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EXCESS COPPER EFFECT ON GROWTH, CHLOROPLAST ULTRASTRUCTURE, OXYGEN-EVOLUTION ACTIVITY AND CHLOROPHYLL FLUORESCENCE IN *GLYCINE MAX* (L.) Merr. CELL SUSPENSIONS

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ABSTRACT

The influence of excess copper on soybean photosynthetic cell suspensions was investigated. The cell suspensions grew well in the presence of 5-20 μM $CuSO_4$ and developed tolerance to even higher levels of $CuSO_4$ (*i.e.*, up to 50 μM), indicating that copper was not toxic to the cells at that high concentrations. Cu-adapted cell suspensions grew faster than the control in limiting light conditions and had higher content of chlorophyll per dry weight of cells. Copper was accumulated within the cells and this event was accompanied by *i)* increase oxygen evolution activity; *ii)* increased number of chloroplasts per cell, smaller chloroplasts, increased thylakoid stacking and grana size; *iii)* higher fluorescence emission of photosystem II antenna complexes; *iv)* stimulation of plastocyanin protein synthesis compared to untreated cells. Microanalysis of cross-sections revealed an increase of copper content in chloroplasts as well as vacuole, cytoplasm and cell wall in Cu-adapted cells. No antagonist interaction between copper and iron uptake took place in these cell suspensions. On the other hand, copper at sub-toxic concentrations stimulated oxygen evolution activity in thylakoids from control cells but this event did not take place in those from Cu-adapted ones. Furthermore, the loss of activity by copper inhibitory action at toxic concentrations was two fold slower in thylakoids from Cu-adapted cells compared with the control ones. The data strongly indicate that copper plays a specific positive role on photosynthesis and stimulates the growth and the oxygen evolution activity in soybean cell suspensions.

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichlorobenzoquinone; D1, protein encoded by the *psbA* gene of the photosystem II reaction center; EDX, energy dispersive X-ray; LHCII, light-harvesting antenna complex of photosystem II; MES, 2-(N-morpholino)ethanesulphonic acid; OECC, oxygen evolving core complex; OEC33, 33 kDa extrinsic protein of photosystem II; PS, photosystem; SOD, superoxide dismutase; SDS, sodium dodecyl sulphate; TEMED, N,N,N',N'-tetramethylethylenediamine.

INTRODUCTION

Copper (Cu) is an essential micronutrient for all photosynthetic organisms (*i.e.,* cyanobacteria, algae and plants) and plays an important role in numerous metabolic processes. In chloroplasts, Cu is a cofactor of several enzymes and metalloproteins that catalyze redox reactions (*i.e.,* plastocyanin, Cu/Zn superoxide dismutase, polyphenol oxidase) (Sadmann and Böger 1983; Droppa and Horváth 1990; Raven et al. 1999). The optimal average composition of Cu in plant tissue is 10 $\mu g\ g^{-1}$ dry weight (Baker and Senef 1995). At concentrations above those required for optimal growth Cu was shown to inhibit growth and to interfere with important cellular processes such as photosynthesis and respiration (Marschner 1995; Prasad and Strzałka 1999, Yruela 2005). Plant grown in the presence of high levels of Cu (3-100 μM) normally showed reduced biomass and chlorotic symptoms. A lower content of chlorophyll (Chl) and alterations of chloroplast structure and thylakoid membrane composition have been found in leaves of spinach, rice (*Oryza sativa L.*), wheat (*Triticum durum* cvs *Adamello* and *Ofanto*), bean (*Phaseolus L.* and *Phaseolus coccineus L.* cv. *Piekny*) in such growth conditions (Baszyński et al. 1988; Lidon and Henriques 1991, 1993; Ciscato et al. 1997; Pätsikkä et al. 1998; Quartacci et al. 2000). In particular, degradation of grana stacking and stroma lamellae, increase in the number and size of plastoglobuli, and appearance of intrathylakoidal inclusions were observed. It was proposed that Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes (Lidon and Henriques 1991; Maksymiec et al. 1994). Pätsikkä et al. (2002) attributed the reduction of Chl content to a Cu-induced Fe deficiency. The substitution of the central Mg ion of chlorophyll by Cu *in vivo* has also been proposed as an important damage mechanism leading to inhibition of photosynthesis (Küpper et al. 1996; 2003). Besides, lipid peroxidations, decrease of

lipid content and changes in fatty acid composition of thylakoid membranes were also shown (Sandmann and Böger 1980; Luna et al. 1994; Maksymiec et al. 1994). As a consequence of those modifications, an alteration of photosystem II (PSII) membrane fluidity was found (Quartacci et al. 2000). The mechanism of Cu toxicity on photosynthetic electron transport has extensively been studied *in vitro* and it was found that PSII is the most sensitive site to Cu toxicity. Both the acceptor and the donor sides of PSII were suggested as the main targets of Cu toxic action (Mohanty et al. 1989; Yruela et al. 1993; Schröder et al. 1994; Jegerschöld et al. 1995). Copper also increased susceptibility to photoinhibition in isolated thylakoids (Pätsikkä et al. 2001) or PSII-enriched membrane preparations (Yruela et al. 1996). Considering copper is an efficient catalyst in the formation of reactive oxygen species (ROS) it was suggested that the toxicity associated with this metal in plants was due, at least in part, to oxidative damage.

On the other hand, it has been reported that Cu is involved in photosynthetic reactions of PSII non-dependent of plastocyanin (a chloroplastic Cu-containing protein) (Lightbody and Krogmann, 1967; Barr and Crane, 1976). These authors reported the probably presence of a Cu-binding site close to the oxygen-evolving complex, which was sensitive to the action of Cu-chelators or the existence of a Cu-protein within PSII, respectively. More recently, Burda et al. (2002) found that Cu in an equimolar concentration to PSII reaction center stimulated *in vitro* the oxygen evolution activity of PSII. Nevertheless, little information in this respect exists *in vivo*.

Most of the studies dealing with the effect of excess Cu in higher plants show that its toxicity on growth and photosynthetic activity are the most general influences of excess Cu in higher plants. Studies on cell cultures from higher plants grown under stress conditions are limited and in particular little information is available on the effects of Cu on cell suspensions. In this respect, the present work is a significant

contribution to this field and may provide relevant information to advance in our knowledge of physiological aspects of leaf cells. The results are discussed in terms of the positive role of Cu on the structure/function of PSII *in vivo*.

MATERIALS AND METHODS

***Cell suspension growth conditions*.-** Photosynthetic cell suspensions from soybean (G*lycine max* var. Corsoy) SB-P line were grown as described by Rogers et al. (1987) with some modifications. These cell suspensions were established in our laboratory in 1990. Cell suspensions were grown in liquid cultures as follows: *a)* photomixotrophically under continuous low light (30 ± 5 μE m^{-2} s^{-1}); *b)* photomixotrophically under continuous normal growth light (65 ± 5 μE m^{-2} s^{-1}); *c)* photoautotrophically under continuous low light (30 ± 5 μE m^{-2} s^{-1}); *d)* photoautotrophically under continuous normal light (65 ± 5 μE m^{-2} s^{-1}). It has been reported that this kind of cultures grow optimally with 65-75 μE m^{-2} s^{-1} (Rogers et al. 1987; Alfonso et al. 1996) and that high light cause photoinhibitory effects in these cell suspensions (personal communication; Alfonso et al. 1996). On the other hand, it is known that Cu is a potential toxic element and its toxicity enhances with light. Thus, first of all we decided to assay a low light regime (30 ± 5 μE m^{-2} s^{-1}) following the light conditions used in previous works studying the copper stress effect on cell cultures (Gori et al. 1998) and compared the results with those obtained using control light regime. To assay the Cu effect on cell growth the media were supplemented with 5, 10, 20, and 50 μM $CuSO_4 \cdot 5H_2O$. Control medium corresponded to 0.1 μM $CuSO_4 \cdot 5H_2O$. To assay the Fe and Zn effect on cell growth the media were supplemented with 10 μM $FeSO_4 \cdot 7H_2O$ and 10 μM $ZnSO_4 \cdot 7H_2O$, respectively. All cell suspensions were grown under an atmosphere with 5% CO_2 at 24ºC on a rotatory shaker (TEQ, model OSFT-LS-R) at 110 rpm in 125 ml Erlenmeyer flasks filled up to 50 ml and illuminated with cool-white fluorescent lamps (Alfonso et al. 1996). It is worth mentioning that this kind of cultures demands for an elevated CO_2 concentration, at least 1% CO_2 (Rogers et al. 1987; Roitsch and Sinha, 2002) and

that CO_2 was not directly bubbled into the flasks. Cells used for physiological and biochemical experiments were collected from cultures after 18-21 days of growth.

