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Altered physiology, cell structure, and gene expression of *Theobroma* cacao seedlings subjected to Cu toxicity

Vânia L. Souza
Instituto Federal de Educação, vlsbio@yahoo.com.br

Alex-Alan F. de Almeida
Universidade Estadual de Santa Cruz, alexalan.uesc@gmail.com

Jadiel de S. Souza
jadielsantana_@hotmail.com

Pedro A. O. Mangabeira
Universidade Estadual de Santa Cruz, pmangabeira@uesc.br

Raildo M. de Jesus
Universidade Estadual de Santa Cruz, pirovanicp@gmail.com

See next page for additional authors

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RESEARCH ARTICLE

Altered physiology, cell structure, and gene expression of *Theobroma cacao* seedlings subjected to Cu toxicity

Vânia L. Souza · Alex-Alan F. de Almeida · Jadiel de S. Souza · Pedro A. O. Mangabeira · Raildo M. de Jesus · Carlos P. Pirovani · Dário Ahnert · Virupax C. Baligar · Leandro L. Loguercio

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Abstract Seedlings of *Theobroma cacao* CCN 51 genotype were grown under greenhouse conditions and exposed to increasing concentrations of Cu (0.005, 1, 2, 4, 8, 16, and 32 mg Cu L^{-1}) in nutrient solution. When doses were equal or higher than 8 mg Cu L^{-1}, after 24 h of treatment application, leaf gas exchange was highly affected and changes in chloroplasts thylakoids of leaf mesophyll cells and plasmolysis of cells from the root cortical region were observed. In addition, cell membranes of roots and leaves were damaged. In leaves, 96 h after treatments started, increases in the percentage of electrolyte leakage through membranes were observed with increases of Cu in the nutrient solution. Moreover, there was an increase in the concentration of thiobarbituric acid-reactive substances in roots due to lipid peroxidation of membranes. Chemical analysis showed that increases in Cu concentrations in vegetative organs of *T. cacao* increased with the increase of the metal in the nutrient solution, but there was a greater accumulation of Cu in roots than in shoots. The excess of Cu interfered in the levels of Mn, Zn, Fe, Mg, K, and Ca in different organs of *T. cacao*. Analysis of gene expression via RTq-PCR showed increased levels of *MT2b*, *SODCyt*, and *PER*-1 expression in roots and of *MT2b*, *PSBA*, *PSBO*, *SODCyt*, and *SODChI* in leaves. Hence, it was concluded that Cu in nutrient solution at doses equal or above 8 mg L^{-1} significantly affected leaf gas exchange, cell ultrastructure, and transport of mineral nutrients in seedlings of this *T. cacao* genotype.

Keywords Cacao · Heavy metal · Lipid peroxidation · Photosynthesis · Ultrastructure · Gene expression

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V. L. Souza
Departamento de Ensino, Instituto Federal de Educação, Ciência e Tecnologia da Bahia, Rod. 148, km 04, n. 1800, Vila Esperança, 44900-000 Irecê, BA, Brazil
e-mail: vlsbio@yahoo.com.br

A.-A. F. de Almeida (✉) · J. de S. Souza · P. A. O. Mangabeira · C. P. Pirovani · D. Ahnert · L. L. Loguercio
Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rod. BR 415, km 16, 45662-900 Ilhéus, BA, Brazil
e-mail: alexalan.uesc@gmail.com

J. de S. Souza
e-mail: jadielsantana_@hotmail.com

P. A. O. Mangabeira
e-mail: pmangabeira@uesc.br

C. P. Pirovani
e-mail: pirovanicp@gmail.com

D. Ahnert
e-mail: darioa@uesc.br

L. L. Loguercio
e-mail: leandro@uesc.br

R. M. de Jesus
Departamento de Ciências Exatas e Tecnológicas, Universidade Estadual de Santa Cruz, Rod. BR 415, km 16, 45662-900 Ilhéus, BA, Brazil
e-mail: raildomota@gmail.com

V. C. Baligar
USDA-ARS—Sustainable Perennial Crops Laboratory, 10300 Baltimore Avenue, Beltsville, MD, USA
e-mail: v.c.baligar@ars.usda.gov

Introduction

Copper is a heavy metal and micronutrient essential for plant metabolism, occurring under physiological conditions as Cu^{2+} and Cu^{+}. Cu ions are vital components of a variety of enzymes and other proteins, including transcription factors. Thus, for the plant to grow and develop in a healthy manner, Cu has to be taken from the soil, transported across membranes, distributed and compartmentalized in different tissues, with its amounts precisely regulated within the cells (Yruela 2005). Excess of Cu inhibits the activity of several enzymes and interferes with various aspects of plant biochemistry, including photosynthesis, metabolism of fatty

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acids and proteins, respiration, and integrity of membranes (Almeida et al. 2007). The most important of these effects is related to photosynthesis, in which Cu excess leads to changes in the ultrastructure of chloroplasts, causing reduction of electron transport in photosystems I (PS I) and II (PS II); it also interferes with the activity of Calvin cycle enzymes, such as the Rubisco (Panou-Filotheou et al. 2001; Pätsikkä et al. 2002; Shaul 2002). Therefore, decreases in net photosynthetic rate (P_N), stomatal conductance (gs), and transpiration (E) are common in plants exposed to excess of Cu (Moustakas et al. 1997; Vassilev et al. 2002; Shi-Sheng 2007; Cambrollé et al. 2011). In addition, Cu excess promotes the production of free radicals that cause damage to proteins and lipids of cellular membranes, thereby altering their permeability and selectivity for intake/exit of nutrients to/from the cell, which can cause electrolytes leakage (Shi-Sheng 2007). The malondialdehyde (MDA), originated from a reaction between a free radical and a polyunsaturated fatty acid, is commonly used as an indicator of lipid peroxidation in membranes of plant cells under stress conditions by Cu excess (Pätsikkä et al. 2002; Bouazizi et al. 2010; Janas et al. 2010). Despite that Cu accumulates mainly in roots, it interferes with the chemical composition of different plant organs, which levels of nutritional imbalance is species-dependent (Shi-Sheng 2007; Bouazizi et al. 2010; Cambrollé et al. 2011). For instance, in *Glaucium flavum* exposed to excess of Cu, P concentration increased in roots and leaves, whereas Ca and Mg levels decreased in leaves (Cambrollé et al. 2011). Bouazizi et al. (2010) observed a decrease in the levels of Fe, Zn and K in the leaves of *Phaseolus vulgaris*; the same was observed for Fe, K, and Mg contents in seedlings of *Amaranthus tricolor* (Shi-Sheng 2007).

Plants have several potential strategies and mechanisms at the cellular level that may be involved in detoxification and tolerance to stresses caused by heavy metals. Tolerant plants show an increase in several homeostatic mechanisms, which contribute to prevent the metal translocation (Hall 2002), or to keep it in a stable manner. With regards to Cu, a form of protection employed by some plant species consists in increasing metallothionein levels, which act mainly as chelator of the metal in excess (Cobbett and Goldsbrough 2002). Metallothioneins are low molecular weight proteins, rich in cysteine residues that bind to copper and protect the cells against the toxicity of this element (van Hoof et al. 2001). Antioxidant enzymes, such as superoxide dismutases (SODs) and class III peroxidases, represent another mechanism of tolerance to Cu stress. These enzymes act by limiting the formation and by removing the reactive oxygen species (ROS) (Alscher et al. 2002). The SOD enzymes are classified into three groups: Fe SOD, Mn SOD, and Cu–Zn SOD, which are located in different cell compartments (Alscher et al. 2002). The Cu–Zn SODs enzymes are divided into two classes of isoforms, one located in the cytoplasm and the other in the chloroplasts (Kurepa et al. 1997). The class III peroxidases are located in vacuoles and cell walls, belong to a multigene family involved in several physiological processes, and act in a wide variety of substrates, showing moderate specificity for phenols (Almagro et al. 2009).

Seeds of *Theobroma cacao* (Malvaceae) are commercially explored for chocolate production, as well as for other derivatives and by-products such as cosmetics, fine drinks, jams, ice-creams and juices (Almeida and Valle 2010). The southeastern region of Bahia, Brazil, has been the main cacao-growing region in the world for over a hundred years. However, with the outbreak of witches' broom disease of cacao at the end of the 1980s, caused by the basidiomycete *Moniliophthora perniciosa*, an economic, social, and environmental disaster struck this region, as a consequence of a drastic reduction in cacao beans production (Pereira et al. 1989). In the past 15 years, a major control method of this and other diseases (such as the black-pod rot caused by *Phytophthora* spp.), has been the development and use of resistant and tolerant cacao genotypes, coupled with other cultural and chemical control measures under an integrated management approach. A couple of these measures have been the use of phosphate fertilizers and cupric fungicides (Fonseca 1990). Therefore, in this cacao-producing region, the accumulation of Cu in soils, plants and, consequently, in cacao beans is a matter of concern, because the control of black-pod and witches' broom with cupric fungicides has been a common practice throughout all these years, as part of the technological package recommended for this crop (Veloso and Santana 2000). It was found that the content of Cu in these cacao-crop soils (at the Bahian municipalities of Camacan, Ilhéus, Itabuna, Pau Brasil, and Uruçuca) has increased proportionally to the time of its applications. In places where this practice has been systematically employed from 5 to 20 years, the available and total soil Cu contents were found to be above the appropriate levels (Veloso and Santana 2000).

