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Copper microlocalisation and changes in leaf morphology, chloroplast ultrastructure and antioxidative response in white lupin and soybean grown in copper excess

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Abstract The microlocalisation of Cu was examined in the leaves of white lupin and soybean grown hydroponically in the presence of 1.6 or 192 μM Cu, along with its effect on leaf morphology, (ultra)structure and the antioxidative response. The 192 μM dose led to a reduction in the total leaf area and leaf thickness in both species, although more strongly so in white lupin. In the latter species it was also associated with smaller spongy parenchyma cells, and smaller spaces between them, while in the soybean it more strongly reduced the size of the palisade parenchyma and epidermal cells. Energy-dispersive X-ray microanalysis showed that under Cu excess the metal was mainly localised inside the spongy parenchyma cells of the white lupin leaves, and in the lower epidermis cell walls in those of the soybean. Cu excess also promoted ultrastructural chloroplast alterations, reducing the photosynthetic capacity index and the green area of the leaves, especially in the soybean. Despite this, the soybean appeared to be more tolerant to Cu excess than the white lupin, perhaps because i) soybean accumulates

smaller amounts of Cu in the leaves, ii) of the microlocalisation of Cu in the cell walls iii) and because of greater induced thiol, superoxide dismutase and catalase activities investing it with a better antioxidative response.

Keywords Antioxidative defence • Copper excess • Energy-dispersive X-ray microanalysis • *Glycine max* L. • Leaf ultrastructure • *Lupinus albus* L.

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Introduction

High copper concentrations have been recorded in some natural soils, although Cu toxicity is more commonly a problem of those polluted by Cu-rich pig and poultry slurries, fertilizers and fungicides, industrial and urban activities, metal mining and processing, and waste disposal (Yruela 2009). While Cu is an essential element for plant growth and development, it is highly toxic to plants at concentrations of > 20 μg g^{-1} dry weight (DW) (Marschner 1995). In general, Cu excess inhibits seed germination and plant growth by interfering with respiration, nitrogen and protein metabolism, and photosynthesis (Yruela 2009), and by causing the overproduction of reactive oxygen species (ROS). Some plants posses mechanisms - enzymatic or non-enzymatic scavenging systems – whose function it is to prevent the oxidative damage caused by ROS (Sharma and Dietz 2008).

The indirect consequence of changes in metabolism and/or signal transduction caused by toxic metals, as well as the direct interaction of the latter with structural components, can cause damage at the cellular, tissular and organ levels in plants (Barceló and Poschenrieder 2004). The visible symptoms of toxicity include structural/ultrastructural abnormalities, leaf chlorosis and necrosis, stem shortening, browning, and altered root morphology (Marschner 1995). The actual problems encountered are dependent on the species and ecotype in question, the concentration of the toxic metal present, the exposure time, and the properties of the soil (Yruela 2009). Certainly, marked differences in Cu tolerance have been observed in different plants. While *Arabidopsis halleri* (Yruela. 2009), *Elsholtzia haichowensis* (Lou et al. 2004), *Elsholtzia splendens* (Shi et al. 2004), *Phragmites australisrice* (Ali et al. 2002), *Silene*

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3 *vulgaris* and *Thalspi caerulescens* (Yruela. 2009) can tolerate excess Cu to some extent,
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5 *Oryza sativa* (Jones 1998) and *Zea Mays* are much more sensitive (Ali et al. 2002).
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7 White lupin (*Lupinus albus* L.) is a temperate grain legume of great agronomic
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10 potential given its high seed protein content and positive effect on soil fertility. The
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12 ability of white lupin to survive in soils of low pH and low nutrient availability
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14 (Fernández-Pascual et al. 2007), and the species' intrinsic biomass production and
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16 relative tolerance to trace elements such as As, Hg and Cd (Vázquez et al. 2009;
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19 Esteban et al. 2008; Zornoza et al. 2002), suggest it to be a suitable species for use in
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21 the remediation of contaminated soils. Soybean (*Glycine max* L.), however, is the most
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23 economically important of all grain legumes. In some countries it is a major protein
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25 source, and is sometimes grown on As- and Cu-contaminated soils (e.g., in China). It is
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28 also used as a model system for legume–*Rhizobium* research (Reichman 2007).
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30 There are few studies that relate Cu microlocalisation to plant tolerance of this
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32 metal. However, the pattern of Cu microlocalisation in cells and tissues may provide
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34 insights into tolerance mechanisms, and might help explain differences in tolerance
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37 between species. To test this hypothesis, the microlocalisation of Cu was studied in the
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39 leaves of white lupin and soybean by energy-dispersive X-ray microanalysis. The
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41 accompanying (ultra)structural, morphological and physiological alterations, as well as
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43 the enzymatic (APX, CAT and SOD) and non-enzymatic (MDA and –SH) antioxidant
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46 response of the plants, were also examined.
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50 **Materials and methods**
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54 Growth conditions and Cu treatments
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White lupin cv. Marta and soybean cv. Williams seeds were surface-sterilised in 10% v/v sodium hypochlorite for 15 min, rinsed thoroughly with deionised water and germinated on water-moistened filter paper in the dark at 28ºC for 3 days. The seedlings obtained were placed in plastic Riviera pots (three seedlings to each pot) containing 2 L of perlite in the upper compartment and 0.75 L of nutrient solution in the lower. The composition of the nutrient solution, the inoculation of the plants with *Bradyrhizobium,* and the plant growth conditions were similar to those reported in earlier work (Sánchez-Pardo et al. 2012). These young plants were grown in a controlled environment chamber under the following night/day conditions: temperature 20/25°C, photoperiod 11/13 h, and relative humidity 60/40%. The photon flux density during the light periods was 520 µmol m^{-2} s^{-1}. Ten days after sowing, the plants were subjected to one of two Cu treatments (1.6 or 192 µM $CuSO_4.5H_2O$) with four replicates, following a randomised block design. The very high 192 µM dose was chosen to ensure that sufficient quantities of Cu would accumulate and be detectable in the different leaf compartments.

