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Copper microlocalisation, ultrastructural alterations and antioxidant responses in the nodules of white lupin and soybean plants grown under conditions of copper excess

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A B S T R A C T

Copper (Cu) is a heavy metal which, at high concentrations, is toxic to organisms. Some plants, however, possess systems for dealing with excess Cu, such as its cell localisation, and have antioxidant enzymes that help to reduce the oxidative stress. The present work examines the microlocalisation of Cu and the antioxidant enzyme activity present in the nodules of white lupin and soybean plants grown hydroponically for 35 days in the presence of 1.6 μM (control) or 192 μM (excess) Cu. The effect of these conditions on nodule (ultra)structure was also examined. Energy-dispersive X-ray microanalysis showed the cell walls to be the main area of Cu binding in the inner and outer cortex and infected zone of white lupin nodules grown under the excess Cu conditions, while in soybean a high Cu signal was detected inside cells (cytoplasm or vacuoles) both in the inner cortex and infected zone. At the tissue level, an increasing Cu gradient was seen from the outer towards the inner nodule cortex in white lupin nodules, while the opposite was seen in soybean. Cu excess also induced oxidative stress and promoted damage to the ultrastructure of nodules. In the white lupin infected cells, a breakdown of the peribacteroidal membrane was seen, along with an increased number of vesicles in the cytosol of these cells. In the infected cells of the soybean nodules, the bacteroidal membrane became degraded and precipitation was seen within the vacuoles of the infected and uninfected cells. Finally, the white lupin nodules seemed to be more

sensitive to Cu excess than those of soybean, with the nodulation process, N_2 fixation, and the ultrastructure of bacteroids more strongly affected. A less effective antioxidative stress response against Cu was also seen in white lupin than in soybean nodules: the excess copper treatment induced a smaller increase in the total thiol content and ascorbate peroxidase activity in white lupin nodules than in soybean nodules, and promoted a greater reduction in catalase activity.

Keywords: Copper excess Energy-dispersive X-ray microanalysis; *Glycine max* L.; *Lupinus albus* L.; Nodule ultrastructure; Oxidative stress.

Abbreviations: APX, ascorbate peroxidase; B, bacteroid; BL, boundary layer; BM, bacteroidal membrane; CAT, catalase; CW, cell wall; DZ, distribution zone; DW, dry weight; EDXMA, energy-dispersive X-ray microanalysis; ER, endoplasmic reticulum; FW, fresh weight; ic, infected cells; IC, inner cortex; IS: intercellular space; IZ, infected zone; LTSEM, low temperature scanning electron microscopy; M, mitochondria; MC, middle cortex; MDA, malondialdehyde; nic, non-colonised cell; OC, outer cortex; PBM, peribacteroidal membrane; Pe, peroxisome; Pr, precipitate; Pt, plastids; ROS, reactive oxygen species; S, symbiosome; SCL, sclereids; -SH, total thiols; SOD, superoxide dismutase; VB, vascular bundle.

1. Introduction

Heavy metal contamination is a widespread problem with environmental and public health consequences in many parts of the world. High levels of Cu occur naturally in some soils, but mining, smelting, manufacturing, agriculture and waste disposal are responsible for the high concentrations recorded in many others (Yruela, 2009).

Copper is essential for plant growth and development. It is a cofactor of numerous enzymes that take part in the electron transfer reactions of photosynthesis and respiration, and it is involved in carbohydrate distribution, N_2 reduction and fixation oxygen superoxide scavenging, ethylene sensing, cell wall metabolism, lignification and protein synthesis (Marschner, 1995). However, when Cu is present in high concentrations (>20 µg g^{-1} DW) it is highly phytotoxic, the consequence of its interfering with photosynthesis, pigment synthesis and plasma membrane permeability, of its causing metabolic disturbances that inhibit growth and development, and through

its initiation of oxidative damage (Yruela, 2009). In plant cells, Cu^{2+} is easily reduced to Cu^+. In this form it is unstable and tends to be oxidized giving rise to Fenton-type reactions that release reactive oxygen species (ROS) (Navari-Izzo and Quartacci, 2001) such as singlet oxygen, hydrogen peroxide and the hydroxyl radicals. These ROS possess an unpaired electron, and are highly reactive. The balance between the generation and scavenging of ROS is controlled by the antioxidant defence system (Becana et al., 2010); when this cannot cope with the level of ROS production, oxidative damage occurs.

Cu toxicity is associated with a series of visible symptoms, including chlorosis and necrosis of the leaves, short stems, browning of the root, and abnormal root morphology (Marschner, 1995). Visible symptoms of metal toxicity stress in plants are indications of abnormalities at the structural and ultrastructural levels. Changes at the cellular, tissular, and organ level are either the result of direct interaction between the Cu and structural components at these sites, or of the more indirect consequence of change in signal transduction and/or metabolism. Examination at the structural and ultrastructural level helps identify toxic injury sites as well as the consequences for the whole plant and its development (Barceló and Poschenrieder, 2004).

Contaminated soils are generally low in nutrients and organic matter. The inclusion of nitrogen-fixing plants in stabilizing vegetation might, therefore, help in ecosystem development by increasing the soil nitrogen content available, and through the promotion of plant cover (Frérot et al., 2006). Unfortunately, N_2 fixation is sensitive to heavy metal pollution. White lupins, however, are tolerant to a number of heavy metals and show strong biomass production; they are therefore good candidates for planting in contaminated soils (Carpena et al., 2006). Soybean is another candidate, but few studies have examined N_2 fixation by the species in Cu-contaminated soils, despite it being the most economically important of all grain legumes. The present study compares the response of white lupin and soybean to Cu excess to determine: (1) whether nodulation and N_2 fixation are altered by the accumulation of Cu, ultrastructural changes caused by Cu, or by the appearance of oxidative stress, (2) whether nodules that accumulate Cu in the cell wall or vacuole show greater or lesser tolerance to Cu excess (it has been reported that Cd accumulation in nodule cell walls leads to greater Cd tolerance [Carpena et al., 2003]), and (3) whether the induction of the antioxidant response has a protective role under Cu excess, as reported for the nodules of plants

grown under conditions of salinity and drought (Mhadhbi et al., 2004; Nasr-Esfahani et al., 2010).