Taking this context into consideration, the main objectives of this study were to evaluate the tolerable physiological limits of exposure to Cu by *T. cacao* seedlings, when grown in nutrient solution with different concentrations of this element, as well as to assess translocation of Cu to the shoots and the overall changes on plants exposed to high levels of this metal. These objectives were achieved by verifying the variation caused by Cu in leaf gas exchange, ultrastructure of cellular organelles, peroxidation of cell membranes and mineral nutrients composition, both in roots and shoots.

Material and methods

Plant material and growth conditions

The experiment was conducted under greenhouse conditions at the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil. Seeds of the *T. cacao* genotype CCN 51 were

grown in black plastic tubes containing *Pinus* bark and turf+triturated coconut fiber (1:1) as substrate. Approximately 30 days after emergence, plants were transplanted into 35-L plastic trays (eight plants per tray), containing nutrient solution at 1/2 ionic strength, prepared according to Hoagland and Arnon (1950). For acclimation, the plants remained in these conditions for 2 months. Once this period was completed, the treatments with increasing concentrations of Cu (0.005, 1, 2, 4, 8, 16, and 32 mg L^{-1}) in the form of $CuSO_4 \cdot 7H_2O$ were applied. The concentration of 0.005 mg Cu L^{-1} was considered as the control concentration, because Cu is an essential micronutrient for growth and development of plants. During the experimental period, the solutions were monitored daily for pH, which was adjusted to 5.5 using NaOH and/or HCl. The nutrient solution was kept under constant aeration and its level maintained by addition of deionized water. Renewal of the nutrient solution was made weekly.

Leaf gas exchange

During the experimental period, the net photosynthetic rate per unit of leaf area (P_N), stomatal conductance to water vapor (gs) and leaf transpiration (E) were measured at 24, 48, 72, and 96 h *a*fter *a*pplication of *t*reatments (AAT). These variables were assessed, between 0800 and 0900 h, on a mature and completely expanded leaf from the end the orthotropic apex axis of four plants per treatment using a portable photosynthesis system LI-6400 (Li-Cor, Nebraska, USA) equipped with an artificial light source 6400-02B RedBlue.

The values of P_N, gs, and E were used to calculate the intrinsic (P_N/gs) and the instantaneous (WUE=P_N/E) water use efficiencies. For the leaf gas exchange measurements, the artificial light source of the system was adjusted to provide a photosynthetic photon flux density of 800 μmol m^{-2} s^{-1}. The mean values of atmospheric CO_2 concentration, air temperature and air vapor pressure deficit during the leaf gas exchange measurements were 421.47±11.94 μmol mol^{-1}, 26.7±0.07 °C, and 1.56±0.04 kPa, respectively (mean±SD, n=35, corresponding to 7 treatments×5 replications).

Ultrastructural assessment

Ultrastructural analyses were performed using a transmission electron microscopy (TEM) on the root tip and middle portion of the second mature leaf from the apex of the orthotropic axis harvested at 96 h AAT. The plant material was fixed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Samples were submitted to four washes (10 min each) in 0.1 M sodium cacodylate buffer, pH 7.2, and post-fixed in 1 % osmium tetroxide, prepared in the same buffer for 2 h at 4 °C, and followed by dehydration in an ethanol gradient (30, 50, 70, 80, and 90 % ethanol), and by two washes in 100 % ethanol. Then, the samples were embedded in a mixture of 100 % ethanol and LR White resin (Sigma) in the proportions 3:1 (2 h), 1:1 (2 h), 1:3 (overnight), followed by two changes of pure LR White resin of 4 h each, always under slow agitation. The samples were placed in gelatin capsules and covered with pure LR White resin. Polymerization of the resin was completed in 24 h at 60 °C. Ultra thin sections (60–70 nm) were cut on a diamond knife using a ultramicrotome (model EM FC6 LEICA Microsystems), and collected from the knife's water bath on 300-mesh Cu grids. The sections were stained for 15 min with aqueous solution of 5 % uranyl acetate, followed by 20 min with 0.4 % lead citrate (Reynolds 1963). Analyses were done using Morgani™ 268D TEM (FEI Company), with acceleration voltage of 80 kV, equipped with a CCD camera and controlled by software running under Windows OS. At least four grids with three to five sections for each treatment were observed and photographed. The images that best represented the changes in the ultrastructure of *T. cacao* leaf mesophyll and root cells, after application of different concentrations of Cu, were selected.

Electrolyte leakage

To assess the cell membrane stability, the electrolyte leakage technique was used (Bajji et al. 2001). Leaf discs were cut out of plants submitted to different [Cu] in nutrient solution at 96 h AAT, and thoroughly washed in deionized water. Afterwards, the leaf discs were placed in vials containing 10 mL of deionized water at 25 °C for 6 h under constant agitation. The electrical conductivity was measured using a conductivity meter and expressed as percentage of total conductivity, which was obtained after placing the vials with the leaf discs at 90 °C for 2 h.

Lipid peroxidation

Oxidative damage to lipids in cell membranes of roots and leaves were estimated as thiobarbituric acid-reactive substances (TBARS), mainly MDA, according to Cakmak and Horst (1991) with some modifications. For each of the different Cu treatments, 20 mg of lyophilized roots or leaves were homogenized in a mortar with 2 mL of 0.1 % (*w*/*v*) trichloroacetic acid (TCA). Following centrifugation at 10,000×*g* for 5 min, an aliquot of 500 μL from the supernatant was added to 1.5 mL of thiobarbituric acid solution (0.5 % in 20 % TCA). Samples were incubated at 90 °C for 20 min. After heating, the reaction was stopped under ice bath. Centrifugation at 10,000×*g* for 4 min was performed, and then the absorbance of supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of TBARS was calculated from its extinction coefficient of 155 mM^{-1} cm^{-1}.

Mineral nutrients

At the end of the experiment (96 h AAT), three seedlings per tray were collected from the different treatments and separated in roots, stems, and leaves. Soon after, washes were carried out once in tap water, once in 3 % HCl, and twice in deionized water; the different vegetative organs were placed in an oven at 75 °C to obtain dry biomass. Dry plant materials were grinded using a Wiley mill to pass a 20-mesh screen, and analyzed chemically (Anunciação et al. 2011). Concentrations of Cu, Fe, Mn, Zn, Mg, K, and Ca were analyzed in root, stem and leaf dry matter, using inductively coupled plasma–optical emission spectrometer, in a "Varian 710-ES" model.

Quantitative real-time PCR analysis

Samples of roots and second mature leaf from the apex of the orthotropic axis were harvested at 12 and 96 h AAT, frozen in liquid nitrogen and stored at −80 °C. Prior to RNA extraction, the tissues were lyophilized. RNA was extracted from roots and leaves at four different treatments (0.005, 2, 4, and 8 mg Cu L^{-1}) with RNAqueous kit (Ambion®). The RNA purity and integrity was checked by electrophoresis in 1 % agarose gels. The RNA samples were used for cDNA synthesis using Revertaid H-Minus Reverse Transcriptase (Fermentas) and oligo $d(T)_{18}$ primers, according to manufacturer instructions. The reactions were incubated at 65 °C for 5 min, 37 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. The qPCR was performed in a "Real Time PCR" (Applied Biosystems, 7500 model) using non-specific sequence fluorophore SYBR Green I (Fermentas). The abundance of transcripts was analyzed using specific primers (Table 1). To test the quality of these primers, the specificity and identity of the reverse transcription products, the qPCR products were monitored after each PCR, using a melt-curve analysis distinguishing gene-specific from non-specific products. The reaction mix consisted of cDNA template (500 ng), 0.5 μM of each primer, and 10 μL fluorophore SYBR Green I in a final reaction volume of 20 μL. The temperature of PCR products was raised from 55 to 99 °C at a rate of 1 °C/5 s, and the resulting data were analyzed using the LightCycler software. Only a single band with a characteristic melting point was observed for each sample, indicating that the qPCR had produced a specific product for each primer-pair used. To confirm that the qPCR had produced only genes of interest, the PCR products were separated and visualized in agarose gel at 1 %. Threshold cycle (C_T) values were determined using the LightCycler software. Numbers on the relative expression of genes were calculated as a percentage of the control treatments (0.005 mg Cu L^{-1}), using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and β-Tubulin as endogenous control in order to detect changes in transcript number (Table 1). All reactions were prepared in triplicate and performed twice. For each treatment at different times (12 and 96 h AAT), three biological replicas were used for each primer evaluation.

Statistics

The experiment was performed on a completely randomized arrangement, with seven levels of Cu, containing five replicates of eight seedlings each. The results were analyzed based on linear and non-linear mathematical models, with variation of Cu concentration in a nutrient solution as independent variable and leaf gas exchange, percentage of electrolyte leakage, lipid peroxidation, and mineral nutrient as dependent variables. Regression analyses and analyses of variance to define the best fitting model were done utilizing the general linear model procedure of the "Statistical Analysis System" (SAS Institute 1997), using methodology outlined by Steel and Torrie (1980). The coefficients of the equations were tested using t test ($p<0.05$). The statistical analysis of expression levels was performed using ANOVA. Mean comparisons were performed by Tukey's test with statistical significance at $p<0.05$.