After 35 days the plants were divided into leaves, stems and roots, and weighed. They were then washed thoroughly with tap water three times, and then again with deionised water three times. One gram (fresh weight [FW]) of total homogenised leaves per plant were frozen in liquid N_2 and stored at -76°C until analysis. The remaining leaf material was dried at 80°C for 3 days until a constant dry weight (DW) was reached. These dry samples were homogenised and used for element determination.

Copper and Fe concentrations

The concentration of Cu and Fe in leaves was determined by digesting 20 mg DW of homogenised samples with a mixture of HNO_3:H_2O_2:H_2O (3:2:10, v:v:v) for 30 min at 125ºC under a pressure of 1.5 kPa (Lozano-Rodríguez et al.1995). Cu and Fe

concentrations were then determined by atomic absorption spectrophotometry (Perkin-Elmer Analyst 800).

Electron microscopy and energy-dispersive X-ray microanalysis

Small pieces (1 mm^2) of fresh leaf from the 1.6 and 192 μM Cu-treated plants, selected at comparable stages of development, were mounted with adhesive (Gurr®, OCT, BDH, Poole, UK) on aluminium stubs. They were then cryofixed in slush nitrogen (-196ºC) and cryotransferred to a vacuum chamber at -180ºC to be fractured using a cooled stainless steel spike. After placing them in a Zeiss DSM 960 digital scanning electron microscope (Oberkochen, Germany) the samples were subjected to superficial etching under vacuum (-90ºC, 120 s, 2 kV) and gold coated. Fractured leaf material was observed at low temperature employing secondary and back-scattered electrons. Energy-dispersive X-ray microanalysis (EDXMA) was performed in conjunction with low temperature scanning electron microscopy (LTSEM) using a Pentaflet apparatus (Pentaflet, Oxford, UK) at a resolution of 133 eV. Only smooth surfaces were taken for microanalysis, following the recommendations of Hess (1980). Semi-quantitative element analysis was performed using standard ZAF (atomic number, absorption and fluorescence) correction procedures employing Link Isis 3.2 software (Link Isis, Oxford, UK).

Light and electron microscopy

Small pieces of fresh leaf (1 mm^2) from the 1.6 and 192 μM Cu-treated plants, selected at comparable stages of development, were fixed in 2.5% (v/v) glutaraldehyde in 50 mM Na-cacodylate buffer containing sucrose (Fedorova et al. 2005), pH 7.4, and vacuum-infiltrated before dehydrating through a graded ethanol series. They were then

embedded in LR White Resin (London Resin, London, UK) in gelatine capsules, according to de Lorenzo et al. (1998). Polymerisation was allowed to occur for 24 h at 60°C. Sections 1 μm-thick were prepared for light microscopy, and 70 nm-thick for electron microscopy, using a Reicher Ultracut S ultramicrotome fitted with a diamond knife. The semithick sections were stained with 1% (w/v) toluidine blue in aqueous sodium borate for direct examination using a Zeiss Axiophot photomicroscope. The ultrathin sections were post-stained with lead citrate and examined using a STEM LEO 910 electron microscope at an accelerating voltage of 80 kV.

Stress indicators and antioxidant enzymes

The concentrations of malondialdehyde (MDA) (a cytotoxic product of lipid peroxidation normally considered the major 2-thiobarbituric acid-reacting compound) and total thiols (-SH) were examined in samples of leaves homogenised to a fine powder in liquid N_2 using an ice-cooled mortar and pestle. They were then assayed as described by Esteban et al. (2008).

Antioxidant enzyme activity was determined by homogenising 100 mg FW of leaves in 1.5 mL of ice-cooled phosphate buffer (50 mM, pH 7.0, containing 1 mM ethylenediamine tetra-acetic acid (EDTA) and 1% w/v insoluble polyvinylpyrrolidone) and passing the solution through four layers of cheese cloth. The extract obtained was centrifuged at 15,000 × *g* for 15 min at 4ºC. The supernatant was used to measure the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). The protein content of the supernatant was measured according to Lowry et al. (1951).

SOD (EC 1.1.5.1.1) activity was assayed using the method of Srivastava et al. (2006), measuring its ability to inhibit the photochemical reduction of nitro-blue

