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Photosynthetic, antioxidative, molecular and ultrastructural responses of young cacao plants to Cd toxicity in the soil

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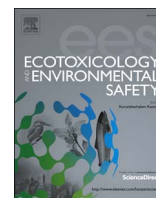*See next page for additional authors*

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Photosynthetic, antioxidative, molecular and ultrastructural responses of young cacao plants to Cd toxicity in the soil

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ABSTRACT

Cadmium (Cd) is a highly toxic metal for plants, even at low concentrations in the soil. The annual production of world cocoa beans is approximately 4 million tons. Most of these fermented and dried beans are used in the manufacture of chocolate. Recent work has shown that the concentration of Cd in these beans has exceeded the critical level (0.6 mg kg^{-1} DM). The objective of this study was to evaluate the toxicity of Cd in young plants of CCN 51 cacao genotype grown in soil with different concentrations of Cd (0, 0.05 and 0.1 g kg^{-1} soil) through photosynthetic, antioxidative, molecular and ultrastructural changes. The increase of Cd concentration in the soil altered mineral nutrient absorption by competition or synergism, changed photosynthetic activity caused by reduction in chloroplastidic pigment content and damage to the photosynthetic machinery evidenced by the *Fv/Fm* ratio and expression of the *psbA* gene and increased GPX activity in the root and SOD in leaves. Additionally, ultrastructural alterations in roots and leaves were also evidenced with the increase of the concentration of Cd in the soil, whose toxicity caused rupture of biomembranes in root and leaf cells, reduction of the number of starch grains in foliar cells, increase of plastoglobules in chloroplasts and presence of multivesiculated bodies in root cells. It was concluded, therefore, that soil Cd toxicity caused damage to the photosynthetic machinery, antioxidative metabolism, gene expression and irreversible damage to root cells ultrastructure of CCN 51 cocoa plants, whose damage intensity depended on the exposure time to the metal.

1. Introduction

Cadmium (Cd) does not have any known biological function in plants and animals, being a toxic element even at low concentrations (Gallego et al., 2012). In the soil, this metal element originates from geogenic processes and/or anthropogenic actions. The main natural sources are volcanoes and meteorites (Tran and Popova, 2013) and the anthropogenic sources are fossil fuels, industrial waste from galvanization, mining, plastics, batteries and phosphate fertilizers (Moradi et al., 2005). In the soil, Cd is found in the inorganic form Cd^{+2}, presenting low adsorption coefficient and high mobility in the soil-plant system (Clemens and Ma, 2016). The bioavailability of this metal in the soil is controlled by the presence of organic matter, redox potential, the concentration of other mineral elements essential for plants, and mainly by the pH (Dong et al., 2007). According to this last author the decrease in soil pH increases the concentration of Cd in plants.

Cadmium toxicity causes inhibition and abnormalities in the overall growth of many plant species, interfering in the physiological and biochemical processes, inhibiting photosynthesis and respiration, and causing irreversible damage to cell structures (Dias et al., 2013; He et al., 2015). Oxidative stress is another effect of Cd, although this metal does not actively participate in the Fenton reactions, which produces reactive oxygen species (O$_2$$^{\cdot-}$, H$_2O_2$, OH$^{\cdot}$, ^{1}O$_2$) and interferes in the antioxidant system (Benavides et al., 2005; Tran and Popova, 2013). Reactive oxygen species (ROS) are byproducts of aerobic metabolism and can act as cellular signals, however, in high concentrations ROS can cause oxidative damage in membranes (lipid peroxidation), lipids, proteins, DNA and RNA molecules, causing autophagy or even apoptosis (Demidchik, 2015; Michaele et al., 2014). On the other hand, there are a variety of proteins that act as detoxifiers, such as Fe-dependent or Cu/Zn Superoxide dismutase (SOD) isoforms, which convert O$_2$$^-$ into H$_2$O$_2$, and ascorbate peroxidase (APX) and guaiacol

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peroxidase (GPX) that convert H_2O_2 to water and oxygen, donating electrons from ascorbate and guaiacol, respectively (Gill and Tuteja, 2010; Choudhury et al., 2016). Cd may increase or reduce the activity of these enzymes, depending on the species (Tran and Popova, 2013).

Phytochelatins (PCs), low molecular weight chelators, are used as the main strategies by plants to reduce the effects of non-essential metals such as Cd (Tran and Popova, 2013). PCs are a family of peptides with general structure of $(\alpha\text{-Glu-Cys})_n\text{-Gly}$, where n ranges from 2 to 11. Due to the presence of the cysteine thiol group, PCs can chelate Cd, protecting the cytosol from free ions of Cd, moving it to the vacuoles. The synthesis of phytochelatins occurs by the enzyme phytochelatin synthetase, which is activated in the presence of metals (Gallego et al., 2012). On the other hand, metallothioneins (MTs), part of another class of metal chelators, are cysteine-rich proteins that inactivate metal ions forming MTs-metal complexes. Metallothioneins are products of mRNA translation which are expressed in response to different types of abiotic stresses (Gallego et al., 2012).