Results

Leaf gas exchange

Leaf gas exchange of *T. cacao* seedlings was highly affected after 24 h exposure to concentrations of 8, 16 and 32 mg Cu L^{-1} in nutrient solution. Decreases of up to 72, 75, and 73 % for P_N, gs, and E, respectively, were observed (Fig. 1a–c). At 96 h AAT for the 32 mg Cu L^{-1} dose, these decreases reached 100, 99, and 98 % for P_N, gs, and E, respectively. On the other hand, for the 4 mg Cu L^{-1} treatment, the decrease in P_N was of 44 % at 96 h AAT. The values of gs and E for the same concentration at 24 h AAT showed lower decreases of 39 and 38 %, respectively, without significant differences ($p<0.05$) for the corresponding values at 96 h AAT.

The intrinsic and instantaneous water use efficiencies (P_N/gs and *WUE*) did not show a significant variation at 24 and 48 h AAT. However, a decline was found at 72 h AAT for both P_N/gs and *WUE* (Fig. 1d, e). These results are striking in demonstrating that seedlings of *T. cacao* were physiologically affected by as short as 24-h exposure to increasing concentrations of Cu in nutrient solution; values up to 2 mg Cu L^{-1} were beneficial, and above it were detrimental. The results showed that the relationship between P_N and gs (Fig. 2) was linear up to a gs value of 0.03 mol H_2O m^{-2} s^{-1} for the 4 mg Cu L^{-1} concentration. The lowest values of this ratio were obtained for concentrations equal and above 8 mg Cu L^{-1}, whereas the highest values of P_N and gs were found for the concentrations of 1 and 2 mg Cu L^{-1}.

Table 1 Pairs of gene-specific primers used in the qRT-PCR analysis

Gene	Acesso	Function	Primer
MT2b	CL9Contig1[b]	Biosynthesis of metallothionein	F—5′-GCAACCCTTGCACTTGTAAATG-3′
			R—5′-CAAGCCATGGCAACTTTATTCTAA-3′
PER-1	CK144296.1[a]	Biosynthesis of peroxidase class III	F—5′=CAGGTGTCGTGGGATCAAGA-3′
			R—5′-TGGAAAAACTACGCCAAATATGC-3′
Cu–Zn SOD$_{Cyt}$	CL94Contig1[b]	Biosynthesis of cytosolic Cu–Zn SOD	F—5′-GATGATGGCTGTGTGAGTTTCTCT-3′
			R—5′-CAACAACAGCTCTTCCAATAATTGA-3′
Cu–Zn SOD$_{Chl}$	CL872Contig1[b]	Biosynthesis of chloroplastic Cu–Zn SOD	F—5′-AATGGATGCATGTCAACAGGAGC-3′
			R—5′-ATGTTTCCCAGGTCACCCGC-3′
PSBO	CL326Contig1[b]	Biosynthesis of PsbO protein	F—5′-GCAAACGCTGAAGGAGTT-3′
			R—5′-GGCTTGAAGGCAAATGAGTC-3′
PSBA	NC_014676.2[c]	Biosynthesis of the PsbA protein or D1 protein	F—5′-GGTTTGCACTTTTACCCGA-3′
			R—5′-CTCATAAGGACCGCCATT-3′
β-Tubulin	GU570572.1[c]	Endogen	F—5′-TGCAACCATGAGTGGTGTCA-3′
			R—5′-CAGACGAGGGAAAGGAATGA-3′

[a] http://cocoagendb.cirad.fr/

[b] http://esttik.cirad.fr/index.html

[c] http://www.ncbi.nlm.nih.gov/

Ultrastructural assessment

The effects of increasing concentrations of Cu on cellular ultrastructure of developing *T. cacao* seedlings were assessed by TEM. Changes in root and leaf mesophyll cortex cells were observed (Figs. 3 and 4), with occurrence of plasmolysis in root cortex cells mainly above 8 mg Cu L^{-1} (Fig. 3a–f), shown by contraction of the plasma membrane and tonoplast shrinkage. Cu treatment apparently led to the rupture of plasma membranes in some cells of the cortical root tissue (Fig. 3g–l).

An evidence that Cu concentrations in nutrient solution between 4 and 8 mg Cu L^{-1} seems to establish the limit from beneficial to detrimental effects in *T. cacao* seedlings was also observed from ultrastructural analysis of chloroplasts (Fig. 4). Organelles of leaf mesophyll cells of seedlings submitted to 0.005 (control), 2, and 4 mg Cu L^{-1} looked normal through TEM. Chloroplasts showed structured and organized thylakoid membranes, normal presence of starch grains and plastoglobuli, and intact double membranes of their envelope (Fig. 4a–c). Contrariwise, from concentrations of 8 mg Cu L^{-1} and above, the chloroplast envelopes and thylakoids became disorganized (Fig. 4d–f), and absence of starch grains in the stroma, swollen thylakoids, and increased interthylakoidal space were verified. Furthermore, the rupture of the outer and inner double-membrane surrounding the chloroplast was found in some samples (Fig. 4d–e).

Electrolyte leakage and lipid peroxidation

The damage to membranes caused by Cu excess can be quantified indirectly by assessing electrolytes leakage. As shown in Fig. 5a, there was an increase in the percentage of electrolyte leakage through leaf mesophyll cells membranes of *T. cacao* seedlings with increasing Cu in the nutrient solution. In order to check if membrane damage also occurred as a result of lipids peroxidation, the concentration of TBARS was determined. Results indicated that there was no significant difference ($p<0.05$) between treatments in relation to lipid peroxidation of leaf mesophyll cell membranes with increasing Cu in nutrient solution; however, in roots, from the 8 mg Cu L^{-1} treatment and above, a significant increase of TBARS concentration was observed (Fig. 5b).

Mineral nutrients

The effects of exposure to increasing Cu concentrations were also studied in terms of variations in mineral composition of vegetative organs of *T. cacao* seedlings. Chemical analysis showed that Cu concentrations in roots and shoots of *T. cacao* was proportional to its increased concentration in the nutrient solution, but the accumulation of Cu in roots was largely higher than in shoots (Fig. 6). For the treatment with the highest concentration of the metal at 96 h AAT, the concentration of Cu in the roots was 1,032 mg Cu kg^{-1} DW, whereas in stem and leaves, the accumulation was only 17 and 6 mg Cu kg^{-1} DW, respectively (Fig. 6). These corresponded to an accumulation of Cu in stems and leaves that was approximately 60× and near 175× smaller than in roots.

The increase in Cu amounts in the nutrient solution interfered with the concentrations of Mn, Zn, Fe, Mg, K, and Ca, both in roots and shoots. There was a sharp decline in Mn and Zn concentrations in roots and stems that level off with