The patterns of Cu microlocalisation in cells and tissues can provide insights into possible mechanisms of toxicity and tolerance. Electron microscopy combined with energy dispersive X-ray microanalysis, laser microprobe mass analysis, electron energy loss spectroscopy, secondary ion spectrometry and or cytochemical methods, provides a powerful tool for investigating these patterns (Barceló and Poschenrieder, 2004). Ultrastructural alterations in leaves and roots promoted by excess Cu have been reported in broad beans (Kasim, 2005), maize (Ouzounidou et al., 1995) and oregano (Panou-Filtheou and Bosabalidis, 2004), as has the response to oxidative stress in these organs (Srivastava et al. 2006; Mashhadi-Akbar-Boojar 2011). However, the literature contains little information on such Cu-induced ultrastructural alterations, the microlocalisation of Cu, or the anti-oxidative response to Cu in root nodules. The specific objectives of the present study were therefore to determine (1) the subcellular localisation of excess Cu in white lupin and soybean nodules, (2) the effect of Cu excess on nodule structure and ultrastructure in these species, and (3) the oxidative stress and anti-oxidative defence responses of these organs. So that sufficient quantities of Cu would accumulate and be detectable in nodules by low-temperature scanning electron microscopy (LTSEM), high doses of Cu (192 μM) were supplied to the plants. To ensure that sufficiently high quantities of Cu would accumulate and be detectable in the nodules by low-temperature scanning electron microscopy (LTSEM), high doses of Cu (192 μM) were supplied to the plants.

2. Materials and methods

2.1. Growth conditions and Cu treatments

Seeds of white lupin (*Lupinus albus* L.) cv. Marta, and of soybean (*Glycine max* L.) cv. Williams, 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 28°C 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 nitrogen-free nutrient solution in the lower compartment. The composition of this solution was: 2.0 mM K_2SO_4, 1.5 mM KH_2PO_4, 0.5 mM KCl, 1.0

mM $CaCl_2$, 2.0 mM $MgSO_4$, 0.1 mM NaCl, 35.9 μM Fe-EDDHA, 32.9 μM $MnSO_4 \cdot H_2O$, 1.6 μM $ZnSO_4 \cdot 7H_2O$, 1.6 μM $CuSO_4 \cdot 5H_2O$, 46.2 μM H_3BO_3, 1.0 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.001 μM $CoCl_2 \cdot 6H_2O$ and 0.001 μM $Ni(NO_3)_2 \cdot 6H_2O$ (pH 5.5-6.0). Deionised water was used for preparing stock nutrient solution, and was added to replace transpiration losses every two days. At the end of every week the entire nutrient solution left in the lower compartment of the Riviera pots was discarded and replaced with newly prepared nutrient solution. Each of the plants was inoculated twice (at sowing and 1 week later) with a 1 mL suspension of either *Bradyrhizobium* sp. (*Lupinus*) ISLU-16 (for the white lupins plants) or *Bradyrhizobium japonicum* USDA-110 (for the soybean plants) in the exponential growth phase (10^8–10^9 cells per mL). Plants were grown in a controlled environment chamber under the following night/day conditions: temperatures 20/25°C, photoperiod 11/13 h, and relative humidity 60/40%. The photon flux density during 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 $CuSO_4$), with four replicates, following a randomised block design − all performed in duplicate. High levels of Cu were supplied to the plant, so that sufficient quantities of Cu would accumulate and be detectable in nodule compartments (Vazquez et al., 2007).

Plants were harvested after 35 days of growth under these conditions. The shoots, roots and nodules of each plant were separated and their fresh weights (FW) recorded; the number of nodules on the roots was also counted. Samples of nodules were frozen in liquid N_2 and stored at -20ºC. The remaining nodules and other plant parts were oven-dried separately at 80ºC until a constant dry weight (DW) was reached.

2.2. Determination of N

The total N concentration of the plant organs was determined using the Dumas combustion method (Leco CHNS-932, St. Joseph, MI, USA) at the beginning and the end of the Cu treatments. Nitrogen fixed was calculated as the total plant N content at harvest, minus the total N content at the start of the treatments.

2.3. Determination of stress indicators and antioxidant enzymes

The concentration of the stress indicators malondialdehyde (MDA) (a cytotoxic product of lipid peroxidation normally considered the major 2-thiobarbituric acid-

reacting compound) and total thiols (–SH) were examined in nodule samples homogenised to a fine powder in liquid N_2 using an ice-cooled mortar and pestle. Maldondialdehyde was determined as previously described (Lozano-Rodríguez et al., 1997). Briefly, 2 mL of TCA–TBA–HCl reagent (15% [w/v] TCA; 0.37% [w/v] TBA and 0.25 mM HCl) was used to extract MDA from root nodules. The extract was then heated in a sand bath at 90ºC for 30 min. After cooling, the flocculent precipitate was removed by centrifugation at 11,000×g for 10 min. The absorbance of the supernatant was measured at 535 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. Total -SH was assayed in 100 mg FW samples of plant material with 0.4 mL of NaOH (1M) containing $NaBH_4$ (1 mg mL^{-1}) and 0.2 mL of deionised water. After centrifugation (11,000×g, 10 min), 0.5 mL of supernatant were added to 0.5 mL of 5,5-dithiobis(2-nitrobenzoic acid) dissolved in neutralising buffer (0.5M potassium phosphate, pH7.2). Absorbance was measured at 410 nm (Jocelyn, 1987).

Antioxidant enzyme activity was determined by homogenising 50 mg of nodules 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). Superoxide dismutase (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 tetrazolium (NBT). The 3 mL reaction mixture contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The test tubes were shaken and placed 30 cm below a 15 W fluorescent lamp. Absorbance was then measured at 560 nm. The activity of SOD was expressed as units mg^{-1} protein. One unit of activity was defined as the amount of protein required to reduce NBT under light to 50% of the initial concentration. Ascorbate peroxidase (EC1.11.1.11) activity was measured by estimating the rate of ascorbate oxidation (extinction coefficient 2.8 mM^{-1} cm^{-1}). The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM sodium ascorbate, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The change in absorbance was monitored at 290 nm (Srivastava et al., 2006) and the enzyme

activity expressed as units mg^{-1} protein. Catalase (EC 1.11.1.6) activity was assayed by measuring the decomposition of hydrogen peroxide. Enzyme extract (50 μL) was added to the reaction mixture containing 1 mL phosphate buffer solution (50 mM, pH 7.0) and 0.1% H$_2$O$_2$. The reduction in the absorbance at 240 nm was then recorded and the enzyme activity calculated using an extinction coefficient of 0.04 mM^{-1} cm^{-1}. One unit of CAT activity was defined as the amount required decomposing 1 μmol of hydrogen peroxide min^{-1} mg^{-1} protein under the assay conditions (Chen et al., 2009).

2.4. Electron microscopy and energy-dispersive X-ray microanalysis

Small pieces of fresh nodules (1 mm^2) from the 1.6 and 192 μM Cu-treated plants, selected at comparable stages of development, were mounted on a clamp holder with adhesive (Gurr®, OCT, BDH, Poole, UK). 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 inside 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 nodules were observed at low temperature and 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 results were obtained using standard ZAF (atomic number, absorption and fluorescence) correction procedures employing Link Isis 3.2 software (Link Isis, Oxford, UK).