Theobroma cacao L. is cultivated in tropical regions worldwide to produce beans that are used for confection of chocolate and cocoa butter as well as cosmetics and foodstuffs (Almeida and Valle, 2007). The species shows high genetic variability for physiological and morphological characteristics (Daymond et al., 2002; Bartley, 2005). Among the cacao genotypes, the CCN 51 clonal cultivar has been outstanding in production because it is self-pollinated, more resistant to diseases, more robust, and has large seeds with high fat content (cocoa butter) (Boza et al., 2014). The world annual production of cocoa beans is estimated at more than 4 million tons (ICCO, 2016 nª4). The critical concentration of Cd in cocoa beans, established by the European Union is 0.6 mg kg^{-1} (Mounicou et al., 2003); however, there are studies showing that seeds from different origins in the world exceed this limit, raising concerns for human health (Huamani-Yupanqui et al., 2012). A recent study in Ecuador demonstrated the presence of Cd in cocoa beans and in the cultivated soils (Chavez et al., 2015). In addition to the parent materials responsible for soil formation, the application of phosphate fertilizers is considered one of the main sources of Cd in agricultural soils, which may be higher than $130 \text{ mg Cd kg}^{-1}$ soil (Jiao et al., 2012; Chavez et al., 2015).

In this study we evaluated Cd toxicity in young plants of CCN 51 cultivated in soil with different concentrations of Cd [control (without addition of Cd), 0.05 and 0.1 g Cd kg^{-1} soil] by means of photosynthetic, antioxidative, molecular and ultrastructural changes in the plants.

2. Material and methods

2.1. Greenhouse and Seeds

The experiment was conducted in a greenhouse at Santa Cruz State University Campus (UESC), Ilhéus, Bahia, Brazil (14°47′ S, 39°10′ W). Cacao seeds of CCN-51 were germinated and grown in sandy soil (pH 4.7) as substrate, without addition of Cd (control) and with Cd concentrations (0.05 and 0.1 g kg^{-1} soil) in the form of $CdCl_2$ in black plastic pots (4 L). Seeds of CCN 51 were obtained by self-pollination of clonal accessions at the Cacao Germplasm Bank of the Cacao Research Center of the "Comissão Executiva do Plano da Lavoura Cacaueira" (CEPLAC), Ilhéus, Bahia, Brazil. During the 120 days of the experiment, the plants were irrigated with deionized water to keep soil moisture at field capacity. Soil characteristics and fertilization are presented in Supplementary Material.

2.2. Leaf gas exchange

During the experimental period, plants were evaluated at 60, 90 and 120 days after emergence for net photosynthetic rate per unit of leaf area (P_N), stomatal conductance to water vapor (g_s) and leaf transpiration (E) between 08:00 and 9:00 am, on a mature and completely

expanded leaf. Five plants per treatment were assessed using a LI-6400 portable photosynthesis system (Li-Cor, Nebraska, USA) equipped with a 6400-02B RedBlue artificial light source. For the leaf gas exchange measurements, the artificial light source of the system was adjusted to provide a photosynthetic photon flux density (PPFD) of $700 \text{ µmol m}^{-2} \text{ s}^{-1}$. To save each reading, the minimum pre-established time for reading stabilization was 60 s and the maximum 120 s. Also, the reading was saved if the coefficient of variation for the measurements was less than 0.3%. In addition to PPFD, temperature and atmospheric CO_2 within the leaf chamber were maintained constant at 26 °C and $380 \text{ µmol } (CO_2) \text{ mol}^{-1}$, respectively.

2.3. Fluorescence emission

The Chlorophyll (Chl) fluorescence emission was measured simultaneously on the same leaves ($n = 5$) and same period used for gas-exchange measurements, with a portable fluorometer unmodulated (Pocket PEA Chlorophyll Fluorometer - v 1.10 - Hansatech Instruments, Norfolk, UK). To assess the Chl fluorescence emission in dark-adapted leaves, the leaf tissue was placed in the leaf clips for 30 min on each leaf prior to each measurement. Following dark-adaptation, the leaf tissue was illuminated with a weak-modulated measuring beam (3500 µmol photons $\text{m}^{-2} \text{ s}^{-1}$, wavelength of 650 nm, 1 s) to obtain the minimal fluorescence (F_0). A saturating white-light pulse (20 kHz; 6000 µmol $\text{m}^{-2} \text{ s}^{-1}$, 630 nm, 0.8 s) was applied to ensure maximum fluorescence emission (F_m). The maximum quantum yield of photosystem 2 (F_v / F_m) was calculated as $[F_v / F_m = (Fm - F_0) / F_m)]$ (Roháček, 2002; Baker, 2008).

2.4. Cd and nutrients

At the end of the experiment, five seedlings with 120 days of growth were collected from the different treatments and separated into roots, stems, and leaves. Dry plant materials were ground and subjected to nitric-perchloric digestion (3:1). After digestion, Cd, Ca, Mg, Fe, Zn, Cu and Mn were determined by atomic absorption spectrophotometry; P was determined by colorimetry and K by flame emission photometry (Embrapa, 1997). Nitrogen was determined by the Kjeldahl method after sulfosalicylic acid digestion (Jones et al., 1991).