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3 tetrazolium (NBT). The 3 mL reaction mixture contained 40 mM phosphate buffer (pH
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5 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and a suitable
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7 aliquot of enzyme extract. The test tubes were shaken and placed 30 cm below a 15 W
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9 fluorescent lamp. Absorbance was then measured at 560 nm. The activity of SOD was
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11 expressed as units mg^{-1} protein. One unit of activity was defined as the amount of
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13 protein required to reduce NBT under light to 50% of the initial concentration.
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16 APX (EC 1.11.1.11) activity was measured by estimating the rate of ascorbate
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18 oxidation (extinction coefficient 2.8 mM^{-1} cm^{-1}). The 3 mL reaction mixture contained
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20 50 mM phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM sodium ascorbate, 0.1 mM
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22 EDTA and a suitable aliquot of enzyme extract. The change in absorbance was
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24 monitored at 290 nm (Srivastava et al. 2006) and the enzyme activity expressed as units
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26 mg^{-1} protein.
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29 CAT (EC 1.11.1.6) activity was assayed by measuring the decomposition of
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31 H_2O_2. Enzyme extract (100 μL) was added to the reaction mixture containing 1 mL
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33 phosphate buffer solution (50 mM, pH 7.0) and 0.1% H_2O_2. The reduction in the
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35 absorbance at 240 nm was then recorded and the enzyme activity calculated using an
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37 extinction coefficient of 0.04 mM^{-1} cm^{-1}. One unit of CAT activity was defined as the
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39 amount required to decompose 1 μmol of H_2O_2 min^{-1} mg^{-1} protein under the assay
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41 conditions (Chen et al. 2009).
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45 Morphological and physiological leaf variables, and statistical analyses
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49 The calculation of leaf thickness was performed using ImageJ 1.45 software. Leaf area
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51 (LA), the photosynthetic capacity index (PCI) and the green, yellow and red leaf areas
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53 were calculated using optically scanned leaf samples and Foliárea software (Muñoz-
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55 Guerra 2002).
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3 The data presented are the means ± standard errors (S.E.) of four independent
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5 replicates. To ensure that the assumptions for statistical analysis were fulfilled, the
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7 equality of variances and the normality of the data were tested. Differences between
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9 means for each variable were tested for significance by one-way ANOVA. Means were
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11 compared using the least significant difference test. Significance was set at $P<0.05$. All
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13 calculations were performed using IBM SPSS v.19.0 software.
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18 Results
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23 Total Cu and Fe concentrations and Cu microlocalisation in leaves
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27 Table 1 shows the leaf Cu and Fe concentrations for the white lupin and soybean plants
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29 grown under the 1.6 and 192 µM Cu conditions. The concentration of Cu in the leaves
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31 of the white lupin and soybean 192 µM Cu-treated plants was 13.5 and 9.8 times that
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33 recorded in the control (1.6 µM plants) respectively. In contrast, the 192 µM Cu
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35 treatment significantly reduced the leaf concentration of Fe – by 91% in white lupin and
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37 63% in soybean.
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40
41 Figure 1 shows the EDXMA results for the leaves of white lupin and soybean
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43 plants exposed to the 1.6 and 192 µM Cu treatments. In white lupin, the latter treatment
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45 increased the Cu signal from the cytoplasm-vacuole of the spongy parenchyma cells to
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47 10 times that of the control 1.6 µM Cu plants. The signal from the cell walls of the
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49 lower epidermal cells increased 1.6 times. In the same species, a decreasing Cu gradient
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51 was observed between the cell walls of the lower epidermal cells to those of the upper
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53 epidermis (Fig. 1c).
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In the soybean leaves, exposure to the 192 μM Cu conditions increased the Cu signal from the cytoplasm-vacuole of the lower epidermal cells to 1.4 times that of the controls. In the cell walls of the palisade parenchyma the signal increased to 1.6 times that recorded in the controls, while in the lower epidermal cells it increased 3.7 times. In contrast, reductions were seen in the Cu signal from the cytoplasm-vacuole of the palisade parenchyma cells (28%), as well as from the walls of the upper epidermal cells (23%). No changes were seen in other tissues (Figs. 1b, d). The leaves of plants subjected to the 1.6 μM Cu treatment showed no clear pattern of Cu distribution, while those treated with 192 μM dose showed a decreasing gradient from the lower epidermal cells towards the cell walls of the upper epidermal cells (Figs. 1b, d).

Leaf variables

Table 2 shows the leaf biomass, LA, LT, number of leaves (LN), PCI, and the green, yellow and red leaf areas for white lupin and soybean plants grown under the 1.6 and 192 μM Cu conditions. The high dose reduced the leaf biomass by 77% in white lupin and 69% in soybean. In white lupin plants exposed to the 192 μM Cu treatment, the LA, LT, LN and PCI were reduced by 68%, 29%, 47% and 36% respectively with respect to the controls, while the yellow leaf area increased significantly (10 times). However, no differences were seen between the treatments in terms of green and red leaf area. In the leaves of the 192 μM Cu-treated soybean plants, the LA, LT and LN, the PCI and the green leaf area were reduced significantly by 64%, 27%, 29%, 77% and 29% respectively compared to the controls. In contrast, the high Cu dose increased the yellow and red leaf areas 11 and 7.5 times respectively.

Leaf and chloroplast (ultra)structure

Figures 2 and 3 show the effects of the 1.6 and 192 μM Cu treatments on the structure of the white lupin and soybean leaves, as determined by light microscopy. The leaves of the white lupin plants showed a well-organised structure after both treatments (Fig. 2a, b, c). However, the 192 μM treatment leaves were thinner, and the mesophyll cells and intercellular spaces were smaller than in the controls; this was more apparent in the spongy parenchyma than the palisade parenchyma (Fig. 2b). Further, the spongy and palisade parenchyma cells of the 192 μM Cu-treated plants showed a reduction in the number and size of chloroplasts, and of large starch granules (Fig. 2d). In some cells of the palisade parenchyma, the chloroplasts appeared more distant from the cell wall, a likely consequence of the separation of the latter from the plasma membrane (Figs. 2e, f). In soybean, the leaves of the 192 μM Cu-treated plants were slightly thinner and the palisade parenchyma cells and abaxial and adaxial epidermal cells were smaller (Figs. 3a, b). The spongy parenchyma cells were not affected. The packing of both types of mesophyll cell also remained unaffected (Figs. 3b, c, d).

Figures 4 and 5 show the effects of the 192 μM treatment on the ultrastructure of the white lupin and soybean leaves. Figure 4a shows the ultrastructure of a chloroplast from the leaf of a control white lupin plant; note the parallel arrangement of the grana and intergrana with respect to the chloroplast axis. White lupin plants treated with 192 μM Cu showed three types of chloroplast in their leaves: 1) approximately a 40% had a single starch granule, an unaffected thylakoid structure, but a very electron-dense stroma; such chloroplasts were slightly distant from the cell wall (Fig. 4b); 2) those with more than two starch granules and showing incipient changes in the ultrastructure of the grana and intergrana, plus a loss of their normal parallel alignment with the main axis

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3 (40%); these also showed a highly electron-dense stroma, a separation of chloroplast membrane (indicated by arrow) and a greater distance between the chloroplast membrane and cell wall (Fig. 4c); and 3) chloroplasts completely filled with starch, with a totally disorganized thylakoid structure, and showing clear signs of degradation (major deteriorations in the mitochondria [Fig. 4d] and cytoplasmic senescence vesicles [Fig. 4c, arrowheads] were also seen in lesser extent [20%]).

The mesophyll cells of the 192 μM Cu-treated soybean plants also showed an increase in the number of starch granules (between 2-7, mean = 5). In addition, they showed bulging of the chloroplast membrane and a loss of the parallel orientation of the grana and intergrana with respect to the chloroplast axis (Fig. 5b). The number of grana was reduced, as was the number of thylakoids per granum. Some thylakoids became swollen, leading to the appearance of plastoglobuli (Fig. 5c). Many were seen in the interior of the chloroplasts; these sign signs of early senescence are characteristic of degraded tissue (Fig. 5c, d).