Cell growth.- Cell suspension growth was measured by monitoring cell-packed volume and Chl concentration during the first 21 consecutive days of culture after the transfer (Alfonso et al. 1996). Cell-packed volume determination consisted in transferring 1 ml of cell suspension to an eppendorf tube and measuring the cell volume after sedimentation. Chlorophyll concentration was measured as described by Arnon et al. (1949).

Isolation of thylakoid membranes.- Soybean cells from 18-day-old cultures were collected, filtered through a layer of Miracloth (Boehringer) and weighted. Cells were then resuspended in buffer containing 400 mM NaCl, 2 mM $MgCl_2$, 0.2% (w/v) sucrose, and 20 mM Tricine, pH 8.0 at a cell to buffer ratio of 1:2 (w/v) and broken with a teflon homogeneizer during 10 min with 2 min delay every 2 min homogeneization to avoid sample heating at 4ºC in darkness. Broken cells were gently stirred for 10 min and centrifuged at 300 x g for 2 min. The supernatant (hereafter called cell extract fraction) was centrifuged at 13,000 x g for 10 min and the resultant sediment resuspended in buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tricine, pH 8.0 and centrifuged at 13,000 x g for 10 min. The pellet (thylakoids) was resuspended in buffer containing 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 50 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH, pH 6.0 and the supernatant fractions were concentrated. For Cu(II) inhibition experiments 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)-NaOH, pH 7.8 instead of Tricine, pH 8.0 was used to avoid chemical interference with Cu (II) (Renganathan and Bose, 1990). The thylakoid fractions were frozen in liquid nitrogen

and stored at -80ºC. Chlorophyll determination was done as described by Arnon et al. (1949).

Oxygen evolution activity.- Oxygen evolution activity was measured with a Clark-type oxygen electrode at 23ºC. The heat-filtered white light intensity on the surface of the cuvette was 3,000 μE m^{-2} s^{-1}. Cells (15 μg Chl ml^{-1}) and thylakoids (10 μg Chl ml^{-1}) were diluted in 3 ml buffer containing 10 mM NaCl, 300 mM sucrose, and 25 mM MES-NaOH, pH 6.5. For cation stimulation and Cu-inhibition effect on oxygen evolution activity experiments thylakoids were incubated with $CaCl_2$, $CuCl_2$, $FeCl_3$, and $ZnCl_2$ for 2 min at 23ºC under constant stirring in the dark before exposing the samples to heat-filtered white light. The temperature was maintained at 23ºC by circulating water from a temperature-controlled waterbath around the Clark cell. Cell and thylakoid activities were measured in the absence and presence of 0.5 mM 2,6-dichlorobenzoquinone (DCBQ) as artificial electron acceptor, respectively. DCBQ was dissolved in ethanol.

Immunoblotting analysis.- The analyses of plastocyanin were done in cell extract fractions. LHCII antenna complex and OEC33 proteins of PSII were assayed in intact and washed thylakoid membranes. Washed thylakoids were prepared from intact thylakoids by washing twice with 250 mM NaCl, 5 mM $MgCl_2$, and 50 mM MES-NaOH, pH 6.0. It is worth mentioning that the surfactant sucrose was eliminated in this buffer. The solutions used contained pefabloc (100 μg ml-1) as a protease inhibitor. The electrophoretic separation of PSII proteins was performed by SDS-PAGE using 12.5% (w/v) acrylamide gels containing 6 M urea according basically to Laemmli (1970). Gels were electroblotted to a nitrocellulose membrane with a BioRad transfer system and the immunodetection was done with a rabbit antibodies

against plastocyanin from *Scenedesmus vacuolatus* (a kind gift from Dr. M.L. Peleato, Biochemistry and Cellular and Molecular Biology Department, Zaragoza University, Zaragoza, Spain), D1 protein of PSII from a synthetic peptide homologous to the N-terminus, and LHCII antenna complex and OEC33 proteins of PSII from spinach (kindly provided by Dr. M. Barón, Estación Experimental del Zaidín, Granada, Spain). A goat anti-rabbit IgG coupled to horseradish peroxidase as a secondary antibody. Bands were revealed by the peroxidase method. The blots were scanned with a Studio Scan II Si (AGFA) and the intensity of the bands was quantified by densitometry using US National Institute of Health Software (NIH IMAGE) available at http://www.ncbi.nih.gov.

Microscopy imaging.- Images were obtained with a confocal laser scanning microscope (Zeiss, Mod. LSM 310) and a transmission electron microscope (Zeiss, Mod. EM 910). Cells were collected from suspension cultures, passed 20 times through a hypodermic syringe to disperse most cell aggregates. For confocal laser scanning microscopy excitation was generated with an argon laser at 488 nm and the resultant emission was filtered through a long pass filter >515 nm. The three-dimensional images were made up of several confocal sections at 0.5-1 μm increments through the sample by computer assisted microscopy. For transmission electron microscopy cells were fixed in 2.5% (w/v) glutaraldehyde (dissolved in 30 mM sodium phosphate buffer, pH 7.4) during 2 h at 25ºC. Then, cells were washed twice with sodium phosphate buffer, pH 7.4 and subsequently treated with 1% (w/v) osmium tetroxide solution, dehydrated through a series of acetone solutions, and embedded in Durcupan ACM (Fluka) epoxy resin. After polymerization during 48 h at 60ºC ultra-thin sections were obtained using a diamond knife ultramicrotome, and post-stained with lead citrate.

Determination of macro and micronutrient elements.- Cells from 20-day-old suspensions were washed twice with 3 mM EDTA and once with destilled H_2O to remove free cations. After washing, cells were filtered through a layer of Miracloth and dried in a ventilated oven at 60ºC for 48 h. Analyses were performed in an atomic absorption spectrometer (UNICAN 969).

Energy-dispersive X-ray microanalysis.- Cells from 20-day-old suspensions were cry fixed using a high-pressure freezer (Leica EMPact; Leica Microsystems, Gladesville, Australia) and were then stored in liquid nitrogen until freeze-substitution. Frozen cells were freeze-substituted with diethyl ether containing 10% acrolein with freshly activated 5Å molecular sieve (Baker Analyzed) for 4 days at -90ºC, 2 days at -70ºC and 1 day at -30ºC. Subsequently, samples were gradually brought to 4ºC by raising the temperature for 1.5 h. Diethyl ether was the freeze-substitution solvent chosen because it does not lead to redistribution of ions, even for highly diffusible elements (Bidwell et al. 2004). The freeze-substituted cells were infiltrated with Araldite resin over 4 days. Araldite contains negligible levels of elements detectable by energy-dispersive spectrometry (Pålsgård et al. 1994). Cell sections were cut dry at 0.25, 0.5 and 1.0 μm using an ultramicrotome (Leica Ultracut UCT) and mounted directly onto titanium grids (75 mesh) that were coated with formvar. Sections were stored in a desiccator under vacuum until analysis to prevent absorption atmospheric water and hence possible redistribution of ions.

Unstained sections were analysed at room temperature in a carbon holder by energy-dispersive X-ray microanalysis using a transmission electron microscope (model H-800-MT, Hitachi) and X-ray EDX Quantum detector (model 3600, Kevex). The detector was interfaced to a signal processing unit (Röntec). The electron

microscope was operated at 100 kV in STEM mode with a spot size 10-15 nm and a beam current 15 μA. The spatial resolution of analysis in a 0.5 μm section at 120 kV was estimated to be better that in a 0.25 μm and 1.0 μm sections. The spectral data from individual cells and compartments in each section were collected using selected area analysis with an acquisition time of 300 s. Analyses were carried out by Quantax 1.5 program (Röntec). Spectra were normalized at titanium peak used as internal standard.

