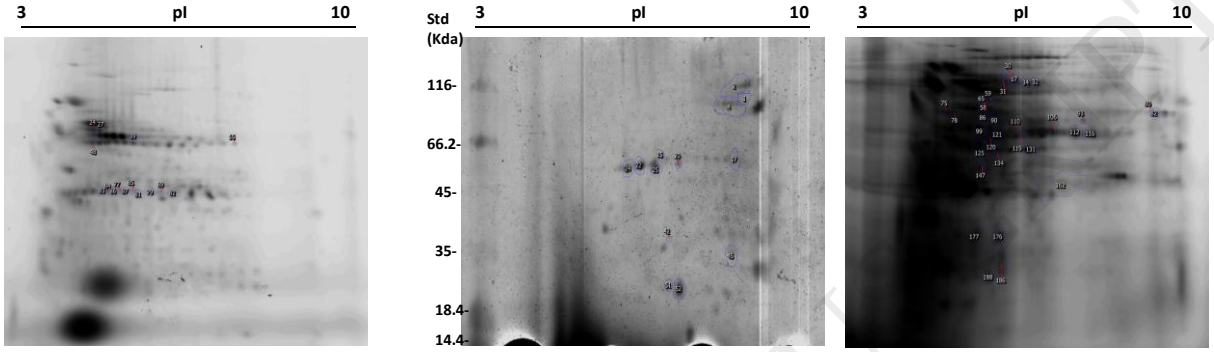
(SH revelation) **(CO revelation)**

Shoot

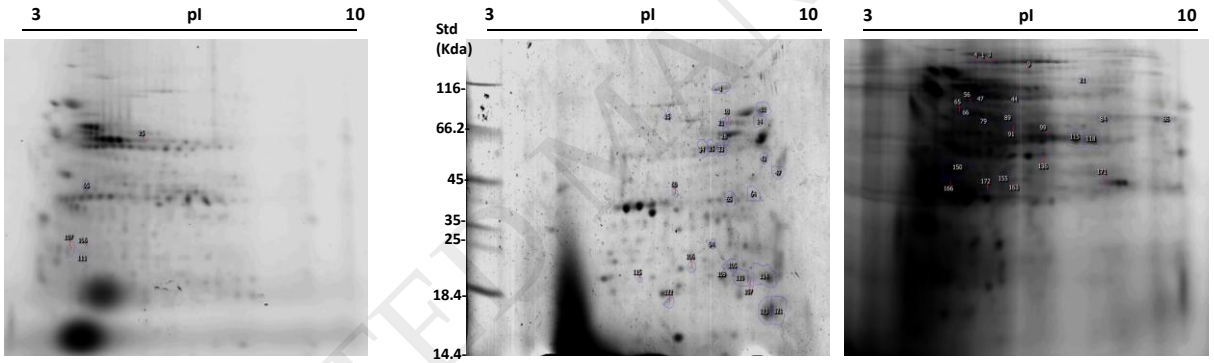
ACCEPTED MANUSCRIPT



H_2O / Cu



Cu / Cu+ IAA



Cu / Cu+GA_3

FLUORESCENCE (IAF)

(SH revelation)

COOMASSIE (CBB)

FLUORESCENCE (FTSC)

(CO revelation)

ACCEPTED MANUSCRIPT

Table 1

Summary of quantitative changes of proteins containing thiol groups (IAF labeling) and carbonyl groups (FTSC labeling) in roots and shoots of pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3" (data from Fig. 7).

Treatment	H_2O/Cu		Cu/Cu+IAA		Cu/Cu+GA_3	
Revelation	IAF (SH)	FTSC (CO)	IAF (SH)	FTSC (CO)	IAF (SH)	FTSC (CO)
	Number of spots modified > 1.5 fold with $p < 0.05$					
Root	25	37	20	23	36	22
Shoot	18	15	15	32	5	25

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 2

Root and shoot copper content of pea seedlings germinated for 3 days in the presence of distilled water (H_2O) or 200 µM $CuCl_2$ and then replaced up to 6 days either by H_2O or 200 µM $CuCl_2$ added combined with 1 µM IAA "Cu+IAA" or 1 µM GA_3 "Cu+GA_3"**.** Values are the averages of four individual measurements. Values ± SE (n=5) followed by a common letter in the same line are not different at the 0.05 level of significance, using ANOVA followed by Tukey's test.

Treatment	Cu (µg/g DW)	
	Root	Shoot
H_2O (Control)	45.9±12.2**a**	22.7±3.5**a**
Cu	88.3±8.5**b**	67.3±10.3**b**
H_2O+Cu	51.5±13.6**a**	41,3±3.6**c**
Cu+IAA	61.8±5.2**a**	37.5±7.41**c**
Cu+GA_3	60.2±7.4**a**	46.5±9.7**c**

ACCEPTED MANUSCRIPT