2.5. Chloroplastidic pigments

After 120 days of growth the content of photosynthetic pigments was determined in the same leaves used for the gas-exchange measurements (n = 5), using methodology described by Hiscox and Israelstam (1979). Chl a, Chl b, total Chl, and carotenoid (car) content were determined using equations described by Wellburn (1994) for DMSO extracts.

2.6. Antioxidants

Samples of roots and leaf tissue from the second mature leaf from the apex were harvested at 120 days of growth, frozen in liquid nitrogen and lyophilized. The activity of the enzymes guaiacol peroxidase (GPX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1) was determined according to methodologies proposed by Pirovani et al. (2008), Nakano and Asada (1981) and Giannopolitis and Ries (1977), respectively. The sample and standard readings were done with a UV–visible spectrophotometer (SpectraMax Paradigm Multi-Mode Microplate Reader, Molecular Device, USA).

2.7. Gene Expression

Samples of roots and leaf tissue from the second mature leaf from the apex were harvested at 120 days of growth frozen in liquid nitrogen

Table 1
Gene-specific primer pairs that were used in qRT-PCR analysis.

Gene	Acess	Function	Primer
Cu-Zn sodcyt	CL94Conting1[a]	Biosynthesis of cytosolic Cu-Zn SOD	F—5′-GATGATGGCTGTGTGAGTTTCTCT-3′
			R—5′-CAACAACAGCTCTTCCAATAATTGA-3′
Cu–Zn sodchl	CL872Contig1[a]	Biosynthesis of chloroplastic Cu–Zn SOD	F—5′-AATGGATGCATGTCAACAGGAGC−3′
			R—5′-ATGTTTCCCAGGTCACCCGC-3′
phyt	XM_007050160[b]	Heavy metal-detoxifying	F—5′- TTCAGGCACGGTAATTAGTAATGG -3′
			R—5′- GGATGCATGCCACAACAATTAT -3′
mt2b	CL9Contig1[a]	Biosynthesis of metallothionein	F—5′-GCAACCCTTGCACTTGTAAATG-3′
			R—5′-CAAGCCATGGCAACTTTATTCTAA-3′
psbA	NC_014676.2[c]	Biosynthesis of the PsbA protein or D1 protein	F—5′-GGTTTGCACTTTTACCCGA−3′
			R—5′-CTCATAAGGACCGCCATT-3′
psbO	CL326Contig1[a]	Biosynthesis of PsbO protein	F—5′-GCAAACGCTGAAGGAGTT-3′
			R—5′-GGCTTGAAGGCAAATGAGTC-3′
β-Tubulin	GU570572.1[c]	Endogen	F—5′-TGCAACCATGAGTGGTGTCA-3′
			R—5′-CAGACGAGGGAAAGGAATGA-3′
act	XM_018128615[c]	Endogen	F—5′- TCCTCTTCCAGCCATCTCTC-3′
			R—5′-TCTCCTTGCTCATTCGGTCT-3′

[a] http://esttik.cirad.fr/index.html
[b] http://cocoagendb.cirad.fr/
[c] http://www.ncbi.nlm.nih.gov/

and stored at − 80 °C. Prior to RNA extraction, the tissues were lyophilized. RNA was extracted from roots and leaves of the three different treatments (control, 0.05 and 0.1 mg kg^{-1} Cd) with RNAqueous kit (Ambion®). 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 qPCR was performed in a "Real Time PCR" System (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. Threshold cycle (Ct) values were determined using the LightCycler software. Numbers on the relative expression of genes were calculated as a percentage of the control treatments, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and actin and β-Tubulin as endogenous control in order to detect changes.

2.8. Ultrastructure

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 harvested at 120 days. 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 LRWhite 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 by a Diamond knife using an 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 cacao leaf *meso*phyll and root cells, under concentrations of Cd were selected.

2.9. Statistical analysis

The experiment was performed on a completely randomized arrangement, with 2 Cd concentrations (0.05 and 0.1 g Cd kg^{-1} soil) + control (without addition of Cd in the soil), containing five replicates of each treatment. Analysis of variances (ANOVA) were carried out to analyze the treatments and Tukey test ($p < 0.05$) was performed to separate the averages.

3. Results

3.1. Cd accumulation

Cadmium content in the root, stem and leaf of the CCN 51 cacao plants increased with increasing Cd concentrations in the soil (Table 2). The Cd content accumulated in the leaves and roots of the control plants were 0 and 1.15 mg Cd g^{-1} DW, respectively. On the other hand, in the treatment corresponding to 0.05 g Cd kg^{-1} soil, the accumulated Cd content in roots and leaves were 218 and 235 mg Cd g^{-1} DW, respectively. In the treatment of 0.1 g Cd kg^{-1} the contents were 345 and 388 mg Cd g^{-1} DW, respectively. In addition, the lowest Cd content, in both concentrations of Cd applied to the soil, was accumulated in the stem and the highest in the leaves. (Table 2).