Fluorescence measurements.- Thylakoid membranes isolated from 18-day-old suspensions were resuspended in 400 mM sucrose, 20 mM NaCl, 5 mM $MgCl_2$, and 50 mM MES-NaOH, pH 6.0, placed in a 3-mm quartz tube and frozen in liquid nitrogen. Fluorescence spectra were obtained at 77 K by exciting the samples with a 1,000 W ORIEL 66187 tungsten halogen lamp and a double 0.22 m SPEX 1680B monochromator. Excitation was carried out at 470 nm. Fluorescence was detected through a 0.5 JARREL-ASH monochromator with a Hamamatsu R928 photomultiplier tube. All the measurements were corrected from the system response. The spectral linewidths (FWHM) for the excitation and the emission were 3.6 nm and 1.92 nm, respectively.

RESULTS

Soybean cell suspension growth

The influence of Cu on soybean photosynthetic cell suspension growth was investigated under two different conditions; *i.e.*, low-sucrose content medium (photomixotrophic growth) and minimal medium with CO_2 as the sole carbon source (photoautotrophic growth). Addition of 5, 10, and 20 μM $CuSO_4$ to the control media that already contained a sufficient amount of Cu for culture growth did not reduce cell growth rate under both 30 ± 5 μE m^{-2} s^{-1} (low light) and 65 ± 5 μE m^{-2} s^{-1} (control light) illumination regimes. Control and Cu-treated cell cultures showed the same doubling time and Chl content rate during the first 22 days of growth (equivalent to one transfer, data not shown). Interestingly, a different feature was observed after two transfer in media containing excess $CuSO_4$ (5-20μM) and under low light illumination. In these light conditions cells grew faster either photomixotrophically (Fig. 1A) or photoautotrophically (Fig. 1C). It is worth mentioning that the average volume of individual cell did not change with Cu treatment (data not shown). During the growth exponential phase the Chl content of these Cu-treated cultures was also 2.5-3.0 fold that of control suspensions (Fig. 1B,D). These findings were independent on the presence of sucrose in the medium, therefore the participation of a sucrose-dependent mechanism in this faster growth could be discounted. No enhancement of growth rate was observed in the same conditions under 65 ± 5 μE m^{-2} s^{-1} which corresponded to the normal illumination in our cell suspension growth conditions (Alfonso et al. 1996). Control and Cu-treated cells exhibited similar growth rate and Chl content under normal illumination grown either photomixotrophically (Fig. 1E,F) or photoautotrophically growth (data not shown). Higher $CuSO_4$ concentrations up to 50 μM were somewhat toxic as reflected by a change of colour and slower growth

(Fig. 1A,B), but tolerance to up 50 μM $CuSO_4$ was achieved in cultures previously grown in 20 μM $CuSO_4$ for twenty transfers (Fig. 1A,B, dashed line). Since the results indicate that Cu stimulates the growth of soybean cell suspensions after two transfer under limiting illumination, the rest of the results of the present work correspond to those obtained under these conditions of growth (hereafter called Cu-adapted cells) unless stated otherwise.

Oxygen evolution activity from cell suspensions and thylakoids

Oxygen evolution activity measurements were performed in cell suspensions and thylakoids from those cells. Cells from control and Cu-adapted 18-day-old cultures exhibited 57±5 and 74±5 μmol O_2 mg^{-1} Chl h^{-1}, respectively, with no artificial electron acceptor added to the reaction mixture. Similar activity values have been reported in intact chloroplasts and alga cells (Rai et al. 1991). A similar difference ratio was observed in thylakoids from the cell suspensions. Indeed, thylakoids from cells exposed to high content Cu showed on average a 30-45% increase of oxygen evolution activity compared to the control using 2,6-dichloro-*p*-benzoquinone (DCBQ) as artificial electron acceptor to PSII (Table 1). Since no modifications in the PSII/PSI ratio took place in such growth conditions as indicated by the Chl*a*/Chl*b* ratio, the observed results could respond to a specific positive role of Cu on the structure/function of the PSII. This point was investigated in thylakoid membranes isolated from control and Cu-adapted cell suspensions. The results showed a 20% increase of oxygen evolution activity when control thylakoids were incubated with 1.0 μM Cu(II) (Fig. 2A). This oxygen evolution stimulation was somewhat lower than that observed *in vivo* maybe due to different cation accessibility in isolated thylakoids and intact cells. In order to know whether this phenomenon is specific to Cu(II) or the

response to a more general cation effect the oxygen evolution activity was also measured in control thylakoids incubated with other cations such as Ca(II), Fe(III), and Zn(II). It is known that Ca participates in the oxygen-evolving complex and stimulates its activity. On the other hand, Fe and Zn can inhibit the oxygen evolution activity by production of reactive oxygen species (ROS) through Fenton's reaction and by dissociation of extrinsic proteins of the oxygen-evolving complex, respectively (Rashid et al. 1994; Yruela et al. 1996). All the cations assayed stimulated the thylakoid oxygen evolution activity up to a maximum of 15-18% (Fig. 2A) though the range of cation concentrations varied for each element being in the same order of magnitude for Ca(II) and Cu(II), somewhat lower for Fe(III) and higher for Zn(II). Interestingly, *in vitro* cation stimulation was not found in thylakoids isolated from Cu-adapted cells (data not shown) indicating that Cu already has a specific positive role on oxygen evolution activity *in vivo*.

The effect of addition of toxic Cu(II) concentrations (10-100 μM) on the oxygen evolution activity of thylakoid preparations was also examined (Fig. 2B). A 50% inactivation occurred with ~ 24 μM Cu(II) and ~50 μM Cu(II) in thylakoids from control and Cu-adapted cells, respectively. The 50% inactivation in thylakoids from control cells was similar to that previously reported in spinach thylakoids (Yruela et al. 2000). The loss of activity by Cu(II) inhibitory action at toxic concentrations was two fold slower in thylakoids from Cu-adapted cells compared with those from control ones.

Effect of Cu on the OEC33 protein

The composition of LHCII antenna complex, D1 protein, and OEC33 extrinsic protein of PSII in thylakoid preparations from control and Cu-adapted cells before and after twice washing steps was assayed (for details see Materials and Methods). The immunoblot analyses showed that the OEC33 extrinsic protein partially released

after washing of thylakoids from control cells (Fig. 3, *lane* 5). A minor OEC33 loss was detected in thylakoids from cells grown with 5 μM Cu(II) (Fig. 3, *lane* 6). Indeed, no effect of washing steps on OEC33 protein was observed in thylakoids from cells grown with 10 μM and 20 μM Cu(II) (Fig. 3, *lane* 7,8). LHCII antenna complex and D1 protein of PSII composition did not vary in all the conditions assayed. Assuming that ratio of OEC33/ LHCII proteins is 1:1 in intact PSII preparations, the OEC33/LHCII ratio calculated was 0.63±0.11, 0.89±0.16, 1.0±0.18 and 1.0±0.14 in control (*lane 5*) and 5 μM, 10 μM, 20 μM Cu-adapted (*lanes 6-8*) thylakoids, respectively, after washing. A ratio value of 1.0 was calculated for either control or Cu-adapted thylakoids assayed before washing steps (Fig. 3 *lanes 1-4*). OEC33/D1 ratios varied with the same trend.