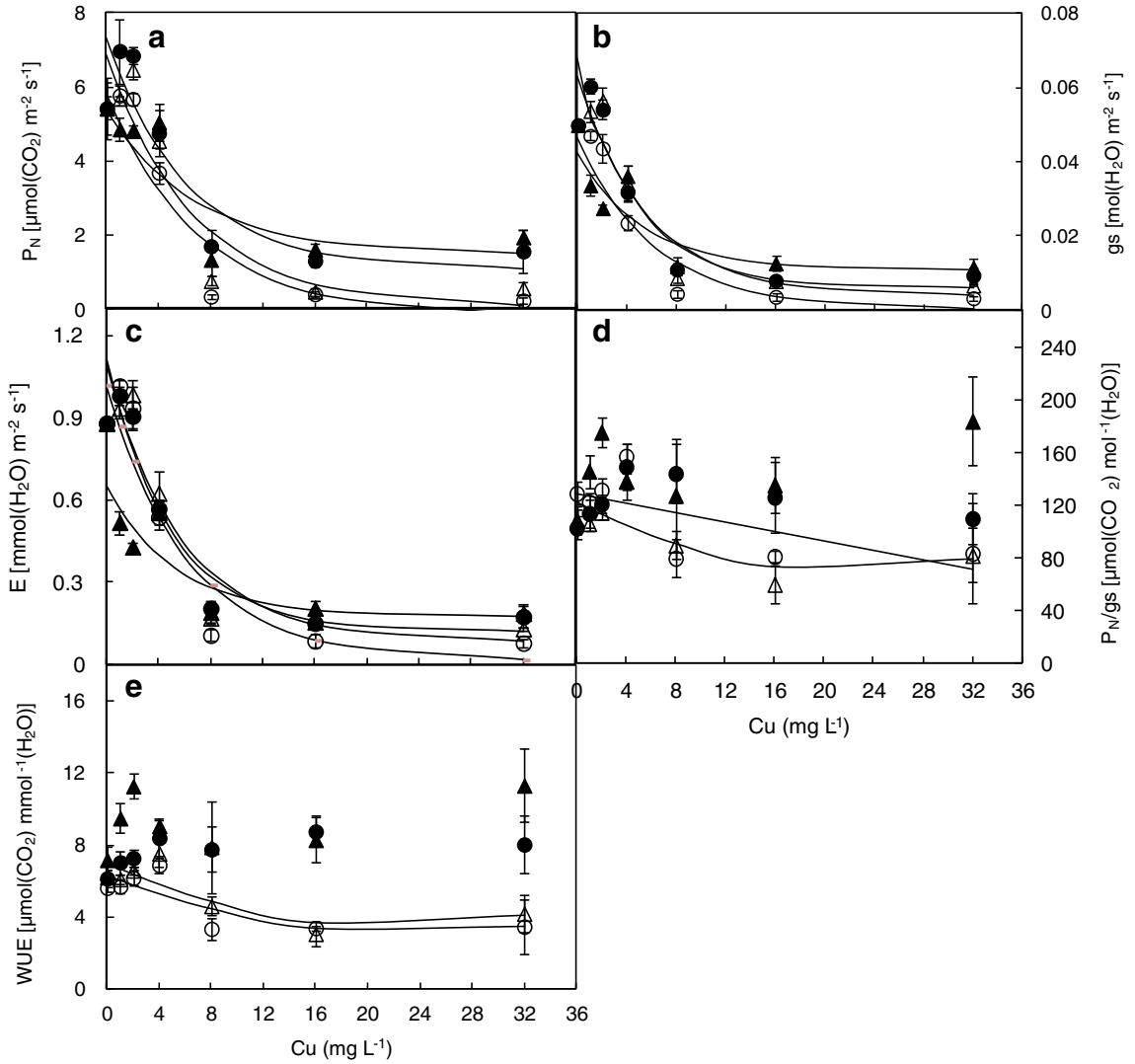

Fig. 1 Changes in **a** net photosynthetic rate (P_N), **b** stomatal conductance to water vapor (gs), **c** transpiration (*E*), **d** ratio of the intrinsic water use efficiency (P_N/gs), and **e** instantaneous water use efficiency (*WUE*) in leaves of young plants of *T. cacao* exposed to different concentrations of Cu in nutrient solution during 24 (*filled upright triangle*), 48 (*filled circle*), 72 (*open upright triangle*), and 96 h (*open circle*), *n*=4, ±SE. The regression curve equations were: $\widehat{y}_{24} = 1.45 + 3.96\exp(-0.14x)$ $(r^2 = 0.66)$, $\widehat{y}_{48} = 1.03 + 6.3\exp(-0.15x)$ $(r^2 = 0.75)$, $y_{72} = 0.03 + 6.84\,ex(-0.14x)$ $(r^2 = 0.87)$, $\widehat{y}_{96} = 0.22 + 6.07\exp(-0.14x)$ $(r^2 = 0.76)$ for P_N; $\widehat{y}_{24} = 0.01 + 0.031\exp(-0.18x)$ $(r^2 = 0.71)$, $\widehat{y}_{48} = 0.006 + 0.062\exp(-0.20x)$ $(r^2 = 0.88)$, $\widehat{y}_{72} = 0.003 + 0.059\exp(-0.17x)$ $(r^2 = 0.86)$, $\widehat{y}_{96} = 0.006 + 0.062\exp(-0.20x)$ $(r^2 = 0.74)$ for gs; $\widehat{y}_{24} = 0.17 + 0.47\exp(-0.18x)$ $(r^2 = 0.71)$, $\widehat{y}_{48} = 0.11 + 1.004\exp(-0.20x)$ $(r^2 = 0.89)$, $\widehat{y}_{72} = 0.07 + 1.01\exp(-0.17x)$ $(r^2 = 0.87)$, $\widehat{y}_{96} = 0.01 + 1.009\exp(-0.16x)$ $(r^2 = 0.77)$ for *E*; $\widehat{y}_{24} = 142.54$, $\widehat{y}_{48} = 126.38$, $\widehat{y}_{72} = 122.44 - 4.75x + 0.11x^2 (r^2 = pt0.57)$, $\widehat{y}_{96} = 128.97 - 1.8x$ $(r^2 = 0.46)$ for P_N/gs; $\widehat{y}_{24} = 9.30$, $\widehat{y}_{48} = 6.73$, $\widehat{y}_{72} = 6.97 - 0.32x + 0.0073x^2 (r^2 = 0.70)$, $\widehat{y}_{96} = 6.30 - 0.28x + 0.006x^2 (r^2 = 0.66)$ for *WUE*. The measurements were made at 800 μmol m^{-2} s^{-1} PPFD

the increase of Cu in the nutrient solution (Fig. 7). The lowest concentrations of Mn and Zn in the roots were 49 and 38 mg-kg^{-1} DW, representing a decline of 81 and 55 %, respectively, when compared to control plants. In the stems, the lowest concentrations of these micronutrients were higher than in roots, with values of 115 mg Mn kg^{-1} DW and 79 mg Zn kg^{-1} DW representing a decrease of 52 and 40 %, respectively, when compared to controls. On the other hand, Fe increased in roots and leaves with increasing Cu amounts in the nutrient solution (Fig. 7), with 95 and 61 % increases, respectively. The maximum values observed for roots and leaves were 477 and 79 mg Fe kg^{-1} DW, while the control plants had values of 244 and 49 mg Fe kg^{-1} DW, respectively.

The reduction observed in Mg both for stems and leaves was also proportional to the increased exposure to Cu. The maximum and minimum levels of Mg in the stems were 9.42 and 7.16 g-Mg kg^{-1} DW, respectively, whereas in leaves they were 5.24 and 3.83 g Mg kg^{-1} DW, respectively, representing a decrease of 24 % in stems and 26 % in leaves (Fig. 8). There was a decrease in K concentrations in roots and stems as Cu increased in the nutrient solution. The minimum values of K found in roots and stems were 15 and 16 g K kg^{-1} DW, respectively, representing

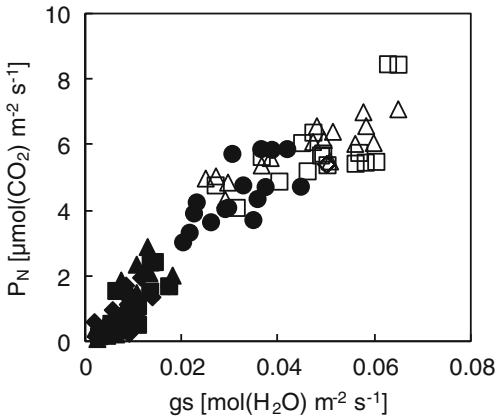

Fig. 2 Relationship between net photosynthetic rate per unit leaf area (P_N) and stomatal conductance to water vapor (gs) in young plants of *T. cacao* submitted to the concentrations 0.005 (*open circle*), 1 (*open square*), 2 (*open upright triangle*), 4 (*filled circle*), 8 (*filled square*), 16 (*filled diamond*), and 32 (*filled upright triangle*) mg Cu L^{-1} in nutrient solution during 24, 48, 72, and 96 h (n=4). The measurements were made at 800 µmol m^{-2} s^{-1} PPFD

a decrease of 33 and 17 % compared to control (Fig. 8). The Ca concentrations increased up to 22 % in root tissues at the highest Cu concentration in the nutrient solution (Fig. 8). Conversely, in stems, there was a decrease of 24 % in Ca concentration in relation to control plants.

Quantitative real-time PCR analysis

From the morpho-physiological changes in roots and leaves of seedlings when exposed to increasing concentrations of Cu, it was possible to select the doses that best represented the behavior of *T. cacao* to toxic Cu levels to allow assessment of gene expression at the transcriptional level. A significant increase (p<0.05) in the transcription levels of the *MT2b*, *SODCyt* and *PER*-1 genes in roots, and *PSBA* and *PSBO* genes in leaves was observed at 12 h AAT for the concentration 8 mg Cu L^{-1}, when compared to controls (Fig. 9a–e). However, at 96 h ATT in the roots, only the expression of the *PER*-1 gene remained two times greater than the control (Fig. 9c); in leaves, compared to control, *PSBA* expression increased for all concentrations of Cu (Fig. 9d)