3.2. Nutrients

The increase in soil Cd concentrations caused significant changes in the absorption and accumulation of macro and micronutrients in the different organs of the CCN 51 plants (Table 2). At the concentration corresponding to 0.05 g Cd kg^{-1} soil, N accumulation was higher in the roots and stems (45% and 24%, respectively) in relation to the control. On the other hand, in the concentration of 0.1 g Cd kg^{-1} soil, a 63% increase in N was observed in the stem, while in the roots and leaves the values were not detectable. On the other hand, P concentration values were not significantly different for roots and stems, which increased by 31% and 73% in the concentrations of 0.05 and 0.1 g Cd kg^{-1}, respectively; while the leaves only showed variation when the plants were subjected to the concentration 0.1 g Cd kg^{-1} soil, with an increase

Table 2
Accumulation of Cd and changes in the concentrations of macronutrient and micronutrient in roots, stems and leaves of CCN 51 cacao plants submitted to different concentrations of Cd in soil, 120 days after seeding. Mean values of five replicates (± SE). Letters indicate comparisons between treatments by Tukey test (p < 0.05).

Parameter	Cd (g kg⁻¹ soil)		
	0	0.05	0.1
Toxic element (mg g⁻¹ DW)			
Cd			
Root	1.15 ± 0.35 c	218.65 ± 12.29 b	344.83 ± 15.73 a
Stem	0.54 ± 0.11 c	166.31 ± 13.96 b	263.83 ± 7.51 a
Leaf	0.00 ± 0.00 c	235.60 ± 11.38 b	388.97 ± 10.58 a
Macronutrient (g kg⁻¹ DW)			
N			
Root	22.72 ± 1.62 b	33.16 ± 0.12 a	0.00 ± 0.00 c
Stem	24.70 ± 0.21 c	30.73 ± 0.52 b	40.44 ± 0.45 a
Leaf	31.10 ± 1.70 a	35.79 ± 1.74 a	0.25 ± 0.05 b
P			
Root	0.91 ± 0.03 a	1.15 ± 0.14 a	0.96 ± 0.02 a
Stem	1.22 ± 0.03 c	1.60 ± 0.08 b	2.12 ± 0.09 a
Leaf	1.18 ± 0.06 b	1.39 ± 0.08 b	1.74 ± 0.11 a
K			
Root	12.21 ± 0.42 a	10.34 ± 0.38 b	7.69 ± 0.28 c
Stem	9.99 ± 0.37 a	8.52 ± 0.40 b	7.59 ± 0.05 b
Leaf	11.82 ± 0.17 a	12.00 ± 0.59 a	14.07 ± 0.84 a
Ca			
Root	3.62 ± 0.25 a	3.25 ± 0.27 a	2.94 ± 0.21 b
Stem	7.59 ± 0.30 a	6.13 ± 0.19 b	5.83 ± 0.12 b
Leaf	13.01 ± 0.64 b	21.56 ± 1.32 a	16.48 ± 1.60 b
Mg			
Root	4.00 ± 0.10 a	2.77 ± 0.18 b	2.38 ± 0.11 b
Stem	5.35 ± 0.29 a	5.62 ± 0.05 a	5.93 ± 0.11 a
Leaf	4.57 ± 0.08 c	5.89 ± 0.08 b	7.15 ± 0.30 a
Micronutrient (mg kg⁻¹ DW)			
Cu			
Root	5.86 ± 0.63 b	6.83 ± 0.30 b	8.56 ± 0.33 a
Stem	2.87 ± 0.24 c	4.14 ± 0.20 b	5.82 ± 0.10 a
Leaf	3.39 ± 0.20 a	2.84 ± 0.14 a	3.14 ± 0.31 a
Fe			
Root	2034.66 ± 217.80 a	1924.07 ± 171.54 a	2232.25 ± 97.23 a
Stem	394.99 ± 49.63 a	435.45 ± 57.82 a	491.05 ± 17.71 a
Leaf	230.28 ± 18.85 a	132.43 ± 3.20 b	141.04 ± 17.23 b
Zn			
Root	22.96 ± 1.71 a	17.90 ± 0.78 b	16.81 ± 0.60 b
Stem	20.57 ± 0.50 a	19.14 ± 0.24 a	21.63 ± 0.44 a
Leaf	23.00 ± 0.90 a	20.78 ± 1.11 a	23.45 ± 0.35 a
Mn			
Root	162.39 ± 18.56 b	169.37 ± 26.34 b	235.71 ± 15.42 a
Stem	228.90 ± 22.82 a	141.84 ± 15.08 b	135.12 ± 7.30 b
Leaf	891.18 ± 19.23 b	855.55 ± 22.90 b	993.16 ± 38.09 a

of 47% in relation to the control. There was a reduction in K, Ca and Mg concentrations in roots and stems of plants subjected to soil Cd. In the roots, K concentration ranged from 7.69 g kg⁻¹ DW at the 0.1 g Cd kg⁻¹ soil to 12.2 g kg⁻¹ DW in the control. Ca ranged from, 2.94–3.62 g kg⁻¹ DW and Mg ranged from 2.38 to 4.00 g kg⁻¹ DW at the 0.1 g Cd kg⁻¹ soil and in the control, respectively. In leaves, K concentration did not show a significant difference (p < 0.05), whereas Ca concentration varied only when the plants were subjected to a 0.05 g Cd kg⁻¹ soil, which increased by 65%. However, the Mg concentration increased by 28% and 56% at 0.05 and 0.1 g Cd kg⁻¹ soil, respectively (Table 2).