Cu-uptake by cell suspensions and its intracellular distribution

At this stage of development of the work it would be important to know how much Cu did really enter into the cells used in the above described experiments. Determination of Cu demonstrated that this element is accumulated in the cells during treatments. The Cu concentration increased on average from 6 μg g^{-1} dry weight in the control to 350 μg g^{-1} dry weight in the highest Cu concentration treatment (Table 2). The data showed certain differences between photomixotrophic and photoautotrophic cultures. Cells grown photomixototrophically accumulate much higher Cu upon Cu treatment. We also found that Fe- and Zn-uptake was affected in the same and opposite direction, respectively. The concentration of Fe increased on average 1.4 fold in the cells treated with 20 μM $CuSO_4$ whereas Zn content was on average 2.0 fold lower in that condition. Interactions between micronutrients affecting absorption and bioavailability have also been reported (Sandström 2001). On the

other hand, Mn (Table 2) and Ca, Mg, K and P (data not shown) content was not influenced by Cu accumulation.

Elemental analysis of cell cross-sections revealed that Cu is predominantly localized in the chloroplast of control cells (Fig. 4A,a) and that Cu content increases in this organelle of Cu-adapted cells (Fig. 4B,a). The results also showed that Cu content increases in all the sub-cellular compartments of the Cu-adapted cells analyzed. Overall, Cu appeared to be distributed in chloroplasts, vacuole, cytoplasm and cell wall of Cu-adapted cells (Fig. 4B,b-d). No accumulation of electro-dense material was observed in the cells analyzed. The data also showed an increase of Fe within Cu-adapted cells compared to control ones that preferentially was localized in cytoplasm and wall cell compartments. These results are in agreement with micronutrient content analysis (Table 2).

Morphological and ultrastructural changes in chloroplasts from Cu-adapted cell suspensions

The presence of high Cu concentration was apparently not toxic to the cells but it would be interesting to know if the high accumulation of Cu within the cells induced some changes in the morphology and ultrastructure of chloroplasts. Confocal laser scanning microscopy experiments on 18-day-old cells treated with excess Cu showed that the size of chloroplasts was altered by the treatment. Chloroplasts from Cu-adapted cells were more numerous and smaller than that from control cells and showed brighter autofluorescence compared with control ones (Fig. 5A,B). Transmission electron micrographs of chloroplasts from 18-day-old Cu-adapted cells were consistent with these findings and showed that changes in the organelle morphology were accompanied by ultrastructure modifications (Fig. 5C,D). Cells grown in the presence of excess Cu exhibited more thylakoid stacking than the

control. Analysis of micrographs (Table 3) showed that the thylakoid/chloroplast area ratio was somewhat higher in Cu-adapted cells. A 20% increase of thylakoid area ratio was reached in the presence of 10-20 μM $CuSO_4$. On the other hand, significant changes of granal distribution were observed upon growth in the presence of excess Cu(II) (Fig. 6). Cu-adapted cells had wider grana than control cells. It is worth mentioning that the cells used for transmission electron microscopy were fixed within 12 min period because changes in irradiance can rapidly initiate modifications of granal appression (Rozak et al. 2002).

Other chloroplast characteristics were also modified in Cu-adapted cells. For instance, total Chl content per cell dry weight on average was 25% higher Table 1). The activation of Cu/ZnSOD synthesis (data not shown) and an increase of plastocyanin content (another chloroplastic Cu-containing protein) (Fig. 3) was observed. Thylakoids had a higher content of C18:2 and C18:3 fatty acids while C16:0, C18:0, and C18:1 levels were lower in comparison to control membranes (data not shown). These findings could be related to the higher thylakoid stacking observed in Cu-adapted cells.

Fluorescence spectral changes induced by Cu treatments

Fluorescence spectra at 77K are often used to monitor ultrastructural changes in thylakoid membranes as a response to environmental condition variations (Anderson 1999; Rozak et al. 2002). Thus, the F_{735}/F_{685} and F_{735}/F_{695} ratios can be used as a probe for the amount of antenna Chls unconnected to each photosystem (van Dorssen et al. 1987; Alfonso et al. 1994). Cells treated with excess Cu normally had the lowest F_{735}/F_{685} ratio values whereas higher values were found in control cells, independently of growth conditions (Table 1). Similar variations were found for the F_{735}/F_{695} ratio. The data indicate that the presence of Cu induces a higher

fluorescence emitted mainly by antenna complexes associated to PSII compared to the control.

Effect of Fe and Zn on chloroplast structure and oxygen evolution activity

In order to know if Fe and Zn have a similar influence as Cu *in vivo*, excess of these cations was supplemented to the growth media. Cells grown in medium supplemented with 10 μM $FeSO_4$ or 10 μM $ZnSO_4$ under low light illumination exhibited a slightly faster growth rate compared with the control but slightly slower one compared with those grown in the presence of excess $CuSO_4$ (data not shown). Oxygen evolution activity of thylakoid membranes from Fe- and Zn-treated cells was only about 5-10% higher than the control but 20-25% lower than that measured in thylakoids from Cu-adapted cells (Table 1). On the other hand, no significant differences in Chl*a*/Chl*b* ratio values and F_{735}/F_{685} and F_{735}/F_{695} ratios were found after 18 days of growth but higher Chl content compared to the control was observed (Table 1). Determination of micronutrients (Table 2) showed that Fe is accumulated by cells grown either in the presence of 10 μM $FeSO_4$ or 10 μM $ZnSO_4$ while Cu-uptake is not significantly influenced. Fe accumulation by cells on average was 2-fold the control value. The data also showed that *i)* Zn treatment results in high increase of Fe accumulation (exceeding that under Fe treatment) with concomitant decrease of Zn content and increase of Mn content; *ii)* the increase of Fe accumulation induced by Fe treatment was accompanied by increase in Zn accumulation and decrease of Mn. These changes in Mn level contrast with those taking place with Cu treatment. Analysis of micrographs revealed differences between chloroplasts from cells grown in the presence of excess Cu(II), Fe(II), and Zn(II). Fe- and Zn-treated cells showed that: *i)* the thylakoid per chloroplast area ratio was somewhat lower; and *ii)* grana were slightly narrower compared with those from Cu-adapted cells (Table 3).

DISCUSSION

Our data demonstrated that in contrast to what commonly happens in whole plants the supplementation of excess Cu to the soybean SB-P line cell suspensions caused a high accumulation of this element within the cells but that such Cu content was apparently not toxic. Soybean cell suspensions used in this study grew well in 5-20 μM $CuSO_4$. Indeed, they develop tolerance to even higher levels of Cu (*i.e.*, up to 50 μM). Only a few works have been published concerning to excess Cu effect on plant cell suspensions. Several of them showed tolerance to Cu (80-100 μM) in cell suspension cultures from *Nicotiana plumbaginifolia, Nicotiana tabacum L.,* and *Acer pseudoplatanus L.* (sycamore) (Kishinami and Widholm, 1986; 1987; Turner and Dickinson 1993; Gori et al. 1998; Raeymaekers et al. 2003). These works were limited and they partially report physiological aspects of this subject. Thus, the extensive study presented in this paper with soybean cell suspensions can be useful to understand some of the mechanisms underlying Cu acclimatation or tolerance in cells of higher plants established from leaves.