2.5. Light and electron microscopy

Small pieces of fresh nodules (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 with 8% (w/v) of 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 of 1 μm thickness for light microscopy, and of 70 nm

for electron microscopy, were prepared using a Reicher Ultracut S ultramicrotome fitted with a Diamond knife. The semithick sections of white lupin were stained with 1% (w/v) toluidine blue in aqueous sodium borate, and those of soybean stained with basic fuchsin (0.05% w/v) in 5% (v/v) aquous ethanol, rinsed and let to dry (de Lorenzo et al. 1998) 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.

2.6. Statistical analyses

The data presented are the means ± standard errors (S.E.) of four independent replicates. To ensure that the assumptions for statistical analysis were fulfilled, the equality of variances and the normality of the data were tested. Differences between means for each variable were tested for significance by one-way analysis of variance (ANOVA). Means were compared using the least significant difference test $p < 0.05$. All calculations were performed using SPSS v17.0 software.

3. Results

3.1. Nodulation and total N content

Table 1 shows the results recorded for the nodule biomass variables and total plant N contents of both types of plant grown under the two treatment conditions. In white lupin, the 192 μM Cu treatment reduced the nodule number by 72%, nodule weight by 69%, and total plant N content by 61%, but increased the nodule normalised weight by 30%. In soybean, the nodule number, nodule weight and total plant N content of the 192 μM Cu-treated plants were reduced by 50%, 57% and 53% respectively compared to the control treatment (1.6 μM Cu), but the nodule normalised weight was increased by 16%.

3.2. Copper microlocalisation in nodule tissues

Microprobe examinations were performed at the same sites in nodules from 1.6 and 192 μM Cu-treated plants. In the 1.6 μM Cu-treated plants, the Cu concentrations in the cytoplasm-vacuoles and cell walls of all cells were below the EDXMA detection

limit. Table 2 shows the EDXMA results for the nodules of white lupin and soybean plants exposed to the 192 μM Cu treatment. In white lupin, Cu was mainly localised in the cell walls of the nodule cortical cells, and in those of the infected zone. The walls of the latter cells had about twice the amount as that detected in their cytoplasm-vacuoles. An increasing Cu gradient was seen from the walls of the outer cortical cells to the walls of the inner cortical cells. Further, the Cu signal in the cell walls of the infected zone cells was 1.5 times that measured for the walls of the outer cortex cells. However, the strongest Cu signal was detected in the cytoplasm-vacuoles of the inner cortex cells.

The microlocalisation of Cu in the soybean nodules was greater (1.7-3.5 times) in the cytoplasm-vacuoles of the inner cortex cells and the infected zone than in the cells walls of cells of this area. The opposite was seen in the outer cortex cell walls. A decreasing gradient was seen from the cell walls of the outer cortex cells towards the cell walls of the inner cortex cells (around 3.7 times greater in the first tissue). However, in the cytoplasm-vacuoles of these cells, no significant differences were seen (Table 2).

3.3. Nodule structure and ultrastructure

The nodules of white lupin (Figs. 1A, C) and soybean (Figs. 2A, C) grown under the 1.6 μM Cu conditions showed a well-organised structure. The structures of white lupin and soybean nodules have been previously described by Fernández-Pascual et al. (1992) and Parsons and Day (1990) respectively. Two regions were clearly visible: the cortex and the infected zone. The nodules of white lupins grown under the 192 μM Cu conditions also showed good structural organization. However, some modifications can be observed like some deformations of the cell walls of the outer cortex (or zone 1 according to Iannetta et al. 1993) and occlusions in the intercellular spaces of zone 2 (Fig. 1B). Furthermore cellular disorganisation was apparent in the inner cortex (or zone 3) (Fig. 1D). The infected zone showed a number of senescent cells (Fig. 1D, see arrows).

The nodules of the soybean plants grown under the 192 μM Cu conditions also showed a well-organised structure, although some abnormalities were seen. In the cortex, the most noticeable alterations were the reduction in the size of the sclereid cells as well as a reduction of volume in the distribution zone cells, with an increase in the degree of compaction between them (Fig. 2B). In the infected zone, increased

vacuolisation was seen, and precipitates noted in the interior of these vacuoles in both infected and uninfected cells (Fig. 2D).

Figures 3 and 4 show ultrastructural abnormalities in the infected zone of nodules from white lupin and soybean plants grown under the 192 μM Cu conditions. In those of white lupin, the peribacteroidal membranes appeared degenerated and in some cases even broken, leaving the bacteroids in direct contact with the cytoplasm (Figs. 3B and D [see arrow]). In addition, an increase was seen in the number of vesicles in the cytosol of the infected cells, a sign of early senescence (Figs. 3B, D). The nodules of soybean plants exposed to the 192 μM Cu conditions showed some alterations in both infected and uninfected cells. The infected cells showed an increased electron-dense cytosol and the degradation or separation of the bacteroidal membrane, although the peribacteroidal membrane remained intact (Fig. 4D). In the uninfected cells, a larger number peroxisomes was seen compared to the same cells from plants grown under the 1.6 μM conditions (although they were smaller), along with precipitates in the vacuoles (Fig. 4B).

3.4. Stress indicators and antioxidant enzymes

Table 3 shows the MDA and total –SH contents, plus the activities of SOD, APX and CAT, in nodules of white lupin and soybean plants exposed to the 1.6 and 192 μM Cu treatments. The concentrations of MDA in the nodules of both types of plant grown under the 192 μM Cu conditions were 1.5 times higher than those of the respective 1.6 μM treatments. The total -SH content of the nodules of the white lupin and soybean plants grown under the 192 μM Cu conditions were 1.3 and 1.7 times higher than those recorded in the corresponding control treatment plants.

Exposure to the 192 μM Cu conditions increased the activities of SOD and APX by 7.0 and 1.1 times in white lupin nodules, but significantly reduced the activity of CAT (59%). The activities of SOD and APX in the nodules of the 192 μM Cu-treated soybeans increased 2.8 and 3.0 times respectively compared to the control values. However, no differences were seen between the treatments in terms of CAT activity.