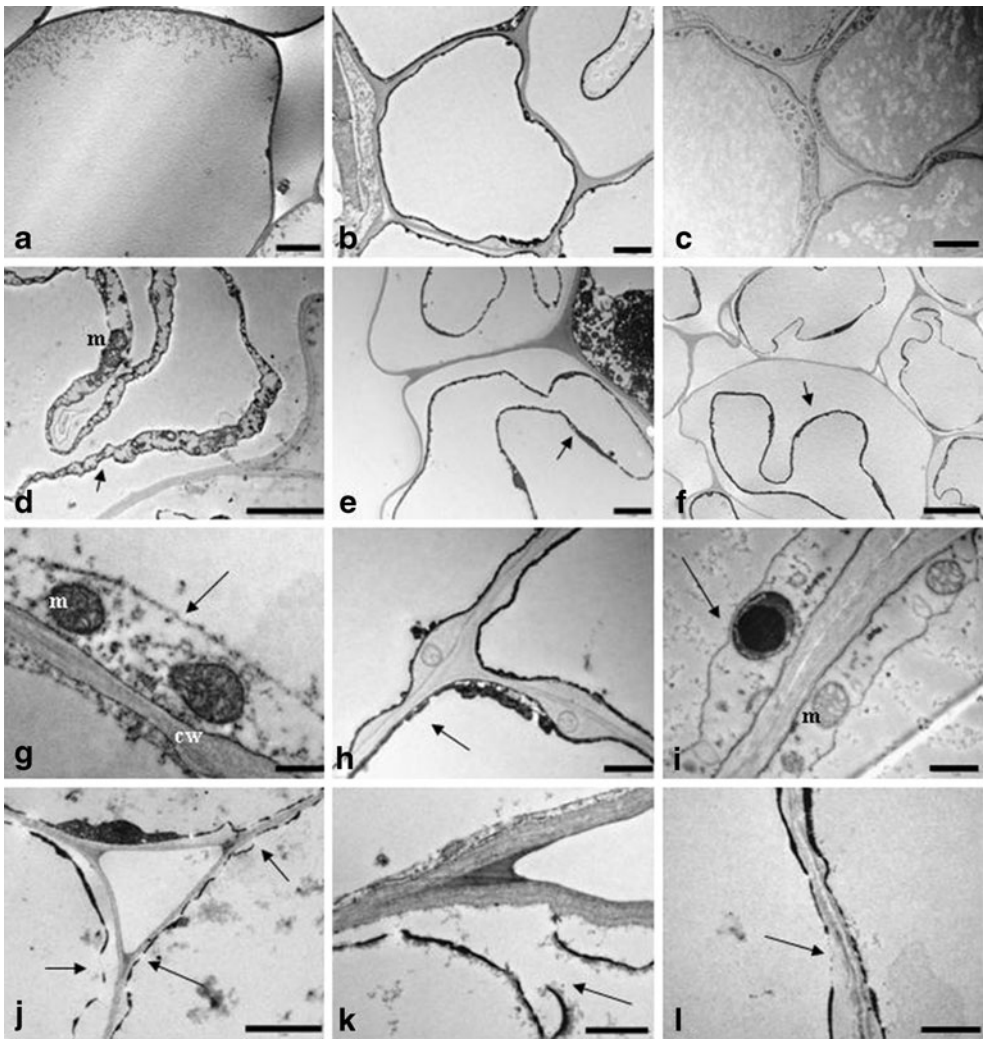

Fig. 3 TEM micrographs of roots cross sections of young *T. cacao* plants exposed to different concentrations of Cu in nutrient solution for 96 h. 0.005 (**a**, **g**), 2 (**b**, **h**), 4 (**c**, **i**), 8 (**d**, **j**), 16 (**e**, **k**) and 32 mg Cu L^{-1} (**f**, **l**). *Arrows* in **d**–**f** indicate shrinkage of the plasma membrane and tonoplast. *Arrows* in **g**–**i** showed intact membrane. *Arrows* in **j**–**l** indicate membrane rupture. *cw* cell wall, *m* mitochondria. *Bars* 0.5 µm (**g**), 1 µm (**h**, **i**, **k**, and **l**), 2 µm (**b**–**e** and **j**), 5 µm (**a**, **c**, and **f**)

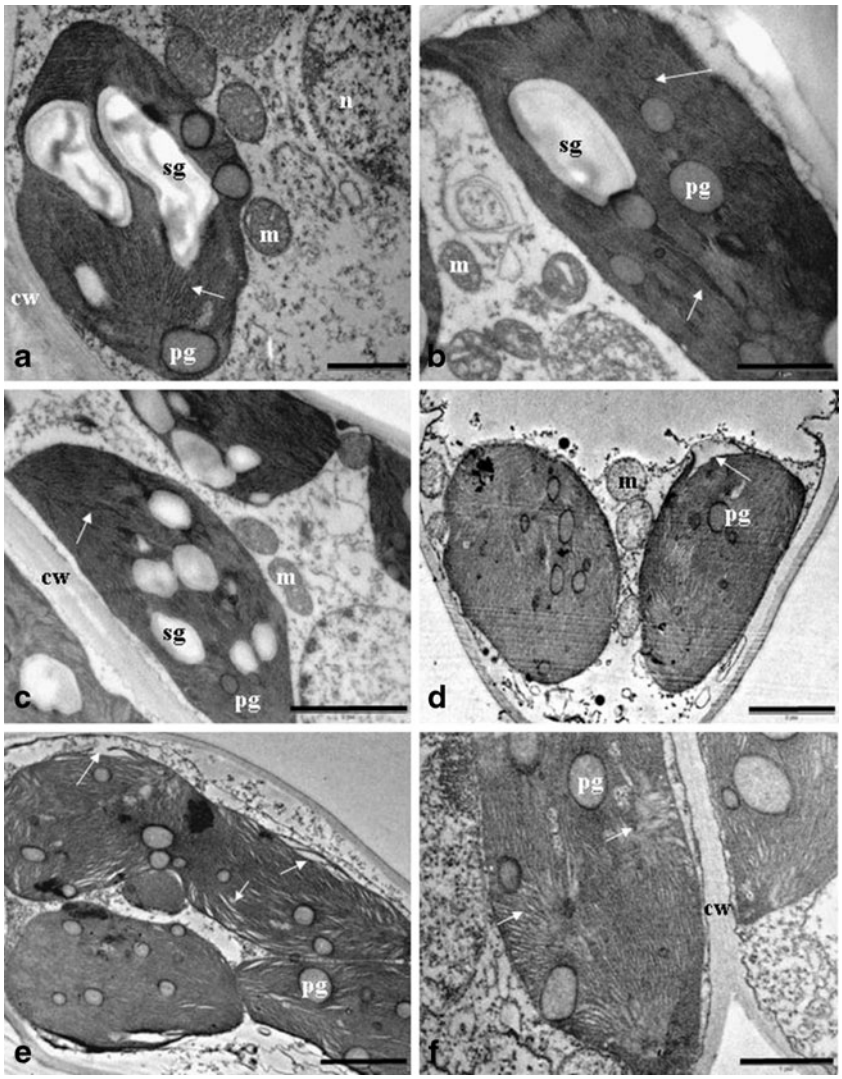

Fig. 4 TEM micrographs of cross sections of leaf mesophyll of young *T. cacao* plants exposed to different concentrations of Cu in nutrient solution for 96 h. 0.005 (**a**), 2 (**b**), 4 (**c**), 8 (**d**), 16 (**e**) and 32 mg Cu L^{-1} (**f**). *Arrows* in **a**, **b**, and **c** indicate the thylakoid membranes. *Arrows* in **d**, **e**, and **f** show rupture of the double membrane surrounding the chloroplast, thylakoids swollen, and increase in intrathylakoidal spaces. *cw* cell wall, *m* mitochondria, *n* nucleus, *pg* plastoglobuli, *sg* starch grains. *Bars* 1 μm (**a**, **b**, and **f**), 2 μm (**c**, **d**, and **e**)

whereas expression of *PSBO* increased only for the low concentrations of 2 and 4 mg Cu L^{-1} (Fig. 9e). Furthermore, transcriptions of *MT2b* and *SODCyt* genes in the leaves at 12 h AAT for the 2 mg Cu L^{-1} dose were repressed when compared to control, whereas at the 4 and 8 mg Cu L^{-1} concentrations, genes transcription increased (Fig. 9f, g). Expression of *MT2b* and *SODCyt* at 96 h AAT, at all concentrations of Cu, was greater than at 12 h AAT, and increased with increasing concentration of Cu in the nutrient solution (Fig. 9f, g). The *SODChl* gene transcripts increased at 12 h AAT for the 8 mg Cu L^{-1} dose, and at 96 h AAT for the concentration 2 mg Cu L^{-1}, whereas its expression levels were suppressed at 96 h AAT for the 8 mg Cu L^{-1} treatment (Fig. 9h).

Discussion

Concerns about the accumulation of Cu in *T. cacao* beans up to levels potentially inappropriate for human consumption have been recently raised in southeastern Bahia, the most important cacao-producing region in Brazil. These concerns resulted from studies showing that a direct proportion exists between increased Cu levels in cacao-growing soils and the number of years of application of Cu-based fungicides (Veloso and Santana 2000). Hence, it became necessary to investigate if such increases in soil Cu levels could lead to corresponding increases in the levels of Cu in cacao tissues, or to an altered physiology that could affect cacao production. Due to a much more precise control of Cu levels in nutrient solution, cacao seedlings were used as experimental models in the present work, aiming to quantify potential Cu increases in tissues, as well as to assess their effects in physiology, cellular ultrastructure, mineral composition and stress-response gene expression.