Soybean Cu-adapted cells showed a higher growth rate than the control under limiting illumination after an adaptive period of one transfer. The increase in measured cell volume can be explained by additional cell division since the volume of individual cell did not change with Cu treatment (data not shown). The higher growth was accompanied by a higher Chl content probably due to a Cu stimulation of Chl synthesis under low light compensating the effect of light limitation. Recently, the role of Cu as a regulatory element of Chl synthesis has been described (Moseley et al. 2002; Tottey et al. 2003). It is well-known that Fe is essential for an optimal formation of the photosynthetic apparatus (Raven et al. 1999). Therefore, the fact that Fe-uptake slightly increased in Cu-adapted cells could in principle explain the significant

increase of Chl content, thylakoid appresion and grana size, and photosynthetic activity observed in these cells. However, the stimulation of the oxygen evolution activity (30-45%) observed in Cu-adapted cells cannot be only explained by the Fe level increase. In this sense our *in vitro* experiments of cation-stimulating effect of PSII activity and *in vivo* experiments supplying excess Cu(II), Fe(II), and Zn(II) to the growth media are in agreement with that. It is worth mentioning that: *i) in vitro* cation stimulation was not found in thylakoids isolated from Cu-adapted cells; *ii)* Fe was accumulated in cells exposed to excess of either Cu(II), Fe(II), or Zn(II) but the oxygen evolution activity of PSII was preferentially activated in thylakoids from Cu-adapted cells where Cu-uptake was significantly increased. Interestingly, no increase of Cu-uptake was observed in cells exposed to excess Fe(II) or Zn(II). Therefore, the data indicate that Cu has a specific positive role in photosynthesis and particularly in the structure/function of PSII. The presence of higher Cu content within chloroplasts of Cu-adapted cells supports this picture. It is clear, therefore, that the high Cu content in these cells is not toxic but modulates photosynthesis at least at levels: Chl synthesis, PSII activity and plastocyanin content. In support of our findings, it has been described that leaves of Cu-deficient plants and *Arabidopsis thaliana* mutants defective in Cu-transport into the chloroplast (Shikanai et al. 2003) exhibited reduced Chl content and photosynthetic electron transport. Cu-adapted cells also showed a higher fluorescence emission of PS II antenna complexes compared to untreated cells in all the conditions assayed. This finding could respond to: *i)* a higher PSII/PSI ratio; or *ii*) a higher level of stacking and aggregation of PSII surrounding antenna complexes due to non-specific electrostatic interactions between membrane and protein negative charged surfaces and Cu(II) cations reducing repulsion effects (Izawa and Good 1966; Chow et al. 1980). The first hypothesis can be discounted since the determined Chl*a*/Chl*b* ratio was 3.0 $\pm$ 0.1 for all the cultures conditions

assayed (Table 1). Thus, our results are more in agreement with the second hypothesis. An increase of fluorescence from the purified LHCII antenna complex has been reported in the presence of cations (Kirchhoff et al. 2003).

It is known that organization of PSII in granal and stromal membranes present important differences. PSII exists in a dimeric form in granal membranes while only a monomeric form of PSII is found in stroma lamellae (Bassi et al. 1995). It has been also found that the polypeptide composition of the oxygen-evolving core complexes (OECC) in both PSII forms is similar except that the level of OEC33 extrinsic protein associated to monomeric PSII forms is lower (Bassi et al. 1995; Hankamer et al. 1997). Furthermore, isolated dimeric OECC preparations are more stable, contain higher levels of Chl and exhibit a higher oxygen-evolution activity than monomeric OECC preparations (Hankamer et al. 1997). Taking into account the above considerations our results would be in agreement with a higher presence of dimeric PSII complexes in the thylakoids of Cu-adapted cells, associated with the higher level of granal membranes observed in these cells. In that case the proportion of monomeric PSII forms should be higher in control cells. Considering this scenario the partial release of the OEC33 extrinsic protein by washing steps in thylakoids from control cells could be explained by the presence of a certain amount of OEC33 proteins not firmly bound to PSII complexes in stromal membranes. The presence of the surfactant sucrose in the buffer might favour the precipitation of these not firmly bound OEC33 proteins in the thylakoid fraction and the washings with buffer without sucrose release that. In this respect, it is worth mentioning that the OEC33 protein is synthesized as a precursor protein in the cytoplasm, it binds to PSII cores or minimal PSII complexes in the stromal regions and then it migrates to the grana (Hashimoto et al. 1997). Nevertheless further experiments are needed to clarify this issue. In the past, the structural role of Cu in PSII was discussed extensively (Barón et al. 1995)

but no definitive conclusions were attained. Accordingly to Burda et al. (2002) we observed that Cu(II) at sub-toxic concentrations increases oxygen evolution activity *in vitro* in isolated thylakoids from control cells but it has no effect on thylakoids from Cu-adapted cells. Interestingly, inhibition of oxygen evolution activity by concentrations of 10 μM Cu and above was 2-fold less in thylakoids from Cu-adapted cells than from control ones. This finding could be explained by: *i)* a Cu-binding site less accessible in the former case probably due to conformational changes in the neighbour of the Cu-binding site; *ii)* an extra metal sequestering or chelating capacity developed in Cu-adapted cells; *iii*) less capacity of Cu ions to damage due to changes in membrane lipids or ultrastructure. Kruk et al. (2003) showed that the OEC33 protein contains a low-affinity binding site for several cations, *i.e.,* Ca, and certain lanthanides and that this metal binding induces protein conformational changes that would stabilize the optimal conformation of PSII reaction center proteins involved in Mn-Ca coordination. In this respect Burda et al. (2001) showed that Ca, lanthanides and low Cu concentrations similarly stimulate *in vitro* the oxygen evolution activity of PSII membranes.

In conclusion, the photosynthetic soybean cell cultures established from leaves used in this study can accumulate high content of Cu in the cells with no apparent toxicity. The high Cu content accumulated by cells stimulates cell growth and Chl synthesis under low irradiance probably due to a Cu stimulation. In addition Cu-adapted cells showed higher photosynthetic activity and some morphological changes in chloroplast and thylakoid ultrastructures. The data support the picture of Cu as a positive element affecting and mediating *in vivo* the structure/function of PSII.

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FIGURE LEGENDS

Figure 1.- Cell volume (A,C,E) and chlorophyll content (B,D,F) of soybean cell cultures growing under low light (A,B,C,D) and control light (E,F) illumination photomixotrophycally (A,B,E,F) and photoautotrophycally (C,D) in the absence and presence of excess Cu(II). ○,●,▲,■,▼ correspond to 0.1 (control), 5, 10, 20, and 50 μM $CuSO_4 \cdot 5H_2O$, respectively. Dashed line corresponds to culture previously adapted to 20 μM Cu(II) and subsequently grown in medium supplemented with 50 μM Cu(II). Data are representative of four independent experiments.

Figure 2.- (A) Oxygen evolution activities of thylakoid membranes from control 18-day-old soybean cells incubated with $CuCl_2$ (0, 0.5, 0.75, 1.0, 1.5 μM), $FeCl_3$ (0, 0.05, 0.1, 0.2, 0.5 μM), $ZnCl_2$ (0, 0.75,1.0, 2.0, 10.0 μM) and $CaCl_2$ (0, 0.05, 0.1, 0.75, 1.0 μM). (B) Inhibition of oxygen evolution activity of thylakoid membranes from control (○) and 5 μM (■), 10 μM (▲), 20 μM (▼) Cu-treated cells in the presence of 10-100 μM $CuCl_2$. The activity was measured in the presence of 0.5 mM DCBQ. One hundred percent activity corresponded to 198, 258, 269, 268 μmol O_2 mg^{-1} Chl h^{-1} in thylakoids from control and Cu-treated cells, respectively. Cells were grown under low light illumination. Data are representative of four independent experiments.

Figure 3.- (A) Immunoblots with antiserum anti-LHCII antenna complex, anti-D1 and anti-OEC33 extrinsic proteins of intact (*lanes* 1-4) and twice washed (*lanes* 5-8) thylakoid membranes. *Lanes 1,5)* control; *2,6)* 5 μM Cu(II); *3,7)* 10 μM Cu(II); *4,8)* 20 μM Cu(II). (B) Immunoblots with anti-plastocyanin serum. *Lanes 1)* control*; 2)* 5 μM Cu(II); *3*) 20 μM Cu(II). The same amount of protein (25 μg in (A) and 16 μg in (B))

was loaded in each gel lane. Cells were grown in medium supplemented with the Cu(II) concentrations specified above. Data are representative of three independent experiments. Other experimental conditions as in Fig. 2.

Figure 4.- Energy dispersive X-ray spectra in the 4.0-10.0 keV range of different sub-cellular compartments of individual cells from 18-old-day cell suspensions grown with 0.1 μM (control) (A) and 10 μM (B) $CuSO_4 \cdot 5H_2O$. *a*) chloroplast; *b*) vacuole; *c*) cytoplasm; *d*) cell wall; *f*) sum of *a,b,c* and *d* spectrum. Spectra were normalized at titanium peak intensity as internal standard. Typically, 8 cells of each type were analysed. Each spectrum represents the mean of four spectra of each cell analysed. Y-axe scale for *f* was double. Other experimental conditions as in Fig. 2.