In relation to the mineral micronutrients, no significant differences were observed for Cu and Zn concentrations in the leaves, Fe and Zn in the stems and Fe in the roots, with the increase of Cd levels in the soil. On the other hand, Cu concentrations increased by 46% in the roots at the level of 0.1 g Cd kg⁻¹ soil, whereas in the stem the increases were 44% and 77% at the 0.05 and 0.1 g Cd kg⁻¹ soil levels, respectively. The Fe concentration in the leaves was reduced by approximately 42% at both levels of Cd in the soil, in relation to the control. In addition, there was a reduction of 15% in the Zn concentration in the roots, in

relation to the control, in the treatments with Cd in the soil. The Mn concentrations in the roots and leaves increased 45% and 11%, respectively, in the 0.1 g Cd kg⁻¹ soil treatment, whereas in the stem there was a 40% reduction in the Mn concentration in both levels of Cd in the soil (Table 2).

3.3. Leaf gas exchange

The increase in soil Cd concentration and the time of exposure to the metal caused reductions in P_N, g_s and E values (Fig. 1). At 60 days of growth there was a significant difference in P_N, g_s and E values in relation to the control. At the concentration of 0.1 g Cd kg⁻¹ soil, the reductions were 34%, 40% and 30% for P_N, g_s and E, respectively. At 90 days of growth the reductions were 40% and 70% (P_N), 33% and 57% (g_s) and 30% and 55% (E), for the concentrations of 0.05 and 0.1 g Cd kg⁻¹ soil, respectively. At 120 days P_N decreased 70% and 80% at the concentration of 0.05 and 0.1 g Cd Kg⁻¹ soil, respectively, compared to control. However, g_s and E values did not show significant differences in both concentrations of Cd in the soil, but decreased by 55% and 60%, respectively, compared to control (Figs. 1A–C).

3.4. Fluorescence

The CCN 51 plants presented significant differences for the fluorescence variables evaluated (Figs. 1D, 1E, 1F). At 60, 90 and 120 days, F_0 was higher in both concentrations of Cd (0.05 and 0.1 g kg⁻¹ soil), compared to control. In addition, a change in F_m values with exposure time to the toxic metal was observed. At 60 days, there was no significant difference between treatments for the F_m values. However, at 90 days there were decreases of 12% and 30% in F_m values, compared to control, at concentrations corresponding to 0.05 and 0.1 g Cd kg⁻¹soil, respectively; while at 120 days, in both soil Cd concentrations, the reduction was approximately 30%, when compared to the control (Figs. 1D and 1E). On the other hand, the values of the F_v/F_m ratio for the plants submitted to 0.05 g Cd kg⁻¹ soil were 0.76, 0.67 and 0.52, while for the plants submitted to 0.1 g Cd kg⁻¹ soil were 0.64, 0.53 and 0.47 at 60, 90 and 120 days, respectively, demonstrating that prolonged exposure to the metal further affected photosystem 2 (PS2) (Fig. 1F).

3.5. Chloroplastidic pigments

The addition of Cd in the soil significantly reduced chloroplastidic pigments content in the leaves of CCN 51 (Table 3). The levels of Chl *a*, Chl *b* and Chl (*a* + *b*) decreased in the same proportion in relation to the control. When the plants were submitted to 0.05 and 0.1 g Cd kg⁻¹ soil, the values were 45% and 75%, respectively. Carotenoid concentrations, however, decreased by 37% and 63% at the 0.05 and 0.1 g Cd kg⁻¹ soil treatments, respectively, compared to control. On the other hand, the Chl *a/b* ratio did not present significant differences in relation to the treatments. However, the concentrations of Cd in the soil caused an increase in the ratio Car/Chl (*a* + *b*) (Table 3).