The inhibitory effects of excessive Cu on photosynthetic processes have been investigated in different plant species (Maksymiec 1997; Pätsikkä et al. 2002; Shi-Sheng 2007; Cambrollé et al. 2011). Results from studies in *Triticum aestivum*

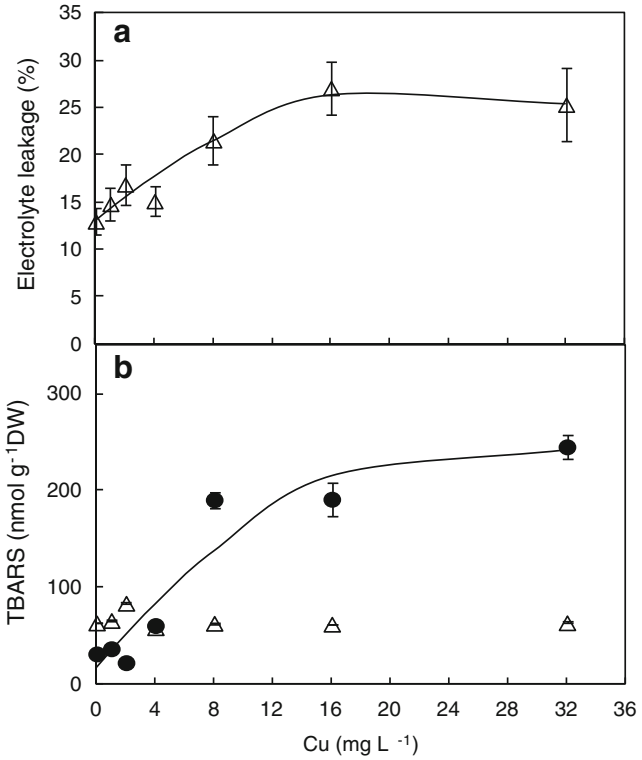

Fig. 5 Electrolyte leakage and lipid peroxidation in leaves and roots of young *T. cacao* plants exposed to different concentrations of Cu in nutrient solution for 96 h, n=4, ±SE. **a** Percentage of electrolyte leakage in leaves, the equation of the regression curve was $\widehat{y} = 12.98 + 1.28x - 0.02x^2 (r^2 = 0.94)$. **b** Concentration of thiobarbituric acid-reactive substances (TBARS) in roots (*filled circle*) and leaves (*open upright triangle*), the equations of regression curves were: $\widehat{y} = 15.88 + 17.84x - 0.33x^2 (r^2 = 0.90)$ for roots; $\widehat{y} = 65.26$ for leaves

(Moustakas et al. 1997), *Hordeum vulgare* (Vassilev et al. 2002), and *G. flavum* (Cambrollé et al. 2011) have shown to be similar to those of *T. cacao*, with observed decreases in leaf gas

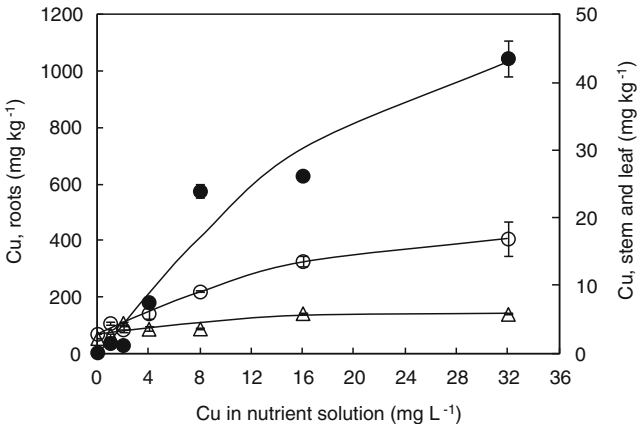

Fig. 6 Variation of Cu concentration in roots (*filled circle*), stems (*open circle*), and leaves (*open upright triangle*) of young *T. cacao* plants exposed to increasing concentrations of Cu in nutrient solution for 96 h, n=3, ±SE. The equations of regression curves were: $\widehat{y} = 1303.212\ (1 - 0.95^x)\ (r^2 = 0.95)$ for roots; $\widehat{y} = 2.78 + 0.89x - 0.014x^2 (r^2 = 0.99)$ for stems; $\widehat{y} = 2.77 + 0.25x - 0.004x^2 (r^2 = 0.77)$ for leaves

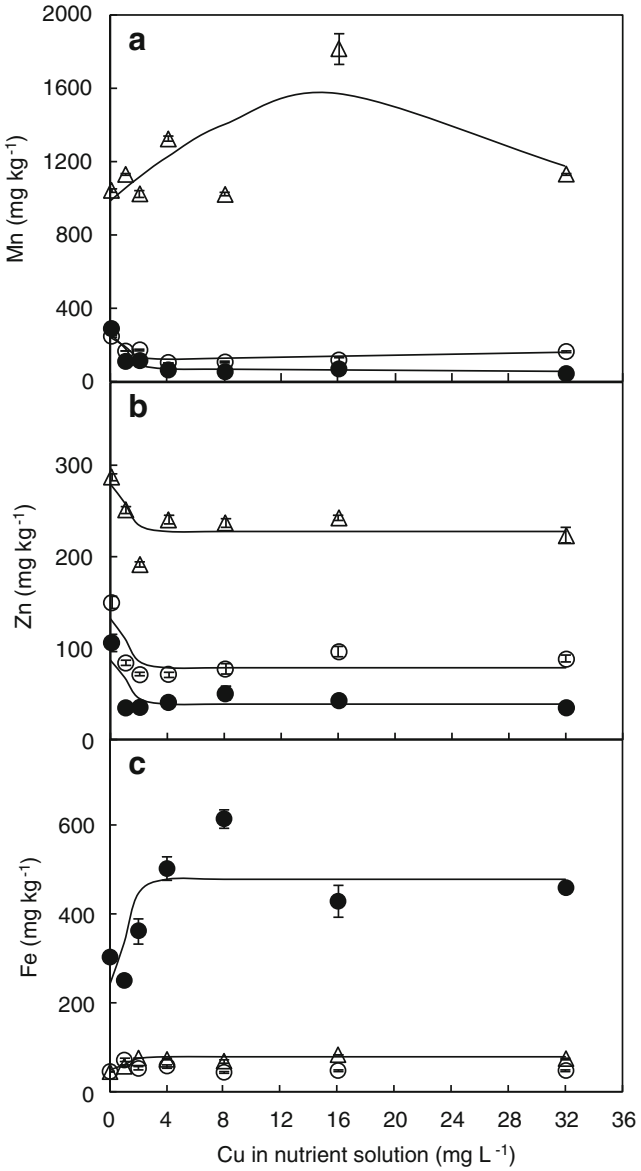

Fig. 7 Changes in the concentrations of Mn, Fe, and Zn in roots (*filled circle*), stems (*open circle*), and leaves (*open upright triangle*) of young *T. cacao* plants exposed to increasing concentrations of Cu in nutrient solution for 96 h, n=3, ±SE. The equations of regression curves for Mn were $\widehat{y} = 64.69 - 0.49x + 188.05\ (\exp(-(x^2)/2))\ (r^2 = 0.84)$ for roots; $\widehat{y} = 109.92 - 1.41x + 130.23\ (\exp(-(x^2)/2))\ (r^2 = 0.79)$ for stems and $\widehat{y} = 980.49 + 67.78x - 1.93x^2\ (r^2 = 0.52)$ for leaves; for Zn were $\widehat{y} = 38.36 + 48.37(\exp(-(x^2)/2))\ (r^2 = 0.56)$ for roots; $\widehat{y} = 78.72 + 54.08(\exp(-(x^2)/2))\ (r^2 = 0.62)$ for stems and $\widehat{y} = 227.10 + 51.71\ (\exp(-(x^2)/2))\ (r^2 = 0.51)$ for leaves; for Fe were $\widehat{y} = 476.58 - 232.53\ (\exp(-(x^2)/2))\ (r^2 = 0.56)$ for roots; $\widehat{y} = 55.73$ for stems and $\widehat{y} = 79.03 - 30.07(\exp(-(x^2)/2))\ (r^2 = 0.86)$ for leaves

exchange parameters after treatment with excess of Cu. In our study, seedlings exposure to Cu concentrations above 8 mg Cu L^{-1} showed to be highly toxic to *T. cacao*, as indicated by the sharp net photosynthetic decline after only 24 h of treatment application. This decrease was interpreted to be directly related to the observed decrease in gs, which indicates that the stomata have closed, with a consequent increase in diffusive resistance to