4. Discussion and conclusions

Several studies have shown that N_2 fixation is very sensitive to a range of abiotic stresses, particularly high environmental trace element concentrations (Carpena et al., 2006). The adverse effect of abiotic stresses on N_2-fixation involve the nodule cell

colonisation process, as well as nodule growth, development and functioning (Carpena et al., 2003). Certainly, high levels of Cu reduce nodule number and weight in *Vigna unguiculata* (Kopittke et al., 2007) and *Vigna radiata* (Manivasagaperumal et al., 2011). In the present work, Cu excess strongly reduced the number and weight of nodules in both species, but especially in white lupin (Table 1). Further, N_2 fixation, estimated as the total plant N content, was reduced in both species under the 192 μM Cu conditions, although more so in the white lupin (Table 1). Georgiev et al. (1996) reported a lower total N content in the nodules and other plant organs of *Robinia pseudoacacia* subjected to Cu excess. A reduction in the amount of N_2 fixed and amino acids present in the nodules of white lupins exposed to Cd has also been reported (Carpena et al., 2003). Exposure to Cd also negatively affects the process of N_2 fixation and assimilation in soybean (Balestrasse et al., 2003).

The inhibition of nodulation and N_2 fixation can be caused by the accumulation of heavy metals in plant tissues, ultrastructural changes in nodule tissues (Carpena et al., 2003) and the negative effect of oxidative stress (Nasr-Esfahani et al., 2010). In the present work, LTSEM-EDXMA showed that Cu was mainly localised in the inner cortex and in the infected zone in white lupin nodules. In contrast, in soybean nodules, the outer cortex was the region that accumulated the highest Cu concentrations. At the cellular level, the greatest amount of Cu was localised in the cell walls in the white lupin nodules treated with 192 μM Cu, while in soybean it deposited in the vacuoles (Table 2). Other authors have reported Cd to be localised mainly in the cell walls in white lupin nodules. This might represent a major mechanism of Cd detoxification in this organ (Carpena et al., 2003).

The accumulation of high concentrations of Cu in the nodules might directly induce abnormalities in the structure and ultrastructure of this organ in both species. Among the structural alterations observed in the plants grown under the 192 μM Cu conditions was the greater vacuolisation of the infected cells than seen in the plants grown under the 1.6 μM Cu conditions, and the presence of precipitates in the vacuoles (Fig. 2D). The appearance of these precipitates coincides with the enhanced Cu signal observed in this organelle. Occlusions of intercellular spaces were also seen in both white lupin and soybean nodules, probably due to the accumulation of glycoprotein (de Lorenzo et al 1998; Figs. 1 and 2). According to Georgiev et al. (1996), an increase in Cu concentration in *Robinia pseudoacacia* nodules can affect the quantity of

glycoproteins accumulated and their properties, reducing the intercellular spaces between cells in the middle cortex. An increase in glycoproteins in the nodule intercellular spaces would limit the diffusion of O_2 to infected zone, as described in Cd-treated white lupin nodules (Carpena et al., 2003). The opposite effect was observed in the presence of glyphosate (de María et al., 2005). The reduction in O_2 concentration in the the infected zone reduces nitrogenase activity, leading to reductions in N_2 fixation, the protein content and the quantity of nitrogenated compounds exported from the nodule (Guasch et al., 2001).

The ultrastructural alteration in the infected cells of the nodules of white lupins grown under the 192 μM Cu conditions included the degradation and in some cases the rupture of the peribacteroidal membrane, and an increased number of vesicles in the cytosol (Fig. 3). Similar results have been reported for white lupin plants grown with Cd (Carpena et al., 2003) and in the presence of high levels of glyphosate (de María et al., 2005). The rupture of the peribacteroidal membrane can affect nodular functioning since solute transport between the cytosol and bacteroid takes place through this membrane (de María et al., 2007). In addition, the process of exchange of substances and signals between the two symbionts may also be altered, limiting the transport of carbohydrates produced by the plant and therefore the amount of N_2 fixed by the bacteroid. Moreover, the integrity of the bacteroid membranes plays an important role in the maintenance of turgor in the non-vacuolated infected cells in white lupin nodules. The rupture or disintegration of these membranes would cause an alteration in osmotic pressure within these cells (de María et al., 2005). In soybean nodules, the excess Cu caused a degeneration of the bacteroidal membrane but the peribacteroidal membrane remained intact (Fig. 4). These alterations have also been observed in naturally senescent white lupin nodules, have been induced by darkness (Hernández-Jiménez et al., 2002), and have been reported in nodules of plants grown with nitrate (de Lorenzo et al., 1990) and in the presence of herbicides (de María et al., 2005). In contrast, in the nodules of soybean plants grown in the presence of Cd, no disruption of the symbiosome is reported, although the effective area for N_2 fixation in the nodule becomes reduced as the Cd concentration increases, as do the number of N_2-fixing cells inside the nodule (Balestrasse et al., 2003).

The alteration of cell membranes can be caused by the induction of ROS production (Hall, 2002). Copper excess may generate oxidative injury with the production of free radicals, which can cause lipid peroxidation (Navari-Izzo and

Quartacci, 2001). The MDA concentration provides an index of lipid peroxidation and, therefore, of oxidative stress. In the present work, supplying Cu doses of 192 μM Cu led to increased nodule MDA concentrations in the same proportion in both species (Table 3). A marked increase in MDA levels has also been reported in nodules from white lupins grown in the presence of Cd (Carpena et al., 2003), although a decline was observed when plants were exposed to dark stress (Hernández-Jiménez et al., 2002). In bean plants, increases in the MDA content have been found in nodules subjected to water stress (Gogorcena et al., 1995).

Damage to cells occurs when the detoxification capacity of the natural antioxidative systems cannot keep pace with the production of ROS. 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). In the present study, the exposure of white lupin and soybean to 192 μM Cu enhanced the production of total -SH in the nodules, more so in soybean than in white lupin (Table 3). Carpena et al. (2003) reported similar results for the nodules of white lupin plants grown in the presence of Cd. In alfalfa nodules the accumulation of total -SH supports the hypothesis that they are involved in a strategy of heavy metal sequestration (Hall, 2002).

Plants can also respond to oxidative stress by stimulating enzymatic antioxidative systems. Copper excess induces an increase in SOD activity in plant organs. Madejón et al. (2009) detected an increase in SOD activity in the apex of Cu-treated roots. According to these authors, Cu tolerance might be related to the enhancement of SOD activity. Cu stress induces an increase of SOD activity in plants. Madejón et al. (2009) detected an increase of SOD activity in roots apex treated with Cu excess. According these authors the Cu tolerance could be related to the enhancement of SOD activity. In the present study, SOD activity was significantly higher in the nodules of the white lupins grown under the 192 μM Cu conditions (Table 3). In soybean this increase was less accentuated. A significant increase in the activity of SOD in response to darkness has been reported in white lupin nodules (Hernández-Jiménez et al., 2002). The strong increase in the activity of this enzyme suggests there is a quick breakdown of superoxide radicals by SOD. This, plus the action APX, GPX and CAT, allows plants

to combat oxidative stress (Srivastava et al., 2006). The results of the present work indicate that, under Cu excess, APX activity is stimulated in both species examined, whereas CAT activity declines in white lupin nodules and does not change in those of soybean (Table 3). The induction of APX activity and its protective role under stress conditions have been reported elsewhere (Matamoros et al., 2003; Nasr-Esfahani et al., 2010). According to Nasr-Esfahani et al. (2010), an efficient antioxidant system is critical for N_2 fixation process. A positive relationship between APX or CAT activity and N_2-fixing capacity exists, with higher enzymatic activities seen in nodules showing higher N_2-fixing capacity. Several authors report a reduction in CAT activity under stress conditions (Mhadhbi et al., 2004; Nasr-Esfahani et al., 2010). According to these authors, CAT activity values may be used as biochemical marker of symbiotic efficiency.