3.6. Antioxidants

The activity of SOD, APX and GPX enzymes was evaluated in roots and leaves of plants subjected to soil Cd treatments. The APX activity in the leaves was three times higher than in the roots, whereas the SOD activity and GPX activity were higher in leaves and roots (Table 3). In relation to the control, the SOD activity was reduced in the roots by 50% and 60% and increased in the leaves by 77% and 55% for the 0.05 and 0.1 g Cd kg⁻¹ soil treatments, respectively. In addition, there was a reduction in APX activity in the roots (35%) and leaves (42%) in both levels of soil Cd. However, GPX activity was observed to be three times increased in the roots in the 0.05 g Cd kg⁻¹ soil, and five times increased in the 0.1 g Cd kg⁻¹ soil level. GPX activity doubled in the
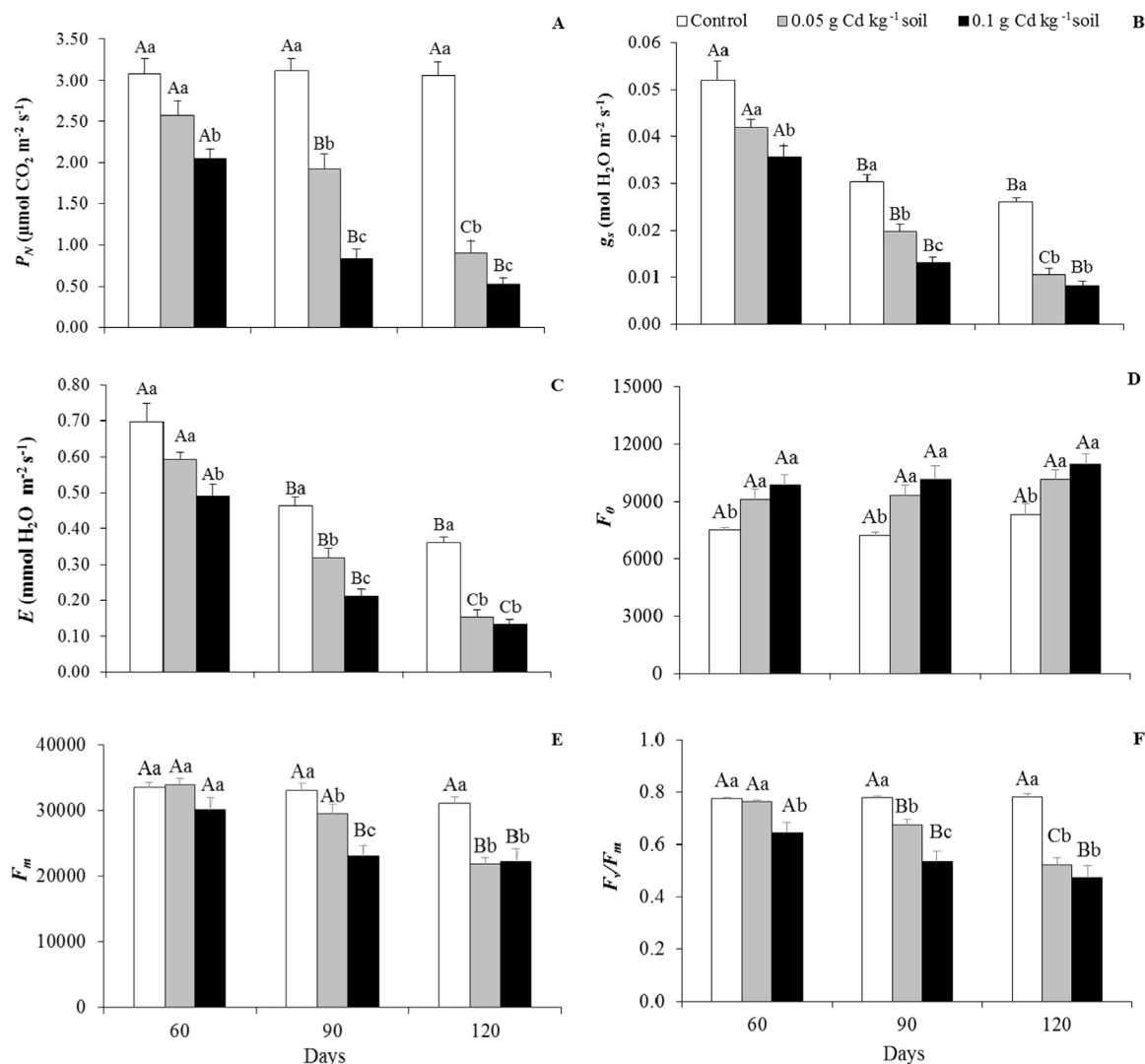
Fig. 1. (A) Net photosynthesis per unit area, (B) stomatal conductance to water vapor leaf, (C) transpiration rate, (D) initial fluorescence, (E) maximal fluorescence and (F) maximum quantum efficiency of photosystem 2, in leaves of CCN 51 cacao plants, submitted to concentrations of Cd in soil 60, 90 and 120 days after emergence. Mean values of four replicates (± SE). Lower case letters indicate comparisons between treatments and capital letters between time in days by Tukey test (p < 0.05).

leaves, for the both soil Cd levels (0.05 and 0.1 g Cd kg^{-1} soil) in relation to the control (Table 3).

3.7. Gene expression

The relative expression of the *Cu-Znsod cyt, phyt* and *met2b* genes in the roots of the CCN 51 plants submitted to soil Cd toxicity was evaluated (Fig. 2). Higher expression of *phyt* and *met2b* genes, about 2-fold higher, was observed at the 0.05 g Cd kg^{-1} soil treatment. In contrast, *Cu-Znsod cyt* and *phyt* genes were repressed at the 0.1 g Cd kg^{-1} soil treatment (Fig. 2A). In addition, the relative expression of the six genes was evaluated at the foliar level, two related to oxidative stress (*Cu-Znsodcyt* and *Cu-Znsodchl*), two associated to metal sequestration in the cytosol (*phyt* and *met*) and two involved in the synthesis of PS2 proteins, one intrinsic (*psba*) and one extrinsic (*psbo*).