Figure 5.- Confocal laser scanning images of cells (A,B) and transmission electron micrographs of chloroplasts (C,D). Control cells and chloroplasts (A,C); cells and chloroplasts in the presence of 10 μM $CuSO_4$ $5H_2O$ (B,D). For confocal images excitation wavelength was 488 nm and focal distance was 1 μm. White bar in A and B correspond to 35.7 μm; black bar in C and D is 1.2 μm; bars in insets are 0.16 μm. Images are representative of four independent experiments. Other experimental conditions as in Fig. 2.

Figure 6.- Granal size distribution changes in chloroplasts from 18-day-old soybean cells grown in photomixotrophic (A) and photoautotrophic (B,C) conditions under low light illumination. Control (black), 5 μM $CuSO_4$ $5H_2O$ (dark gray), 10 μM $CuSO_4 \cdot 5H_2O$ (light gray), 20 μM $CuSO_4 \cdot 5H_2O$ (white), 10 μM $FeSO_4 \cdot 7H_2O$ (right dash), 10

μM $ZnSO_4 \cdot 7H_2O$ (left dash). Data were obtained on average from 10 images from three independent cultures for each condition.

TABLE 1

Photosynthetic parameters of thylakoids from soybean cell suspensions

Samples	Chlorophyll (mg g^{-1} dry weight)	Oxygen evolution (μmol O_2 mg^{-1} Chl h^{-1})	Chl *a*/Chl *b*	F_{735}/F_{685}	F_{735}/F_{695}
Soybean cell suspension					
Photomixotrophic growth					
Control	3.16 ± 0.5	189 ± 7	3.1	3.1	3.0
5 μM Cu(II)	3.93 ± 0.3	243 ± 12	3.1	2.9	2.8
10 μM Cu(II)	3.85 ± 0.6	248 ± 11	3.1	2.4	2.5
20 μM Cu(II)	3.76 ± 1.1	245 ± 14	3.0	2.3	2.4
Photoautotrophic growth					
Control	3.06 ± 0.7	152 ± 9	3.0	2.4	2.6
5 μM Cu(II)	3.68 ± 0.6	216 ± 8	3.0	2.3	2.5
10 μM Cu(II)	3.98 ± 0.6	232 ± 4	3.0	1.7	2.0
20 μM Cu(II)	3.89 ± 0.6	198 ± 9	2.9	1.6	2.0
10 μM Fe(II)	4.24 ± 0.5	184 ± 2	2.9	2.5	2.6
10 μM Zn(II)	4.12 ± 0.6	179 ± 4	2.8	2.5	2.5

[1] Soybean cell cultures were grown in media supplemented with $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ or $ZnSO_4 \cdot 7H_2O$. Cells were analysed after 18 days of growth. Data were obtained from four independent cultures for each condition.

TABLE 2

Micronutrient content [1] in soybean cell suspensions

Samples	Cu	Fe	Mn	Zn
Soybean cell suspensions[2]				
Photomixotrophic growth				
Control	5.9 ± 1.6	282.2 ± 7.0	23.2 ± 0.3	351.1 ± 8.3
5 μM Cu(II)	136.8 ± 2.7	319.5 ± 4.7	23.9 ± 0.2	164.9 ± 3.3
10 μM Cu(II)	209.5 ± 5.3	328.1 ± 4.3	21.9 ± 0.3	161.3 ± 4.3
20 μM Cu(II)	351.2 ± 7.5	389.8 ± 3.9	23.8 ± 0.2	174.3 ± 3.2
Photoautotrophic growth				
Control	9.8 ± 2.3	715.1 ± 9.3	31.3 ± 0.4	395.4 ± 4.3
5 μM Cu(II)	99.4 ± 2.2	754.0 ± 4.7	31.0 ± 0.1	226.0 ± 6.3
10 μM Cu(II)	148.4 ± 4.3	1411.9 ± 8.3	36.4 ± 0.3	197.3 ± 3.3
20 μM Cu(II)	221.2 ± 5.5	1197.5 ± 4.8	40.5 ± 0.2	179.3 ± 5.2
10 μM Fe(II)	9.1 ± 2.0	1372.3 ± 9.3	17.0 ± 0.4	413.7 ± 5.3
10 μM Zn(II)	13.8 ± 2.1	1690.7 ± 9.3	147.5 ± 0.4	299.1 ± 4.3

[1] $\mu g\ g^{-1}$ dry weight

[2] Soybean cell cultures were grown in media supplemented with $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$. Cells were analysed after 20 days of growth. Data were obtained from four independent cultures for each condition.

TABLE 3

Morphometric analysis of chloroplasts from soybean cell suspensions[1]

Measurement	Control	+ Cu(II)			+ Fe(II)	+ Zn(II)
		5 μM	10 μM	20 μM	10 μM	10 μM
Photomixotrophic growth						
Chloroplast surface, μm^2	3.7 ± 0.5	2.9 ± 0.1	2.6 ± 0.3	2.6 ± 0.5	3.5± 0.2	3.7± 0.3
Thylakoid/Plastid surface	0.41 ± 0.08	0.42 ± 0.08	0.48 ± 0.07	0.54± 0.04	0.41± 0.1.	0.39± 0.1.
Photoautotrophic growth						
Chloroplast surface, μm^2	2.9 ± 0.5	2.7 ± 0.6	2.6 ± 0.5	1.7 ± 0.4	3.3 ± 0.8	3.0 ± 0.8
Thylakoid/Plastid surface	0.37 ± 0.03	0.45 ± 0.05	0.46 ± 0.08	0.44± 0.04	0.40 ± 0.05	0.41 ± 0.04

[1] Soybean cell cultures were grown in media supplemented with $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ or $ZnSO_4 \cdot 7H_2O$. Cells were analyzed after 18 days of growth. Data were obtained on average from 10 images from three independent cultures for each condition.