Stress indicators and antioxidant enzymes

Table 3 shows the MDA and total SH contents, as well as the SOD, APX and CAT activities of the leaves of white lupin and soybean plants exposed to the 1.6 and 192 μM Cu treatments. The total SH content of the leaves of the white lupin plants grown under the 192 μM Cu conditions did not vary with respect to the 1.6 μM Cu treatment. However, in soybean, it was 1.6 times that recorded in the corresponding control plants. The concentrations of MDA in the leaves of both types of plant grown under the 192 μM Cu conditions were higher than in the controls: 2.2 times in the white lupin plants and 2.9 times in the soybean plants.

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3 Exposure to the 192 µM Cu conditions reduced the activities of SOD and CAT
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5 by 35% and 45% in the white lupin leaves. However, no differences were seen between
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7 the treatments in terms of APX activity. The activities of SOD and CAT in the leaves of
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9 the 192 µM Cu-treated soybean plants increased to 0.9 and 2.5 times those of the
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11 control plants, while the activity of APX was significantly reduced.
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19 **Discussion**
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21 In many sensitive species, Cu excess inhibits plant growth when leaf concentrations
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23 reach 15-20 mg Cu kg^{-1} DW. However, for most plants, Cu toxicity symptoms appear
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25 when leaf concentrations reach around 30 mg Cu kg^{-1} DW (Marschner 1995). In the
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27 present study, the concentration of Cu in the leaves increased in plants subjected to the
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29 high Cu dose, with the white lupin leaves accumulating about twice that of the soybean
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31 (Table 1). Indeed, the total Cu concentrations found in the leaves of both species fell
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33 within the toxicity range (Reuter and Robinson 1997).
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36 The accumulation of high Cu levels in leaf tissues can cause morphological and
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38 structural disorders, affect many physiological processes, inhibit growth, and sometimes
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40 even hasten the death of a plant (Marschner 1995); it is well known that Cu excess can
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42 inhibit cell elongation and cell division (Panou-Filotheou and Bosabalidis 2004). The
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44 cells of the leaves of bean plants exposed to toxic concentrations of Cu are reported to
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46 be smaller (Kasim 2005). This has been attributed to a reduction in the elasticity of the
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48 cell walls caused by the irreversible inhibition of proteins that regulate this feature
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50 (Kasim 2005). In the present study, reductions in the thickness of soybean and white
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52 lupin leaves were observed under Cu excess. In the white lupin, this reduction seems to
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54 be promoted by a decline in the size of the mesophyll cells (mainly of the spongy
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3 parenchyma) and the spaces between them (Fig. 2). In soybean, the epidermis and
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5 palisade parenchyma cells showed reduced volumes (Fig. 3). The opposite effect has
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7 been observed in oregano leaves grown under Cu excess, due to an increase in the
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9 number of mesophyll cells and their volume (Panou-Filotheou et al. 2001). In turn,
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11 excess Cu adversely affected the LA and LN in both crops studied, although the
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13 reduction was most pronounced in white lupin (Table 2). Elevated levels of Cu have
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15 been associated with similar outcomes in cucumber (Alaoui-Sossé et al. 2004), oregano
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17 (Panou-Filotheou et al. 2001) and wheat (Cook et al. 1997).
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20 In the present work, the leaf tissues most affected were those that accumulated
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22 larger amounts of Cu. In white lupin, the excess Cu was mainly localised in the
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24 cytoplasm-vacuole of the spongy parenchyma cells, whereas in the soybean leaves it
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26 was mainly located in the cell walls of the leaf abaxial epidermal cells (Fig. 3). Other
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28 authors have found Cu to be mainly localised in the upper epidermis and trichomes of
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30 the abaxial epidermis in *Cannabis sativa* leaves (Arru et al. 2004), and in the vascular
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32 tissues of *Avicennia marina* (MacFarlane and Burchett 2000) and *Elsholtzia splendes*
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34 (Shi et al. 2004). The microlocalisation of heavy metals in cells and tissues provides
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36 insight into the possible mechanisms of detoxification, and therefore of tolerance: the
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38 outermost tissues and cell walls would appear to act as a barrier against its harmful
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40 effects. The preferential accumulation of heavy metals in the epidermis would help to
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42 protect mesophyll cells from the buildup and toxicity of metals and maintain the
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44 functionality of mesophyll cells over a wide range of metal concentrations in the leaves
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46 (Küpper et al. 1999).
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49 Alterations in chloroplast structure have been reported in some plants under Cu
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51 excess, such as rice (Lidon and Henriques 1993), bean (Maksymiec et al. 1994), wheat
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53 (Quartacci et al. 2000) and oregano (Panou-Filotheou et al. 2001). In the present work,
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both white lupin and soybean showed changes in the structure of the thylakoids, detachment and/or loss of integrity of the chloroplast membrane, and a degradation of grana stacking and the stroma (Figs. 4, 5). A swelling of the thylakoids and an increase in the number of plastoglobuli were also observed in the soybean plants (Fig. 5). It seems that soybean chloroplasts are more affected by Cu excess than those of white lupin.

According to Maksymiec et al. (1994), Cu interferes with the biosynthesis of the photosynthetic machinery, modifying the pigment and protein composition of the photosynthetic membranes. Low chlorophyll contents and the inhibition of photosynthetic activity have also been observed in the leaves of several species exposed to Cu excess (Yruela 2009). The opposite effect has been reported in Cu-tolerant plants (Borghi et al. 2008). In the present work, Cu excess promoted a reduction of the PCI and the green leaf area, more so in the soybean than in the white lupin plants (Table 2). The reduction in photosynthetic activity may be due to a fall in the biosynthesis of chlorophyll, caused by the destruction of the internal structure of the chloroplast and thylakoid membrane damage (Quartacci et al. 2000). Pätsikkä et al. (2002) attributed the reduction of the chlorophyll content to a Cu-induced Fe deficiency. In the present work, Cu excess caused an intense reduction in the leaf Fe concentration in both species, more so in white lupin than in soybean (Table 1). In both legumes, the Fe concentrations recorded can be considered deficient (Reuter and Robinson 1997). Nevertheless, the white lupin leaves still had a higher Fe concentration under Cu stress than did the soybean plants. Soybean would seem to be sensitive to Fe deficiency. This may explain why the photosynthetic activity and chloroplast structure were more affected than in the white lupin.