In conclusion, excess of Cu promotes (ultra)structural changes in white lupin and soybean nodules, in particular the breakdown of the peribacteroidal membrane in white lupin, the degradation of the bacteroidal membrane in soybean, and the induction of oxidative stress in both species. This affects N_2 fixation and nodule development. However, the nodules of white lupin appeared to be more sensitive to the 192 μM Cu conditions than did the soybean nodules-the nodulation process, N_2 fixation and the ultrastructure of the infected zone being more strongly affected. This could be due to soybean nodules accumulating a smaller amount of Cu in the infected zone than those of the white lupin, and/or or the precipitation of the metal in the cytoplasm-vacuole of these cells. This would reduce the toxicity of this heavy metal and provide a defence against Cu stress. In addition, the antioxidant stress response seen in the nodules of both species increased under the 192 μM Cu conditions, but more so in the soybean. A greater increase in total -SH content and APX activity was seen in this species, along with a smaller reduction in CAT activity than in white lupins. This might reduce the concentration of free Cu and ROS in the nodule cells, which should help protect nodules against Cu stress.

Acknowledgements

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Figure captions

Fig. 1. Photomicrographs of nodule sections showing the cortex and the infected zone in white lupins grown under the 1.6 µM (A, C) and 192 µM Cu conditions (B, D). IC: inner cortex, IZ: infected zone, MC: middle cortex, OC: outer cortex.

Fig. 2. Photomicrographs of nodule sections showing the cortex and the infected zone in soybean grown under the 1.6 µM (A, C) and 192 µM Cu conditions (B, D). BL: Boundary layer, DZ: distribution zone, ic: infected cell, IC: inner cortex, IS: intercellular space, IZ: infected zone, MC: middle cortex, nic: non-colonised cell, OC: outer cortex, Pr: precipitate, SCL: sclereids, VB: vascular bundle.

Fig. 3. Electron photomicrographs of white lupin nodule sections. Nodules from 1.6 µM (A, C) and 192 µM Cu-treated plants (B, D). B: bacteroid, BM: bacteroidal membrane, CW: cell wall, ER: endoplasmic reticulum, ic: infected cell, M: mitochondria, PBM: peribacteroidal membrane, Pe: peroxisome.

Fig. 4. Electron photomicrographs of soybean nodule sections. Nodules from the 1.6 µM (A, C) and 192 µM Cu-treated plants (B, D). B: bacteroid, BM: bacteroidal membrane, CW: cell wall, ic: infected cell, M: mitochondria, nic: non-colonised cell, PBM: peribacteroidal membrane, Pe: peroxisome, Pr: precipitate, Pt: plastids, S: symbiosome.