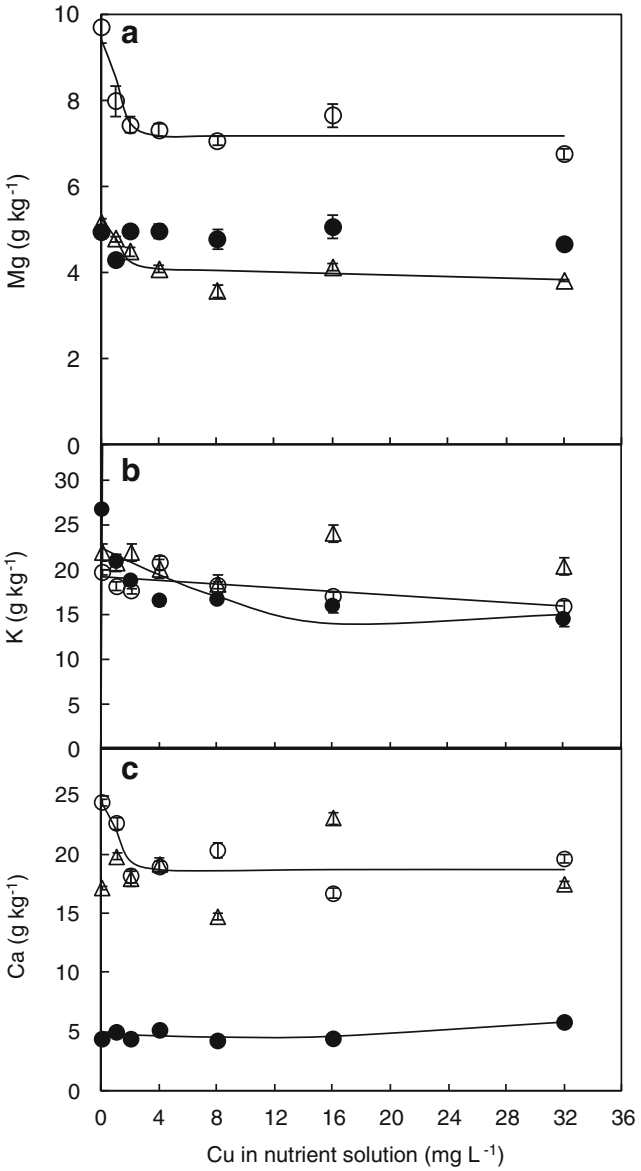

Fig. 8 Variation of the levels of Mg, K, and Ca in roots (*filled circle*), stems (*open circle*) and leaves (*open upright triangle*) of young *T. cacao* plants exposed to increasing concentrations of Cu in nutrient solution for 96 h, n=3, ±SE. The equations of regression curves for Mg were $\hat{y} = 4.73$ for roots; $\hat{y} = 78.72 + 54.08\ (\exp(-(x^2)/2))$ $(r^2 = 0.62)$ for stems and $\hat{y} = 4.60$ for leaves; for K were $\hat{y} = 22.46 - 0.82x + 0.018x^2 (r^2 = 0.65)$ for roots; $\hat{y} = 19.23 - 0.104x (r^2 = 0.54)$ for stems and $\hat{y} = 21.15$ for leaves; for Ca were $\hat{y} = 4.76 - 0.063x + 0.003x^2 (r^2 = 0.65)$ for roots; $\hat{y} = 18.76 + 5.84\ (\exp(-(x^2)/2))$ $(r^2 = 0.77)$ for stems and $\hat{y} = 18.35$ for leaves

CO_2 and reduction in water loss by transpiration, confirmed by the observed reduction in *E* (Figs. 1 and 2). It was found that *T. cacao* plants tolerated exposures up to 4 mg Cu L^{-1}, despite the observed decrease in P_N at 96 h AAT, which can be considered an indirect effect of the decreases in gs and *E* observed as early as 24 h AAT. Exposure to lower Cu concentrations (1 and 2 mg L^{-1}) did not affect the cacao physiology negatively, or alternatively, it might have had a beneficial effect to the plants, possibly as a micronutrient within the favorable limits. At least part of the Cu effects in photosynthesis derives from the fact that this metal is a component of plastocyanin, and as such, has a direct participation in the electron transport between PS II and PS I in the photochemical phase of photosynthesis. In addition, Cu excess can cause changes in proteins and lipids of thylakoid membranes, leading to a susceptibility of PS II to photoinhibition in vivo and to impairment of the electron transport between the two photosystems (Maksymiec 1997; Pätsikkä et al. 1998; 2002).

The reduction in *E* and gs values as a result of stomatal closure promoted by higher Cu concentrations has likely favored the decline in P_N/gs and *WUE* at 72 h AAT (Fig. 1d, e). This was possibly due to an inefficient absorption of water by the roots and a poor translocation of water and mineral nutrients to the shoots, as suggested by the ultrastructural changes in cells of the root tip cortex. Concentrations equal and above 8 mg Cu L^{-1} considerably affected root cells by promoting plasmolysis, likely due to osmotic effects and disruption of the plasma membrane (Fig. 3). Panou-Filotheou and Bosabalidis (2004) have also observed plasmolysis, disintegration of cell membranes and non-recognition of any organelles in root cells of *Origanum vulgare* plants exposed to Cu stress. In leaves of *T. cacao* seedlings exposed to higher levels of Cu, the damage imposed to the thylakoid membranes and chloroplast's double membrane certainly contributed to the observed changes in leaf gas exchange. Storage of starch grains in chloroplasts was not observed, likely due to a sharp decline in carbon assimilation and to mobilization of all assimilated compounds from the current photosynthesis to other metabolic drains. Symptoms of starch grains absence and swelling of chloroplasts double membrane have also been identified in leaves of *O. vulgare* after Cu treatment (Panou-Filotheou et al. 2001). In *Populus tremula* plants grown in soil containing a mixture of heavy metals including Cu, breakages in thylakoid membranes and chloroplast envelope were observed, with extrusion of waste material to the cytoplasm (Hermle et al. 2007). Swelling of thylakoid and increased interthylakoidal spaces were also featured in chloroplasts of *T. aestivum* under Zn-deficiency stress (Peck and McDonald 2010).

The loss of membrane integrity in root and leaf cells of *T. cacao* was evaluated by the levels of lipid peroxidation. TBARS concentrations increased up to 15× in roots for the highest Cu dose, suggesting that, above 4 mg Cu L^{-1}, changes in cell membranes were due to lipids degradation. On the other hand, in leaf mesophyll cells, the damage to chloroplasts (Fig. 4) and the increase in electrolyte leakage (Fig. 5) appear to be related to possible effects of Cu on membrane proteins, because there was no significant difference (p<0.05) between the concentration of TBARS in control and in Cu treatments. However, as we discuss below, Cu was mostly retained in the root system, thereby suggesting that the ultrastructural effects on roots and leaf cells are of a different nature. Excessive

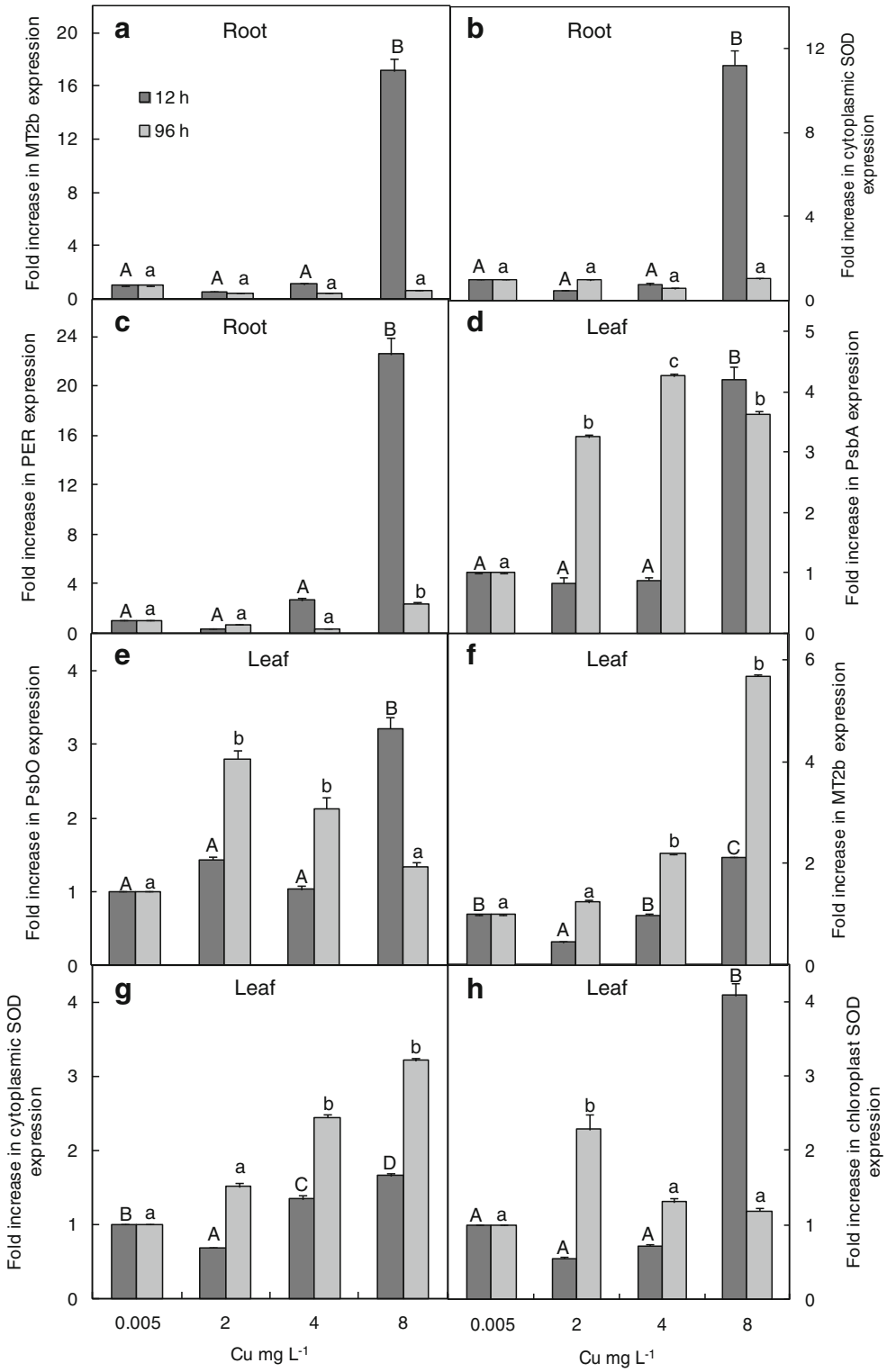

Fig. 9 Amount of transcripts in the roots (**a**–**c**) and leaves (**d**–**h**) of young *T. cacao* plants exposed to increasing concentrations of Cu in nutrient solution for 12 and 96 h (n=3, ±SE). The mRNA levels were quantified by quantitative real-time PCR. The mRNA levels were normalized with respect to tubulin, and are expressed relative to those of control plants that were given a value of 1. *Uppercase letters* compare averages between treatments 12 h AAT and *lowercase letters* compare averages between treatments 96 h AAT. Means followed by the same letter are not significantly different at $p<0.05$, according to Tukey's test

amounts of Cu showed to induce oxidative stress responses, with the formation of ROS that cause lipid peroxidation of cell membranes and increases in TBARS levels (mainly MDA) in plant cells (Pätsikkä et al. 2002; Janas et al. 2010). Moreover, Cu can also damage the membranes by reacting with sulfhydryl groups, which lead to proteins denaturation (Panou-Filotheou and Bosabalidis 2004).