Copper excess catalyze the formation of hydroxyl radicals (OH·) from the non-enzymatic chemical reaction between superoxide ($O_2^{\cdot-}$) and H_2O_2 (Haber-Weiss reaction). These cause lipid peroxidation (Navari-Izzo and Quartacci 2001), damage to the photosynthetic apparatus (Vajpayee et al. 2005), and may also catalyze the degradation of proteins through oxidative modifications and increased proteolytic activity (Romero-Puertas et al. 2002). The degree of damage suffered by cells depends on the rate of ROS formation and the efficiency and capacity of their detoxification and repair mechanisms. The MDA concentration provides an index of lipid peroxidation and, therefore, of oxidative stress. In the present work, Cu excess led to increased leaf MDA concentrations in both species, although more strongly in soybean (Table 3). The differences between species might be attributable to the increased number of plastoglobuli observed in the soybean plants only.

Antioxidants are molecules that inhibit or slow down the oxidation of other molecules, thus stopping the propagation of oxidative chain reactions (Navari-Izzo and Quartacci 2001). Thiol groups play an important role in the cytoplasmic detoxification defence mechanism against heavy metals, but they are also required to counteract the harmful effects of oxidative stress (Noctor and Foller 1998). Plants can respond to oxidative stress by stimulating enzymatic antioxidative systems. The effects of Cu on the activity of antioxidant enzymes and the involvement of these enzymes in the defence of plant tissues against metal-induced damage remain unclear, with differences seen between plant species and tissues, and depending on the concentration and duration of exposure (Chamseddine et al. 2009). Yurekli and Porgali (2006) showed that, in *Phaseolus vulgaris*, both low and high concentrations of Cu lead to an increase in CAT and SOD activities after Cu excess. Chaoui and El Ferjani (2005), in contrast, reported that the exposure of pea plants to 20 μM Cu had no effect on the activity of these

enzymes, although the supply of 100 μM Cu did reduce it. In the present soybean plants, the enzymatic and non-enzymatic scavenging systems were enhanced under Cu excess, whereas antioxidant activity was depressed in the white lupin plants (Table 3). A reduction in antioxidant enzyme activity would result in the accumulation of reactive oxygen species and the recorded reductions in photosynthetic pigments and activity. The scavenging function of both studied enzymes appears to have been impaired by the prolonged period of severe stress (Chamseddine et al. 2009). In addition different enzymatic antioxidant responses shown by the legumes studied may be due to differences in affinities for H_2O_2 between APX and CAT. Mittler (2002), proposed that ‘‘two-way defence systems’’ might be involved, suggesting that ROS could be eliminated through the SOD–CAT pathway or by the ascorbate–glutathione cycle, both of which are considered ROS-scavenging systems”.

In conclusion, Cu excess affects chloroplast ultrastructure and photosynthetic capacity in both soybean and white lupin legumes, especially in the former. This could be due to reduced Fe contents, particularly in the soybean leaves. Despite this, the soybean plants appeared to be more tolerant to Cu excess than the white lupin plants, as shown by their smaller reduction in biomass and the less intense effects on leaf morphology and structure. This could be due to soybean: i) transporting smaller amounts of Cu to the leaves, reducing its accumulation in these organs; ii) the different sites of Cu microlocalisation - soybean leaves localised Cu in the walls of the lower epidermis cells while the white lupin plants accumulated Cu mainly inside of the spongy parenchyma cells, and iii) the induction of a better antioxidative response. Finally, the structural alterations observed seem to bear a relationship with Cu microlocalisation; the tissues most affected were those that localised the largest

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quantities of Cu. It would therefore seem that Cu microlocalisation is an important factor to consider when assessing the response of plants to Cu excess.

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Table legends

Table 1 Concentrations of Cu and Fe (mg kg^{-1} DW) in the leaves of white lupin and soybean grown for 35 days with 1.6 or 192 µM Cu treatments. Data are means ± S.E. (n = 4). Values in the same row followed by different letters differ significantly ($P < 0.05$).

Table 2 Leaf biomass, LA, LT, LN, PCI, and green, yellow and red leaf areas of white lupin and soybean grown for 35 days with 1.6 or 192 µM Cu treatments. Data are means ± S.E. (n = 4). Values in the same row followed by different letters differ significantly ($P < 0.05$).

Table 3 MDA and total -SH contents and activity of SOD, APX and CAT in leaves of white lupin and soybean plants grown for 35 days with 1.6 or 192 µM Cu treatments. Data are means ± S.E. (n = 4). Values in the same row followed by different letters differ significantly ($P < 0.05$).

Figure captions

Fig. 1 EDXMA-determined Cu localisation in transverse sections of leaves of 1.6 and 192 μM Cu-treated plants, viewed by LTSEM. Values are expressed as percentages of the total signal. Data are means ± S.E. ($n = 4$). Different letters to the right of the bars indicate significant differences between Cu treatments ($P < 0.05$).

Fig. 2 Photomicrographs of white lupin leaves from plants grown with 1.6 μM (**a,c**) or 192 μM Cu (**b, d, e, f**). Cl: chloroplast, LE: lower epidermis, PP: palisade parenchyma, SP: spongy parenchyma, S: starch, UE: upper epidermis, VB: vascular bundle

Fig. 3 Photomicrographs of soybean leaves from plants grown with 1.6 μM (**a, c**) or 192 μM (**b, d**) Cu. Cl: chloroplast, LE: lower epidermis, PP: palisade parenchyma, SP: spongy parenchyma, S: starch, UE: upper epidermis, VB: vascular bundle

Fig. 4 Electron micrographs of white lupin leaves from plants grown with 1.6 μM (**a**) or 192 μM (**b-d**) Cu. C: cytosol, Cl: chloroplast, CM: chloroplast membrane, CW: cell wall, G: grana, IG: intergrana, M: mitochondria, Pe: peroxisome, S: starch, T: tonoplast, V: vacuole

Fig. 5 Electron micrographs of soybean leaves from plants grown with 1.6 μM (**a**) and 192 μM (**b-d**) Cu. C: cytosol, Cl: chloroplast, CW: cell wall, ER: endoplasmic reticulum, G: grana, IG: intergrana, N: nucleus, P: plasmalemma, Pe: peroxisome, PG: plastoglobuli, S: starch

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Table 1 Concentrations of Cu and Fe (mg kg^{-1} DW) in the leaves of white lupin and soybean grown for 35 days with 1.6 or 192 µM Cu treatments. Data are means ± S.E. (*n* = 4). Values in the same row followed by different letters differ significantly (*P* < 0.05).