Figure 1
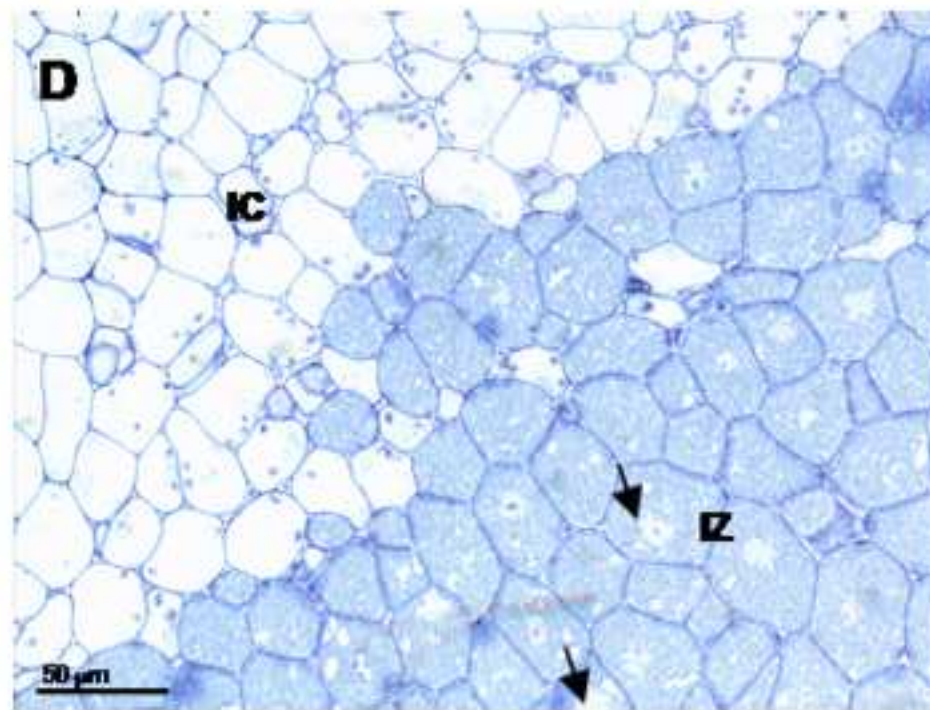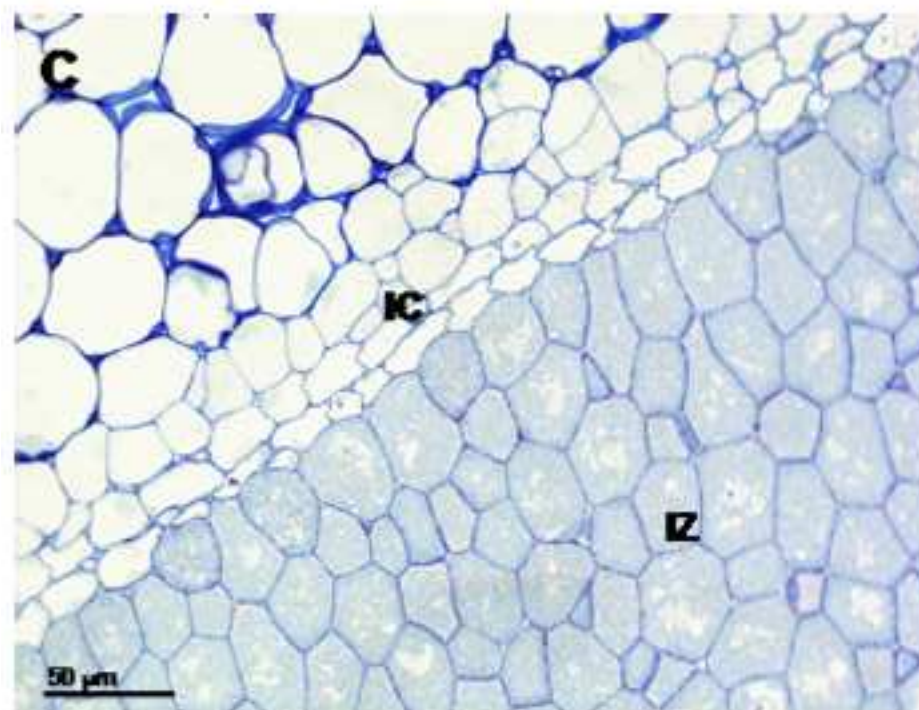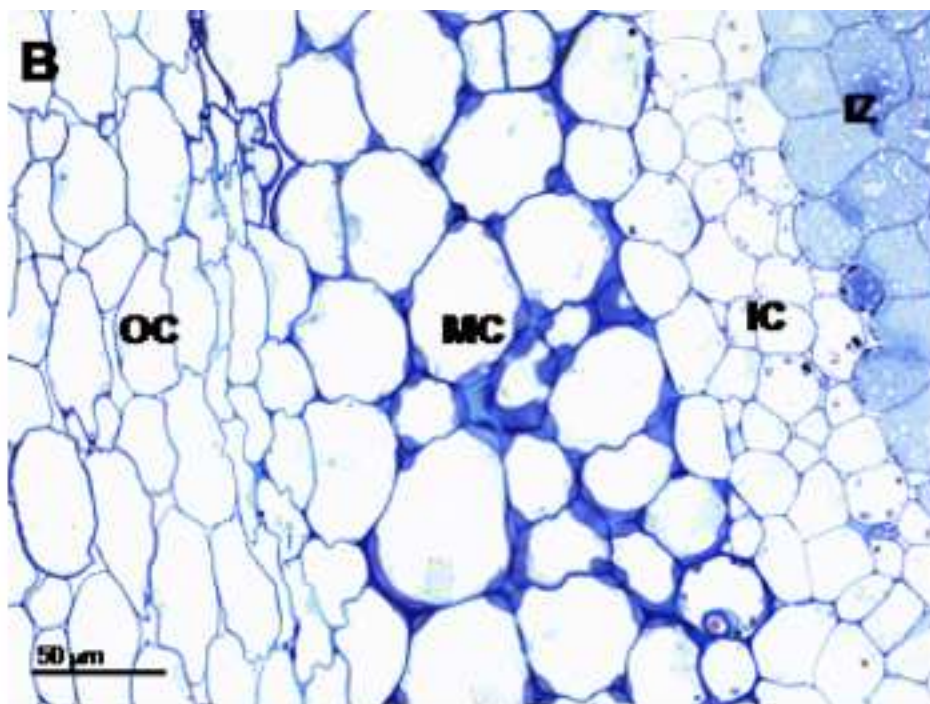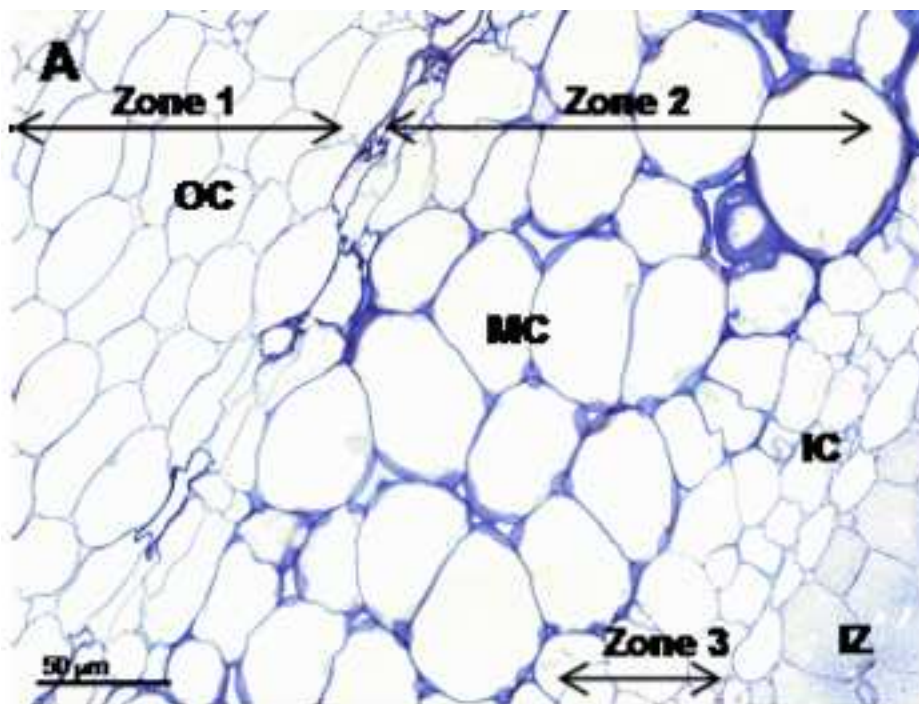[Click here to download high resolution image](#)