The observed low mobility of Cu within the *T. cacao* plant, as suggested by its major accumulation in the root system (Fig. 6), confirms similar results in other studies in

various plant species (Pätsikkä et al. 2002; Panou-Filotheou and Bosabalidis 2004; Shin-Sheng 2007; Bouazizi et al. 2010; Cambrollé et al. 2011). It has been suggested that the preferential accumulation of Cu in roots is the result of a tolerance mechanism in plants to cope with the stress caused by this metallic element at high concentrations (Bouazizi et al. 2010). When such mechanisms of Cu tolerance in the root zone become overloaded, Cu^{+2} is then translocated to shoots through xylem and phloem. When it reaches the leaves, a small proportion of Cu accumulates in the chloroplasts, which is likely responsible for the ultrastructural damages observed (Figs. 3 and 4). Taken together, these results suggest that the transport of this metal is a highly regulated process, even in plants exposed to Cu excess (Pätsikkä et al. 2002). Patterns of stress-response gene expression obtained (and discussed further below) come into support of this view. A crop-cultivating soil is a physicochemically and biologically dynamic entity, such that distribution and availability of any element to plants tend not to occur in a uniform and homogenous manner; soil patches of different sizes, composition and locations compose a mosaic that will display distinct forms and concentrations of the various soil elements available to plants. Hence, in a cacao production field, we can reasonably speculate that the protection against excess of Cu in adult plants operates at the level of morphological and gene expression changes in the root system, likely to avoid absorption and/or prevent this element to translocate to more sensitive tissues/cells of the plant.

Despite its low mobility, and accumulation mainly in the root system, Cu affects the transport of other essential mineral nutrients for plant metabolism (Shaul 2002; Xiong et al. 2002), as shown in this study. The uptake of Mn and Zn by roots of *T. cacao* seedlings was significantly affected with increasing Cu in the nutrient solution. However, the levels of these elements in leaves were practically unchanged, possibly due to the fact that they are critical for photosynthetic processes. Mn is an essential cofactor in the water oxidation pathway of PS II (Dučić and Polle 2005). Zn occurs in high concentrations in biological systems when compared with other micronutrients. Zn helps maintaining the structural integrity and controlling the permeability of cell membranes, it is critically important for cells protection from ROS-induced damages (Cakmak 2000). Thus, the decrease of Zn in *T. cacao* roots may have contributed to the disruption and lipid peroxidation of cell membranes, as observed by ultrastructural analysis (Figs. 3 and 4) and TBARS quantification (Fig. 5). Moreover, in many monocots and eudicots, Zn deficiency causes accumulation of Fe in roots and shoots, which is associated with increased lipid peroxidation and damage to the chlorophyll molecules in plants under stressing conditions (Cakmak 2000). The Cu-associated increase in Fe content that occurred in roots and leaves of the studied seedlings was likely coupled to the decline in Zn and may have also influenced the damage observed in cell membranes and in photosynthetic rates.

The Mg element in plants has the ability to interact with nucleophilic ligands (Shaul 2002). It is the central atom of the chlorophyll molecule and is an important element for ribosomal subunits aggregation during protein biosynthesis. In chloroplasts, Mg^{2+} is required to activate Rubisco, thereby resulting in CO_2 fixation (Shaul 2002). When plant species are submitted to excessive amounts of Cu, Mg can be chemically replaced by this element in the chlorophyll molecule, leading to a decline in photosynthesis (Kupper et al. 1998). Hence, the decrease in Mg concentration in shoots of *T. cacao* and its replacement by Cu in chlorophyll may have contributed to the decline in assimilation rates of CO_2 in this species. With regards to K, a decline in its levels often indicates efflux of this element across a plasma membrane, which was likely damaged by the lipid peroxidation promoted by Cu excess, resulting in loss of membrane selectivity and increase in its permeability (Murphy et al. 1999; Janas et al. 2010). Toxic levels of Cu can also inhibit membrane ATPases and promote increased cytoplasmic concentrations of Ca, due to blockage in ATP-dependent pumping of Ca to the outside of the cell. The Cu-induced increase of cytoplasmic Ca levels triggers various catabolic processes by activation of phospholipases, which result in the formation of free radicals (Maksymiec 1997).

Transcriptional analysis of gene expression by qPCR suggests that the antioxidant defense systems and abiotic stress tolerance in seedlings were sharply stimulated by Cu excess in nutrient solution. Up-regulation of metallothioneins in plants has been constantly related to Cu homeostasis by detoxification and protection against oxidative stress (Cobbett and Goldsbrough 2002; Cozza et al. 2012). The increased expression of *MT2b* only 12 h AAT implies that the protection strategy adopted by *T. cacao* is to tolerate and accumulate high levels of Cu in the root system, in order to ensure the maintenance of metabolism in leaves. Studies with *Silene vulgaris* tolerant to Cu have shown higher levels of mRNA transcripts for *MT2b* (van Hoof et al. 2001). On their turn, the increased expression of *SODCyt* and *PER*-1 in roots, and *SODChl* and S*ODCyt* in leaves of *T. cacao* suggest the existence of a defense mechanism against oxidative stress promoted by Cu, by the expression of genes encoding antioxidant enzymes (Lee et al. 2007). The SOD enzymes constitute the first line of cellular defense against ROS (Alscher et al. 2002), whereas the class III peroxidases are involved in the formation of lignin and suberin, as well as in maintaining adequate levels of ROS (Almagro et al. 2009).

The gene *psbO* encodes a protein that is extrinsic to the oxygen evolution center located in PS II, being considered as the main protein responsible for water photolysis (De Las Rivas et al. 2004). Mutants of *Arabidopsis thaliana* that lack the *psbO* gene have shown a decreased activity of the PS II (Murakami et al. 2002). In turn, *psbA* is located in the chloroplast genome and encodes a protein, PsbA or D1 that is intrinsic to the PS II (Nelson and Yocum 2006). Since

stresses caused by heavy metals promote reduction of *psbA* gene transcripts, and so, interfere with the operation of PS II (Geiken et al. 1998; Allakhverdiev et al. 2002), the decreases in *T. cacao* leaf gas exchange is likely related to damage to PS II as described for other plant species subjected to stress by Cu (Maksymiec 1997; Pätsikkä et al. 2002). The exposure of *T. cacao* seedlings to 8 mg Cu L^{-1} stimulated an increase in *PSBA* and *PSBO* transcripts at 12 h AAT, likely to prevent damage to PS II. However, at 96 h AAT, their expression was lower than in the 2 and 4 mg Cu L^{-1} doses (Fig. 9), suggesting that inhibition in their transcription has likely started from this level on. This would be in agreement with the fact that there was no recovery of the leaf gas exchange after treatments with higher Cu doses, as previously discussed (Fig. 1). The levels of transcripts of *PSBA* and *PSBO* at 96 h AAT increased for the 2 and 4 mg Cu L^{-1}, suggesting that *T. cacao* tolerates well up to these Cu concentrations.

Despite that exposure to Cu was only during a very short period of time, not addressing effects of a long-term stress, or acclimation/adaptation types of response, all the damages identified at the cellular level can be regarded as irreversible to those cells and able to affect downstream processes and metabolisms of the plant, as long as the Cu stress is maintained (Lichtentaler 1996). From the present studies with seedlings, we conclude that the *T. cacao* plants are likely capable of tolerating Cu concentrations from 4 to 8 mg Cu L^{-1} at their root system. Cu doses above these levels (at least in conditions that surround the entire root system in a homogeneous and constant manner, as provided by nutrient solutions) appear to be very toxic, remarkably interfering with the overall metabolism of this species. As a tolerance strategy, Cu was mainly accumulated in roots and remained mostly restricted to this organ, indicating a low mobility within the plant. In this sense, this plant species appears to be a rhizofilterer of this metal. In spite of the increase expression of genes involved in tolerance to stresses, such as that caused by Cu, the excess of this metallic element in the roots caused the loss of integrity of cell membranes through lipid peroxidation, leading to an imbalanced uptake and translocation of water and mineral nutrients to shoots. These events irreversibly compromised the ultrastructure of chloroplasts and the leaf gas exchange. Taken together, all these alterations observed in *T. cacao* seedlings subjected to increasing levels of Cu suggest that plants grown in soils with high levels of copper can be negatively affected in their growth and metabolism. Our results also indicate that the major deleterious effects of high levels of Cu at the root system is more likely related to potential losses in cacao productivity due to the physiological and the ultrastructural changes observed, rather than to the accumulation of Cu in shoot tissues at toxic levels (although this effect should not be neglected). Therefore, accumulation of Cu in the cacao beans at toxic levels for consumption appears not to become a problem, as environmental pollution by copper will likely strike directly on cacao production resulting in the lack of beans available for consumption.

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