There was a six-fold increase in *Cu-Znsod cyt* gene expression and a repression of the *Cu-Znsod chl* gene in both treatments with addition of Cd in the soil (0.05 and 0.1 g Cd kg^{-1} soil). In contrast, the *phyt* and *met2b* genes were repressed at the level of 0.1 g Cd kg^{-1} soil, while at the 0.05 g Cd kg^{-1} soil an induction of the *phyt* gene was observed, an increase of 40%. It was also verified that 0.05 g Cd kg^{-1} soil showed a significant change in the expression of the *psba* gene, whose increase was 2-fold, whereas the *psbo* gene was repressed five times at the level

of 0.1 g Cd kg^{-1} soil (Fig. 2B).

3.8. Ultrastructure

The TEM analysis allowed observing ultrastructural changes in the roots and leaves of the CCN 51 cacao plants caused by Cd toxicity. In the roots of the control plants, cellular organelles, such as nucleus, mitochondria and vacuole, together with the cell wall, remained intact and without anomalies (Fig. 3A). In contrast, at the concentration of 0.05 g Cd kg^{-1} soil, there was disorganization of the cytoplasmic membrane (Fig. 3B) and rupture of mitochondrial membranes (Figs. 3B and 3C), whereas at the concentration of 0.1 g Cd kg^{-1} soil the cellular damage was more accentuated (Fig. 3D), mitochondrial disorganization (Figs. 3B, C and E), presence of multivesiculated bodies with double membranes, an indication of autophagy, and signs of rupture in the outer membrane (Fig. 3F).

The electromicrographs of *meso*phyll cells of leaves of the control treatment presented chloroplasts with a large number of starch grains, a nucleus, mitochondria and normal cell walls (Fig. 3G). On the other hand, in the leaf *meso*phyll cells of plants grown at levels of 0.05 and 0.1 g Cd kg^{-1} soil, there was disorganization of thylakoid and mitochondrial membranes (Figs. 3G, J and L). At the 0.05 g Cd kg^{-1} soil treatment, there was a reduction in the number of starch grains and

Table 3

Content of chloroplastidic pigments in leaves, and enzymatic activity of SOD (superoxide dismutase), APX (ascorbate peroxidase), GPX (guaiacol peroxidase) in roots and leaves of CCN 51 cacao plants, submitted to three concentrations of Cd in soil 120 days after emergence. Mean values of five replicates ($\pm$ SE). Letters indicate comparisons between treatments by Tukey test (p < 0.05).

| Parameter	Cd (g kg^{-1} soil)		
	0	**0.05**	**0.1**
Pigment			
Chl a (mg m^{-2})	258.85 ± 8.86 a	139.99 ± 9.13 b	63.75 ± 4.75 c
Chl b (mg m^{-2})	76.11 ± 2.54 a	42.30 ± 2.68 b	20.15 ± 1.52 c
Chl $(a+b)$ (mg m^{-2})	334.96 ± 11.18 a	182.29 ± 11.62 b	83.90 ± 6.09 c
Car (mg m^{-2})	58.57 ± 1.91 a	36.42 ± 1.76 b	21.63 ± 1.02 c
Chl (a/b)	3.41 ± 0.06 a	3.33 ± 0.09 a	3.29 ± 0.14 a
Car /Chl $(a+b)$	0.18 ± 0.00 c	0.21 ± 0.01 b	0.28 ± 0.01 a
Enzimatic activity			
SOD (U mg^{-1} DW)			
Root	2.86 ± 0.10 a	1.45 ± 0.05 b	1.11 ± 0.08 c
Leaf	2.19 ± 0.04 c	3.89 ± 0.01 a	3.4 ± 0.17 b
APX [mol (AsA) g^{-1} (DW) min^{-1}]			
root	14.01 ± 0.45 a	10.53 ± 0.68 b	9.76 ± 0.34 b
leaf	49.97 ± 0.21 a	30.59 ± 0.93 b	28.02 ± 0.46 b
GPX [μmol(guaiacol) g^{-1}(DW) min^{-1}]			
Root	1.40 ± 0.25 c	5.26 ± 0.30 b	9.10 ± 0.05 a
Leaf	3.08 ± 0.03 c	6.44 ± 0.19 a	6.41 ± 0.09 a
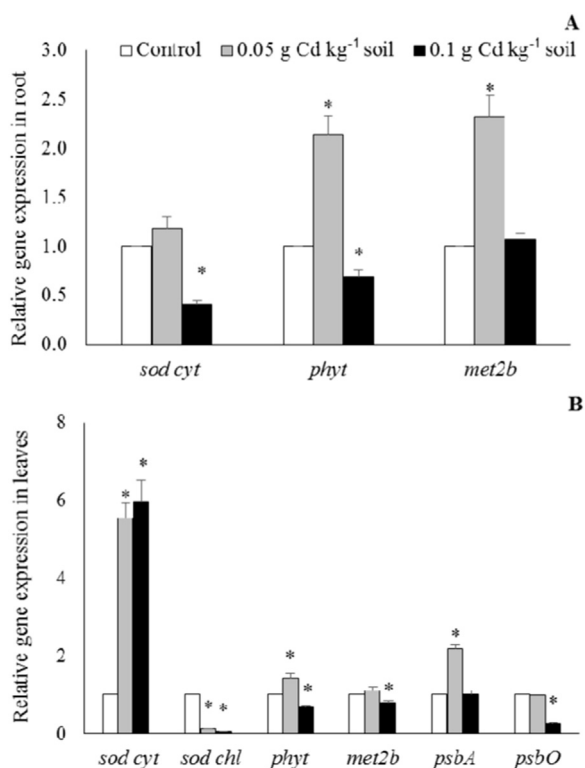
Fig. 2. Relative expression of gene in roots (A) and leaves (B) of CCN 51 cacao plants, submitted to three concentrations of Cd in soil 120 days after emergence. Mean values of four replicates ($\pm$ SE). Statistical significance was determined by Tukey test (p < 0.05).