Figure 2
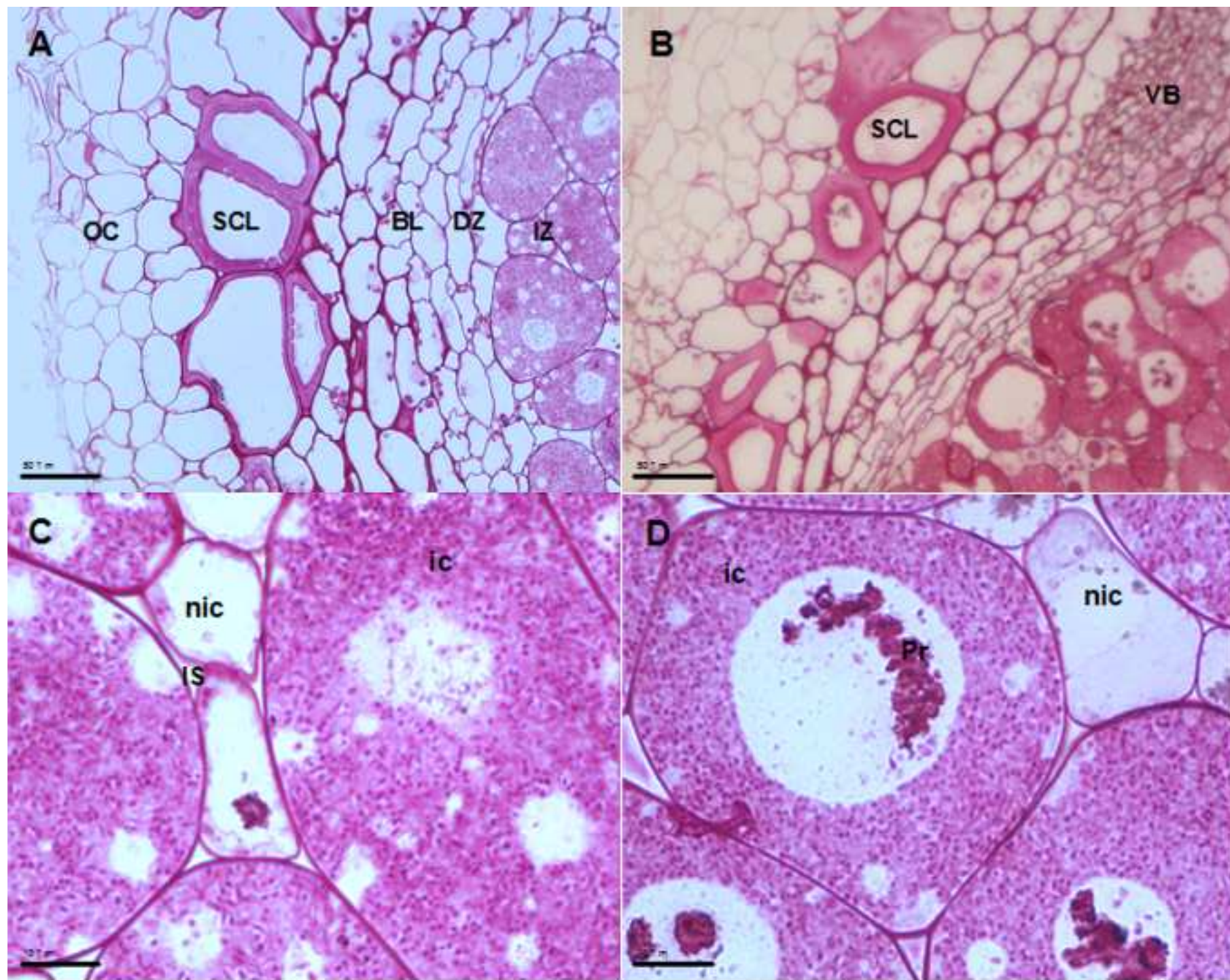**Click here to download high resolution image**

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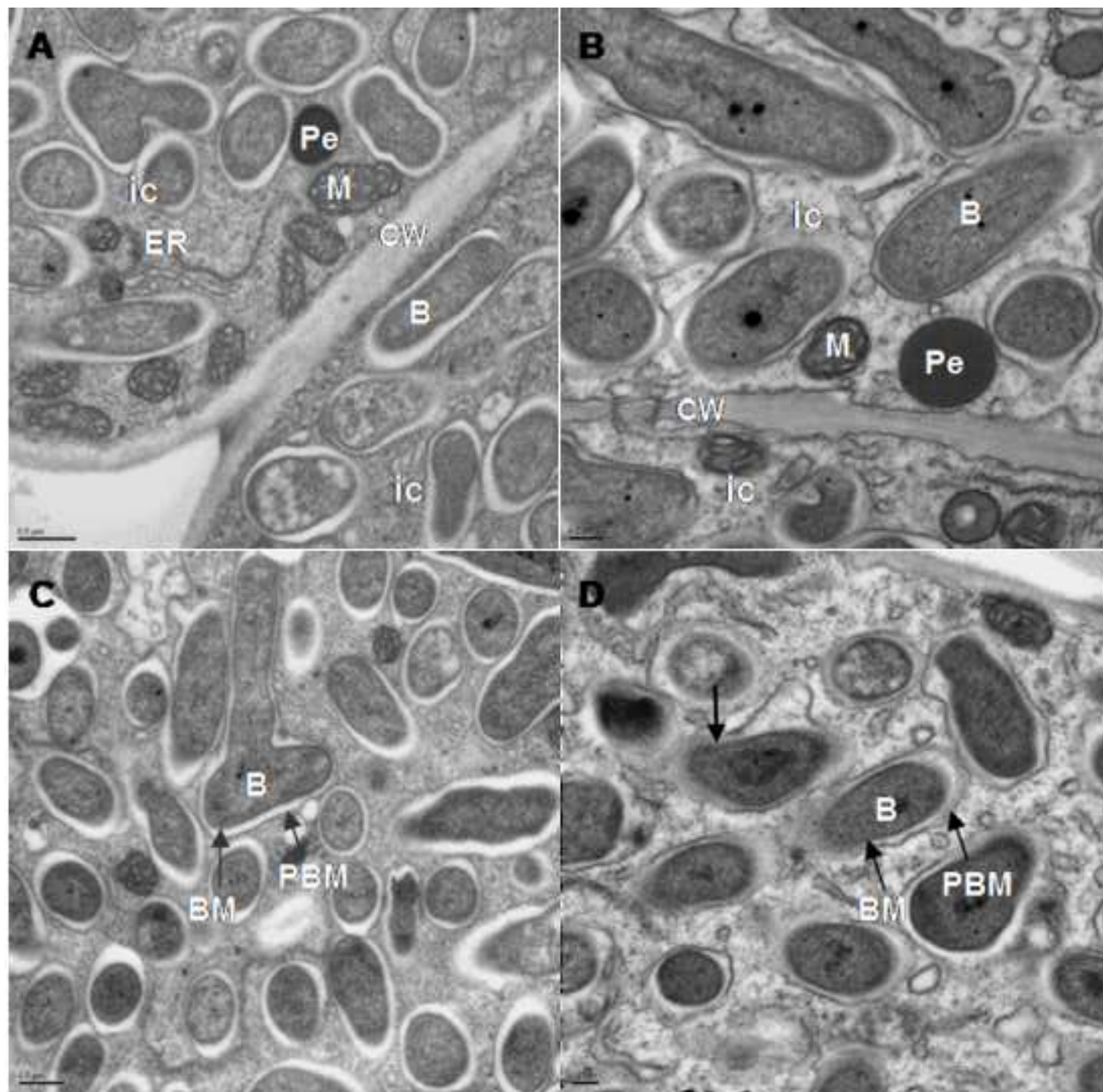**Click here to download high resolution image**