	Cu treatments (µM)	
	1.6	192
	White lupin	
Cu	9.79 ± 0.17^{a}	132.08 ± 1.53^{b}
Fe	239.98 ± 4.48^{a}	21.85 ± 0.53^{b}
	Soybean	
Cu	6.86 ± 0.39^{a}	67.11 ± 1.76^{b}
Fe	44.40 ± 0.93^{a}	16.60 ± 0.80^{b}

	Cu treatments (μM)	
	1.6	192
	White lupin	
Biomass (g FW plant^{-1})	3.68 ± 0.10[a]	0.84 ± 0.10[b]
LA (cm^2 plant^{-1})	1003.3 ± 25.1[a]	325.9 ± 15.4[b]
LT (μm)	203.93 ± 3.08[a]	145.39 ± 2.89[b]
LN	34.76 ± 0.28[a]	12.69 ± 0.80[b]
PCI	458.4 ± 41.1[a]	294.1 ± 22.6[b]
Green area (%)	91.59 ± 0.42[a]	90.08 ± 1.36[a]
Yellow area (%)	0.18 ± 0.01[a]	1.86 ± 0.20[b]
Red area (%)	8.23 ± 0.42[a]	8.06 ± 0.55[a]
	Soybean	
Biomass (g FW plant^{-1})	3.43 ± 0.31[a]	1.06 ± 0.07[b]
LA (cm^2 plant^{-1})	1285.0 ± 83.1[a]	468.7 ± 32.4[b]
LT (μm)	131.69 ± 1.93[a]	96.12 ± 1.47[b]
LN	24.50 ± 0.75[a]	17.42 ± 0.85[b]
PCI	2379.0 ± 79.1[a]	538.2 ± 88.0[b]
Green area (%)	95.77 ± 0.63[a]	68.30 ± 1.72[b]
Yellow area (%)	0.02 ± 0.00[a]	0.22 ± 0.03[b]
Red area (%)	4.20 ± 0.63[a]	31.48 ± 1.84[b]

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3 **Table 3** MDA and total -SH contents and activity of SOD, APX and CAT in leaves of white lupin
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5 and soybean plants grown for 35 days with 1.6 or 192 µM Cu treatments. Data are means ±
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7 S.E. (n = 4). Values in the same row followed by different letters differ significantly ($P < 0.05$).
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	Cu treatments (µM)	
	1.6	192
	White lupin	
MDA (nmol g^{-1} FW)	13.66 ± 1.50[a]	29.79 ± 1.26[b]
-SH (nmol g^{-1} FW)	473.85 ± 18.52[a]	511.19 ± 24.49[a]
SOD (units mg^{-1} protein)	5.73 ± 0.38[a]	3.72 ± 0.07[b]
APX (units mg^{-1} protein)	2.93 ± 0.08[a]	2.63 ± 0.17[a]
CAT (units mg^{-1} protein)	84.66 ± 1.88[a]	46.21 ± 3.30[b]
	Soybean	
MDA (nmol g^{-1} FW)	31.50 ± 3.32[a]	91.28 ± 3.51[b]
-SH (nmol g^{-1} FW)	620.22 ± 19.63[a]	963.68±80.72[b]
SOD (units mg^{-1} protein)	2.08 ± 0.08[a]	2.51 ± 0.09[b]
APX (units mg^{-1} protein)	7.78 ± 0.29[a]	6.47 ± 0.05[b]
CAT (units mg^{-1} protein)	18.73 ± 3.87[a]	47.15 ±0.51[b]

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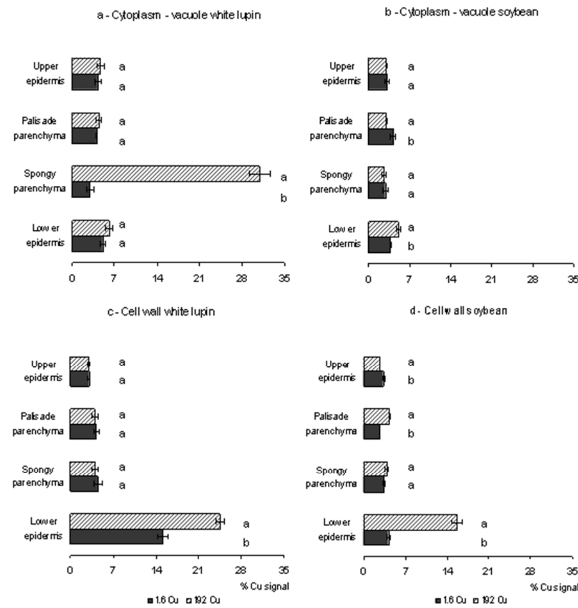

Fig. 1 EDXMA-determined Cu localisation in transverse sections of leaves of 1.6 and 192 µM Cu-treated plants, viewed by LTSEM. Values are expressed as percentages of the total signal. Data are means ± S.E. (n = 4). Different letters to the right of the bars indicate significant differences between Cu treatments ($P < 0.05$).