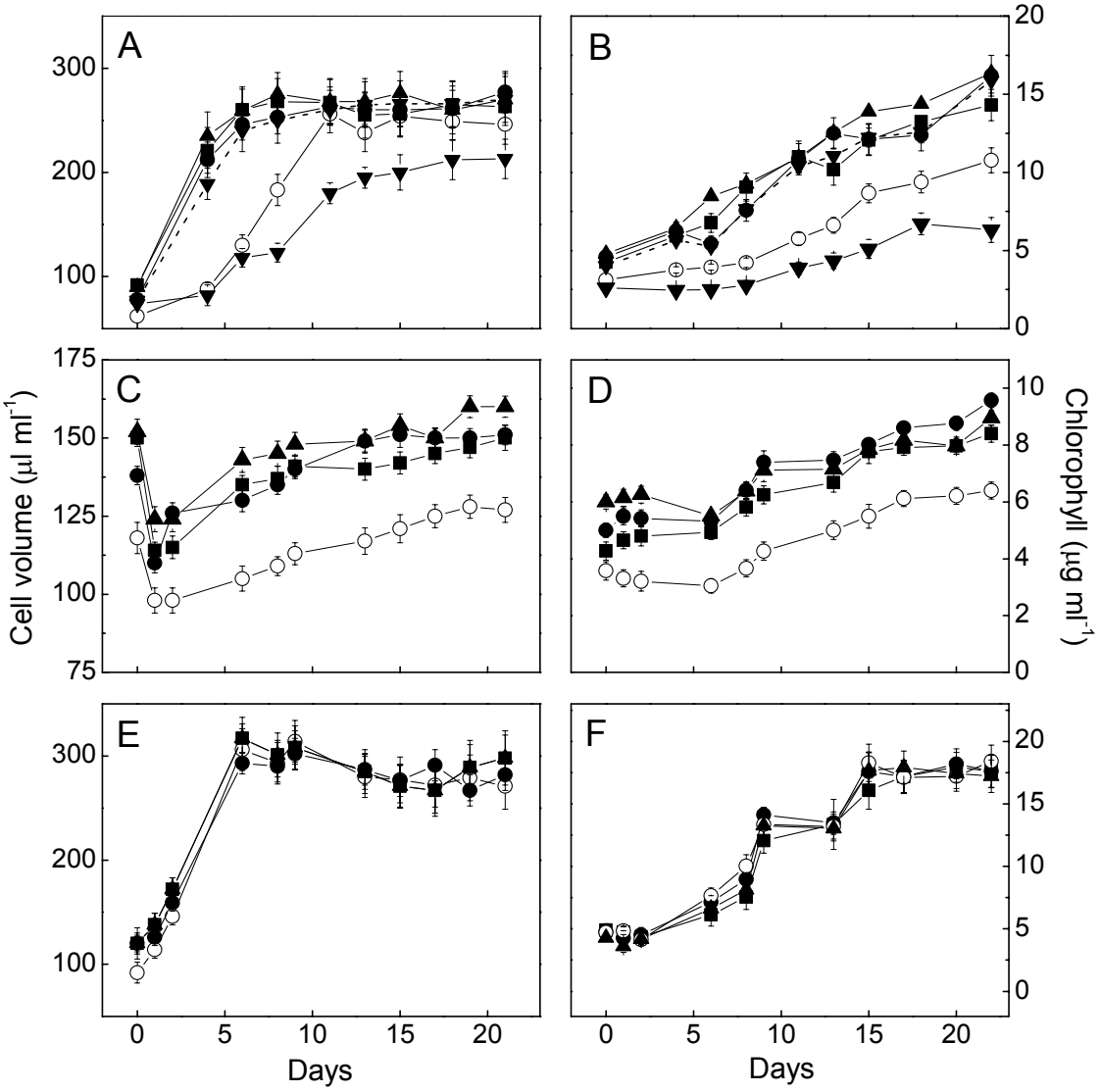

Fig. 1

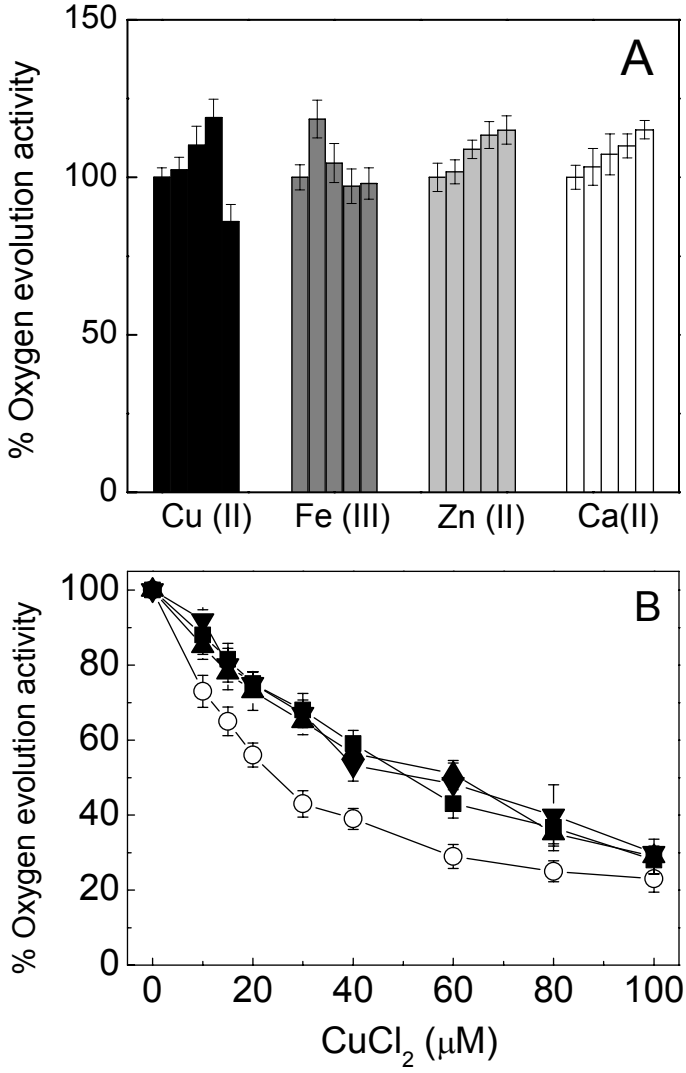
A
% Oxygen evolution activity
150
100
50
0
Cu (II)
Fe (III)
Zn (II)
Ca(II)
B
% Oxygen evolution activity
100
80
60
40
20
0
0
20
40
60
80
100
CuCl2 (μM)