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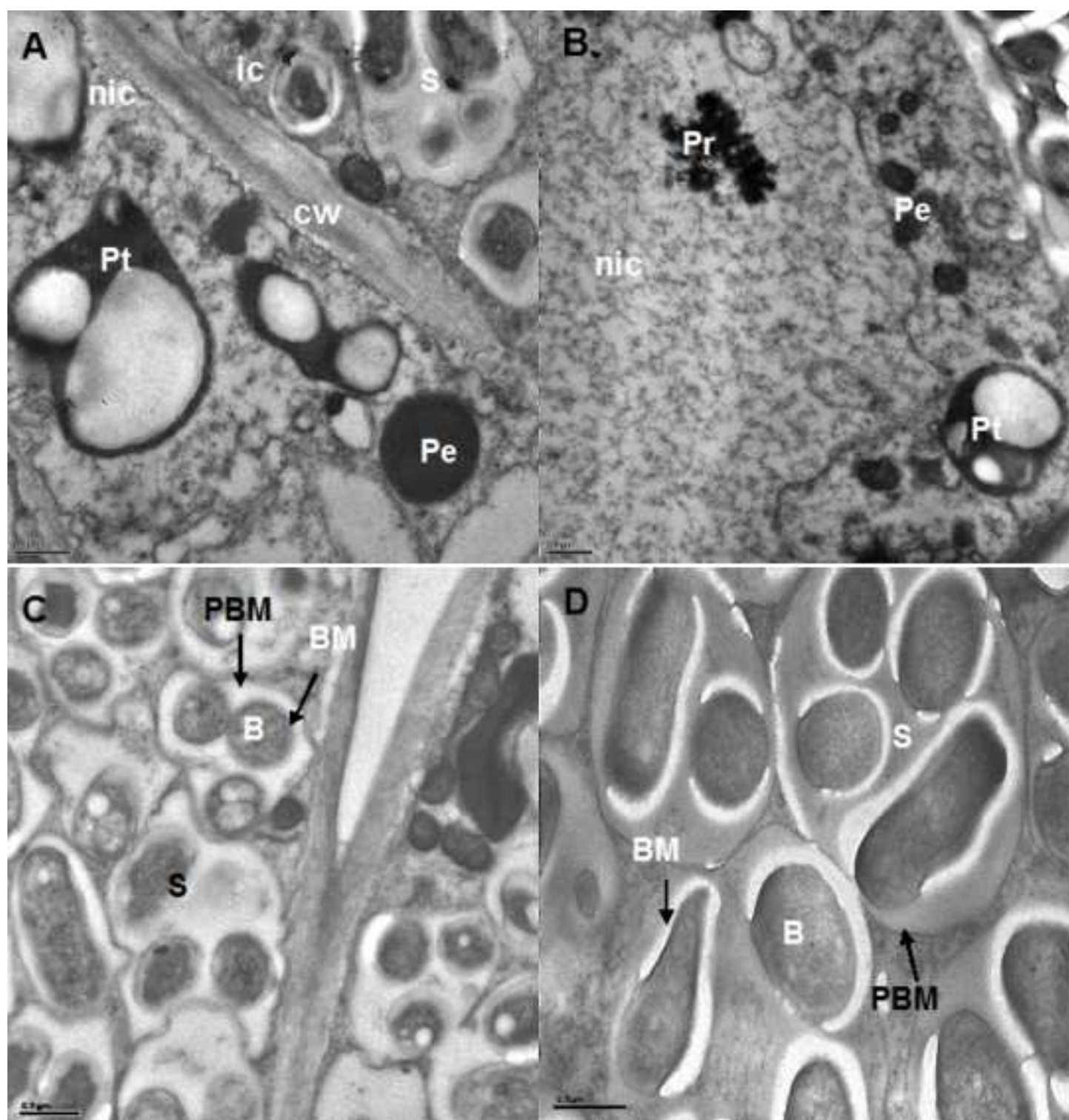Click here to download high resolution image

Table 1
Nodule biomass variables and total plant N content of white lupin and soybean plants grown for 35 days under the 1.6 and 192 μM Cu treatment conditions. 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	1.6	192
	White lupin		*Soybean*	
Nodule number per plant	34.67 ± 2.95^a	9.78 ± 0.71^b	13.78 ± 0.71^a	7.56 ± 0.17^b
Nodule weight (mg FW per plant)	488.33 ± 28.17^a	152.50 ± 21.87^b	265.56 ± 9.77^a	113.33 ± 7.26^b
Nodule normalised weight (mg DW nodule mg DW^{-1} shoot)	0.10 ± 0.01^a	0.13 ± 0.01^b	0.25 ± 0.01^a	0.29 ± 0.01^b
Total plant N content (mmol per plant)	1.93 ± 0.06^a	0.76 ± 0.02^b	3.14 ± 0.18^a	1.47 ± 0.05^b

Table 2
EDXMA-determined Cu localisation in transverse sections of 192 μM Cu-treated nodules viewed by LTSEM. Values are expressed as percentages of the total signal. Data are means ± S.E. ($n = 4$). Values in the same column followed by different letters differ significantly ($p < 0.05$).

Sample points	% Cu signal	
	Cell wall	Cytoplasm-Vacuole
White lupin		
Outer cortex cells	3.98 ± 0.23^a	2.52 ± 0.09^a
Inner cortex cells	5.20 ± 0.29^b	4.20 ± 0.07^c
Infected zone	6.16 ± 0.40^c	3.26 ± 0.33^b
Soybean		
Outer cortex cells	3.69 ± 0.57^a	2.63 ± 0.31^a
Inner cortex cells	1.77 ± 0.09^b	3.09 ± 0.34^a
Infected zone	nd	3.52 ± 0.28^a

nd: not detected

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

	Cu doses (μM)			
	1.6	192	1.6	192
	White lupin		*Soybean*	
MDA (nmol g^{-1} FW)	73.53 ± 2.44^a	108.48 ± 5.90^b	62.55 ± 1.68^a	91.56 ± 3.67^b
-SH (nmol g^{-1} FW)	799.22 ± 25.10^a	1067.52 ± 13.45^b	782.13 ± 23.45^a	1353.52 ± 22.75^b
SOD (Units mg^{-1} protein)	0.34 ± 0.09^a	2.38 ± 0.17^b	0.38 ± 0.04^a	1.06 ± 0.15^b
APX (Units mg^{-1} protein)	25.99 ± 0.81^a	29.11 ± 0.78^b	5.52 ± 0.44^a	16.60 ± 0.49^b
CAT (Units mg^{-1} protein)	50.10 ± 2.54^a	20.60 ± 1.36^b	3.22 ± 0.52^a	2.05 ± 0.32^a