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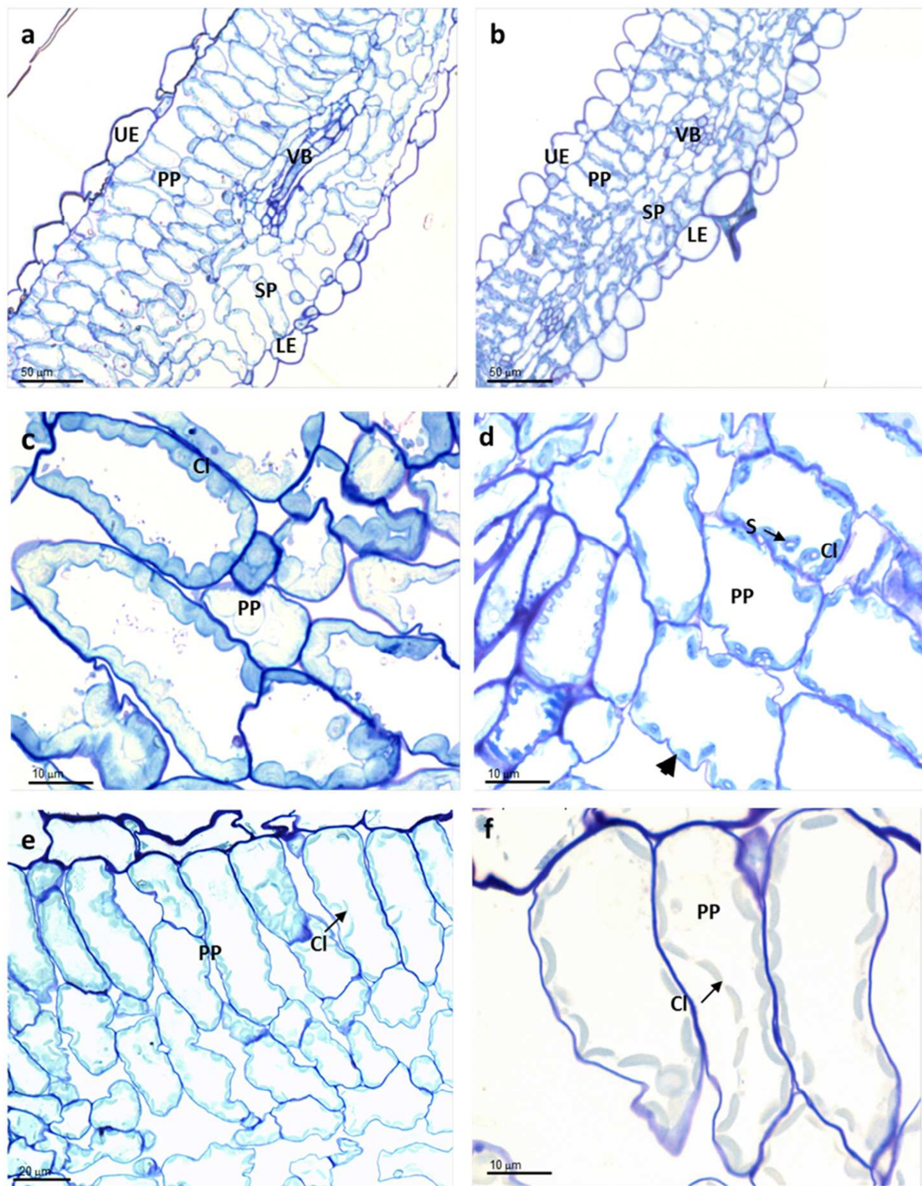

Fig. 2 Photomicrographs of white lupin leaves from plants grown with 1.6 μM (a,c) or 192 μM Cu (b, d, e, f). Cl: chloroplast, LE: lower epidermis, PP: palisade parenchyma, SP: spongy parenchyma, S: starch, UE: upper epidermis, VB: vascular bundle

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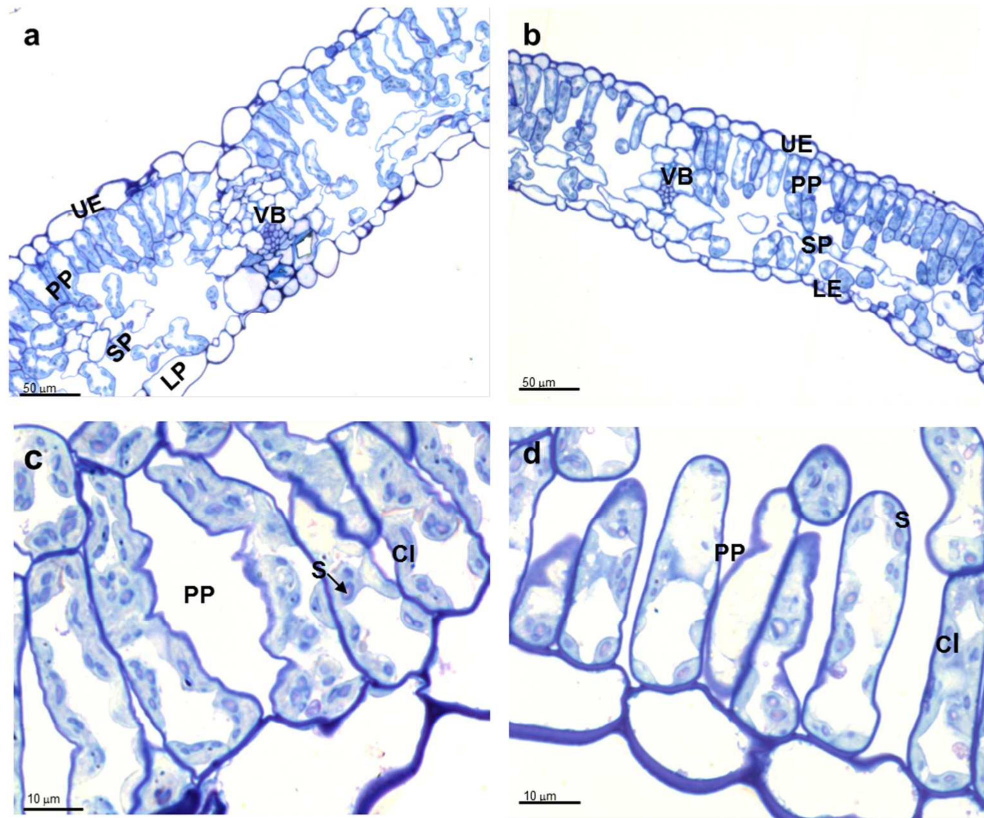