Fig. 2

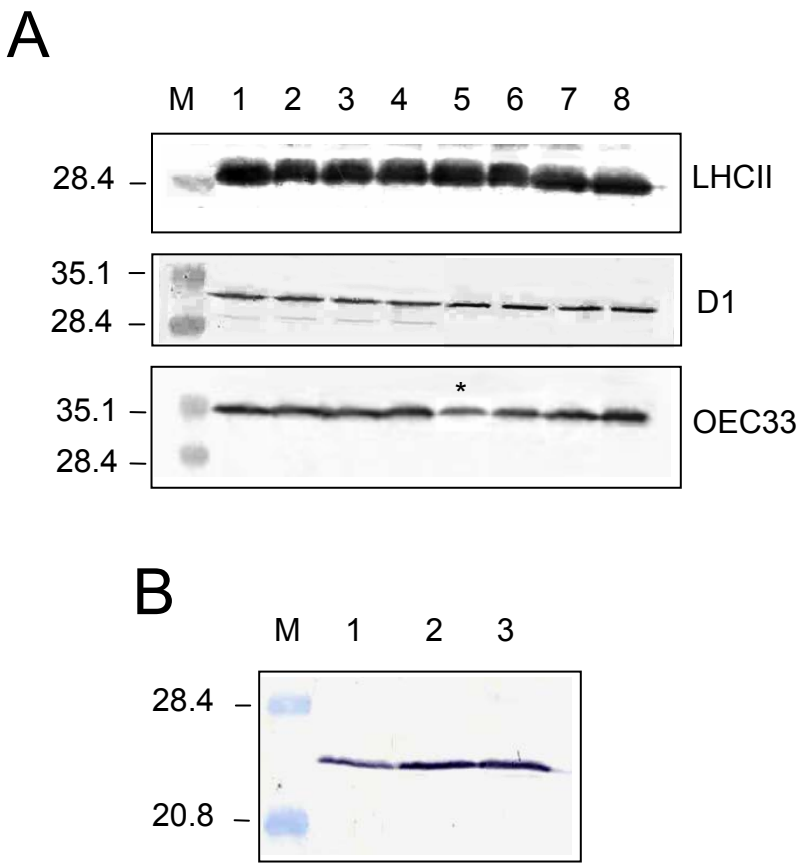

Fig. 3

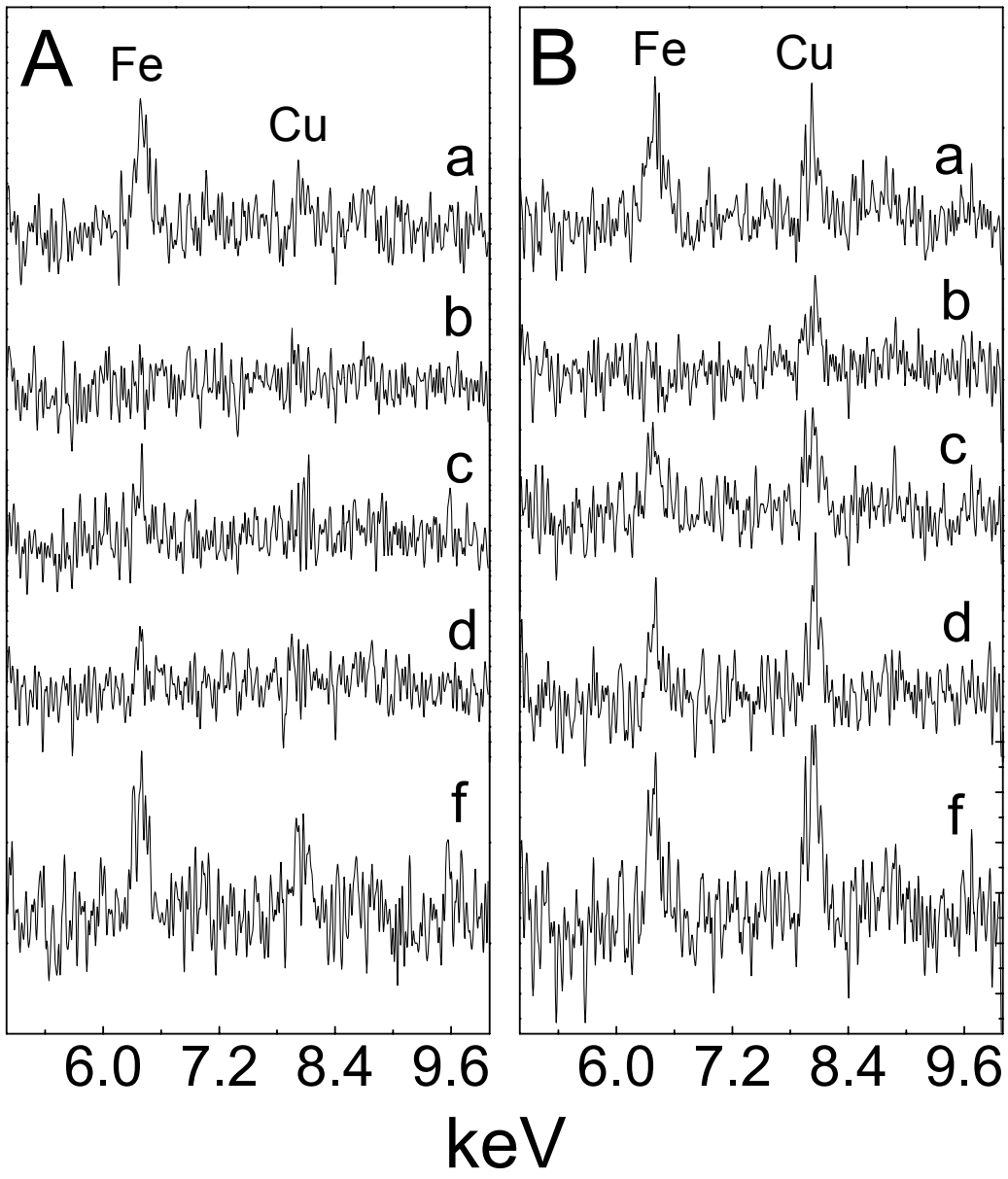

Fig. 4

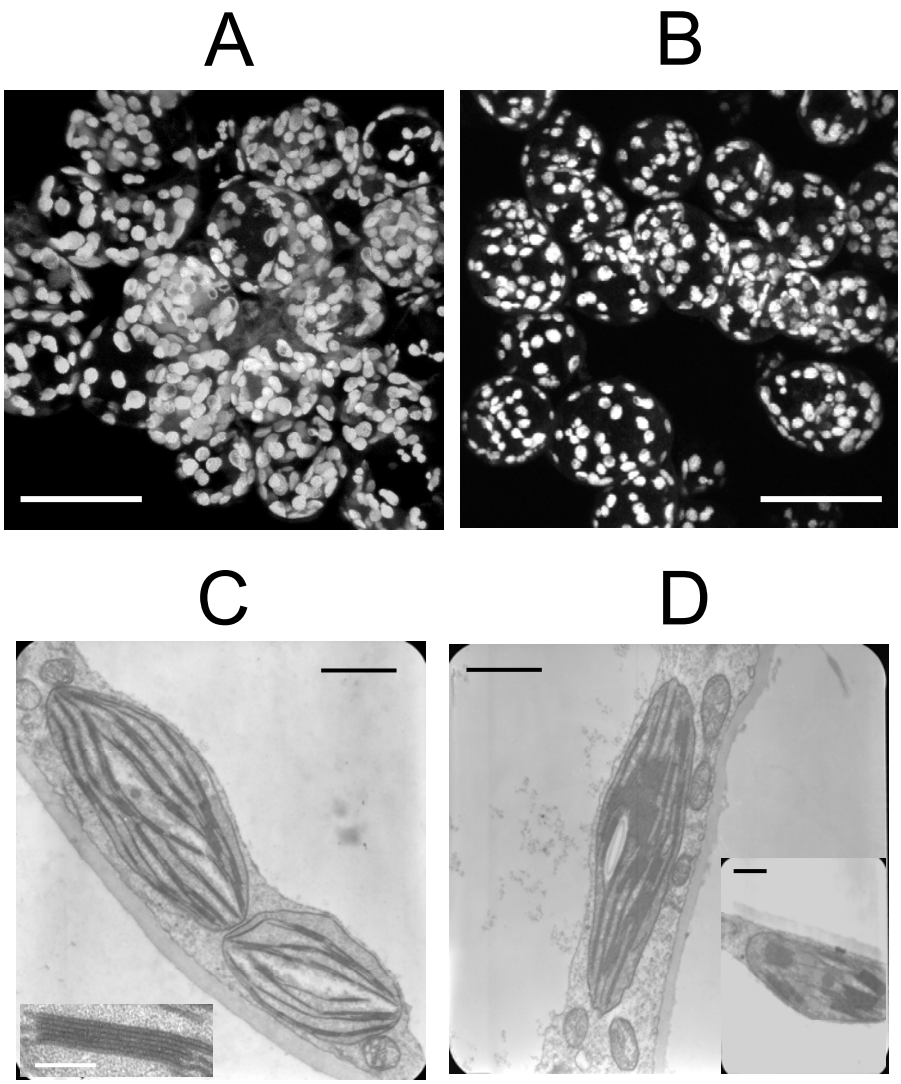

Fig. 5

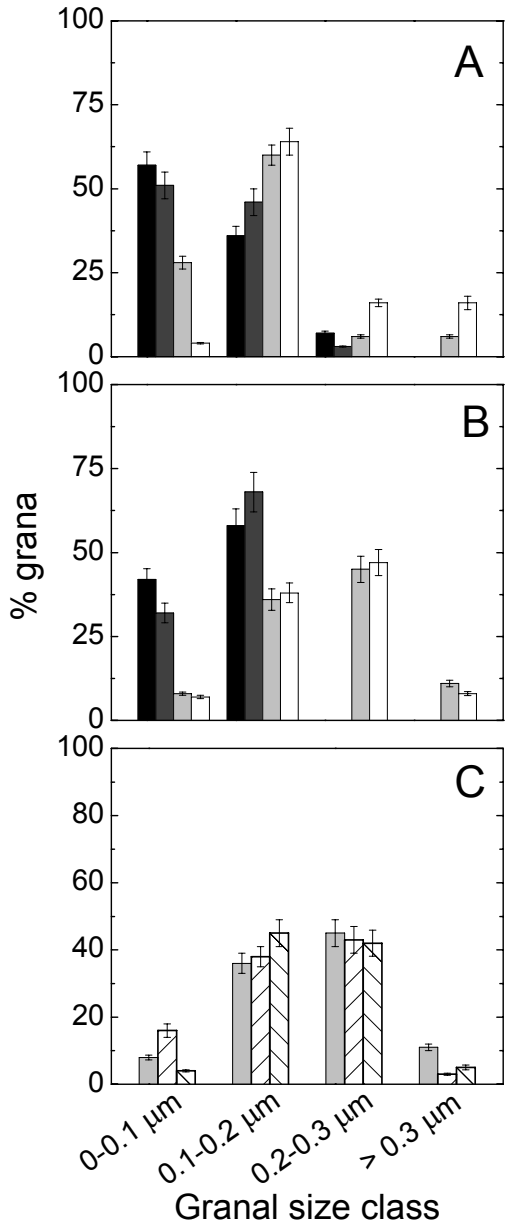
A
B
C
100
75
50
25
0
80
60
40
20
% grana
0-0.1 µm
0.1-0.2 µm
0.2-0.3 µm
> 0.3 µm
Granal size class

Fig. 6