Fig. 3 Photomicrographs of soybean leaves from plants grown with 1.6 µM (a, c) or 192 µM (b, d) Cu. Cl: chloroplast, LE: lower epidermis, PP: palisade parenchyma, SP: spongy parenchyma, S: starch, UE: upper epidermis, VB: vascular bundle
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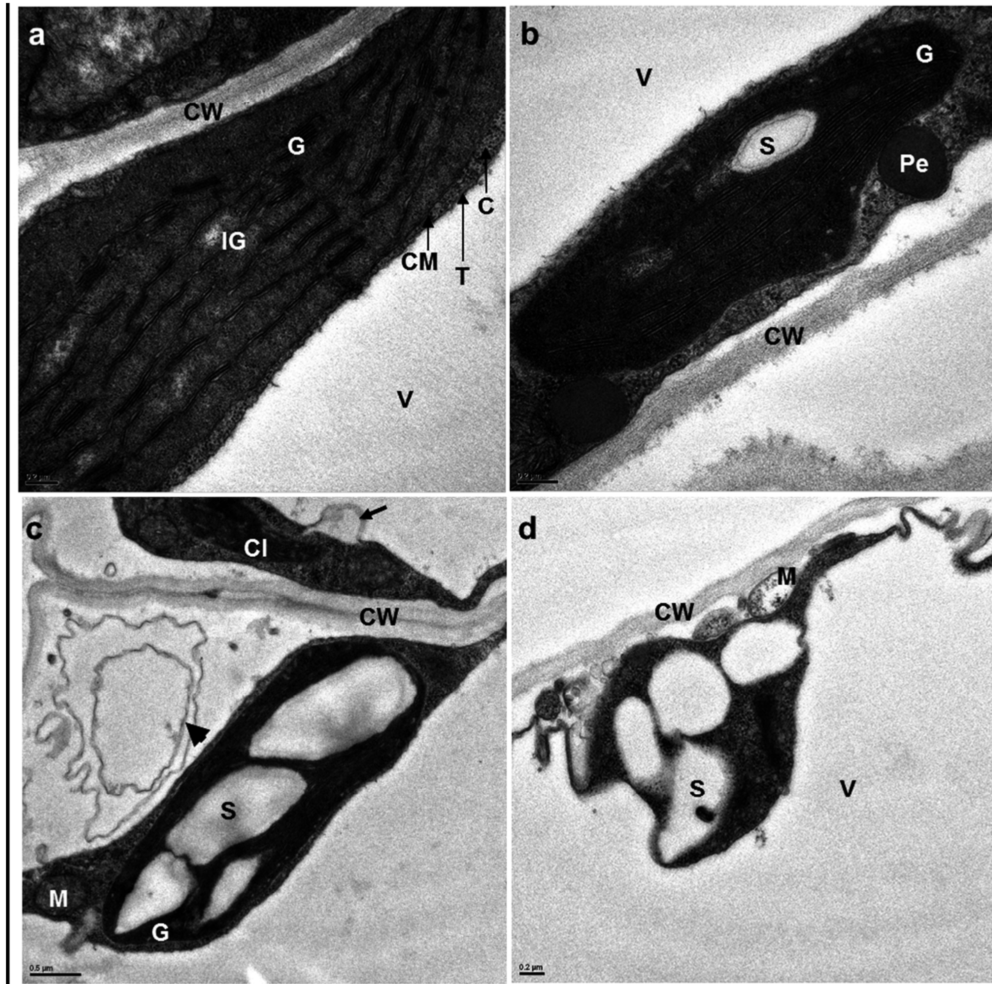

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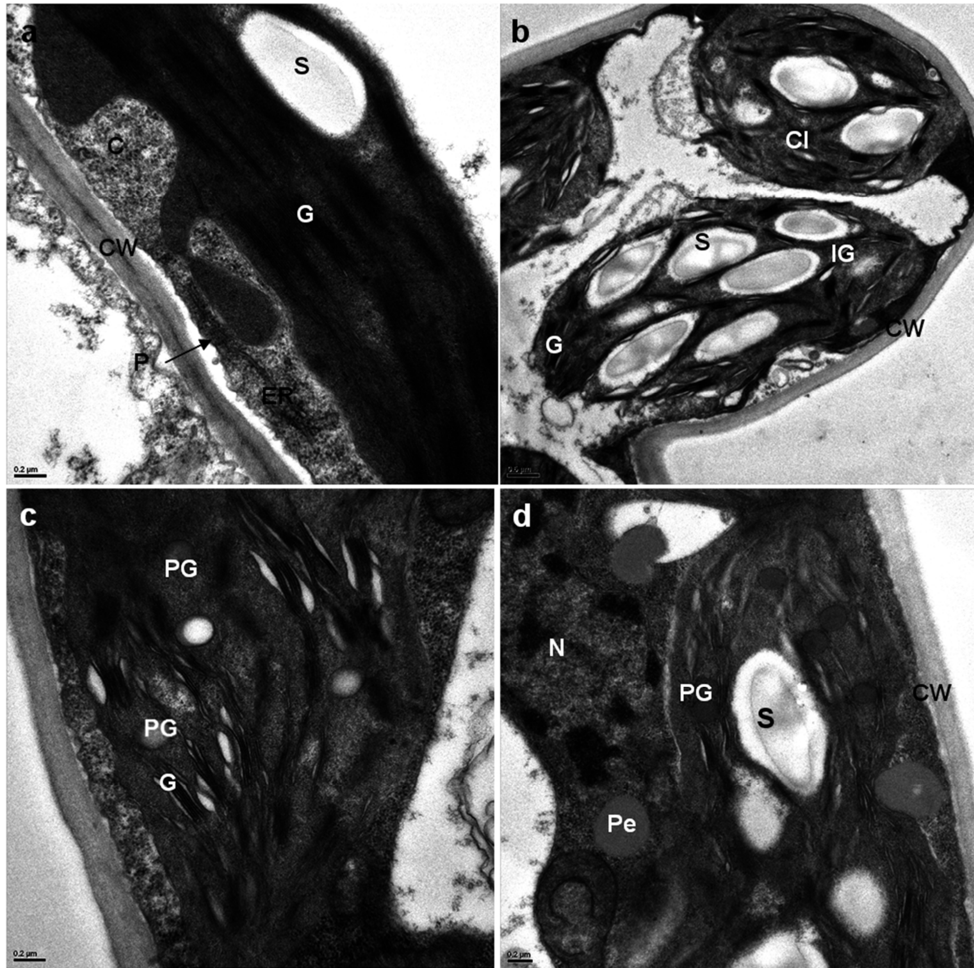

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