emergence of plastoglobules (Fig. 3H), whereas in plants grown at 0.1 g Cd kg^{-1} soil the number of plastoglobules increased in relation to the 0.05 g Cd kg^{-1} soil (Fig. 3K) and no starch grains were present (Figs. 3J, K and L).

4. Discussion

4.1. Cd and mineral nutrients

Cd toxicity depends on the concentration, exposure time and soil physical and chemical characteristics, such as pH, cation exchange capacity and organic matter content (Bhargava et al., 2012; He et al., 2015). Additions of 0.05 and 0.1 g Cd kg^{-1} soil were toxic to cacao plants. The results showed that Cd was absorbed by the root system and accumulated equally in all organs of the cacao plant, demonstrating a high translocation rate in cacao. A higher accumulation of metals in the root is characteristic of intolerant plants, whereas the ability to translocate metals to the aerial part is recognized as a tolerance factor (Verbruggen et al., 2009).

Cadmium is absorbed by the root through bivalent cation transporters found in the plasmatic membrane, such as ZIP (Zrt/Irt Protein), Zn and Fe transporters (Parmar et al., 2013). Besides, it can compete with Ca^{+2}, Mg^{+2}, Mn^{+2} and Cu^{+2} transporters (Clemens, 2006). For the CCN 51 plants this competition affected the absorption of Zn and Mg, and reduced Fe translocation. Mn is an essential cofactor in the oxidation pathway of the water molecule at PS2 (Photosystem 2) (Ducic and Polle, 2005). The higher absorption of Mn may be related to mechanisms that reduce the toxic effects of Cd, as reported by Sarwar et al. (2010). Cu can be assimilated as a bivalent or monovalent cation, and the ferric reductase enzyme is responsible for the conversion of Cu^{+2} into Cu^{+}. The presence of Cd can optimize the action of this enzyme, justifying a higher absorption of copper (Mwamba et al., 2016). Previous studies have demonstrated that cocoa seedlings are able to tolerate 4–8 mg Cu L^{-1} in the root system, so the concentrations observed in this work do not indicate additional toxicity (Souza et al., 2013). Beside that, Cd can be a substitute for Ca^{+2} in calmodulin- protein dependent signaling pathways, such as enzymatic activation and gene expression control (DalCorso et al., 2013). Thus, changes in Ca content may interfere in the response of cacao plants to Cd toxicity, since the translocation factor for Ca was found to be higher in the presence of Cd.

At the highest concentration of Cd (0.1 g kg^{-1} soil), there was no detection of N in plant tissues. Probably, there were low levels of N in the plant *in vivo*, which were further reduced in the drying process of the plant material, through volatilization. N has a linear relationship with the content of chloroplastidic pigments and is part of the chemical composition of several molecules, such as proteins, chlorophylls and nucleic acids. In addition, the observed decrease of K concentration in cacao roots was also observed in plants of *Genipa americana* subjected to increasing levels of Cd in nutrient solution (Souza et al., 2011). Our data demonstrated that the Cd did not interfere in the P concentration in the roots, however, its translocation was affected, causing an increase in P concentration in the stem and leaves.

4.2. Cd and photosynthesis

Cd accumulation in leaves of cacao plants caused severe damage to photosynthesis as described in the literature (Clemens, 2006). This effect may be caused by a decrease in the expression of important genes, such as *psbA*, inactivation of enzymes involved with carboxylation, lipid peroxidation and antioxidant defense disorders (Gallego et al., 2012). In the present work, the damages were influenced by the concentration and time of exposure to the toxicity of Cd.

The *Fv/Fm* ratio is used as a stress indicator, since this ratio evaluates the efficiency and integrity of PS2 (Baker, 2008). The observed reduction for this variable in leaves of cacao plants was caused by the decrease in F_0 and *Fm* values, indicating that there was damage in the PS2 reaction center (Baker, 2008). In a recent study evaluating the toxicity of Cd in *Populus* plants grown in nutrient solution with increasing concentrations of Cd, the authors verified low variation in the values of the *Fv/Fm* ratio (Ge et al., 2015; Jiao et al., 2015). But, it is worthwhile to mention that this study was conducted under hydroponic
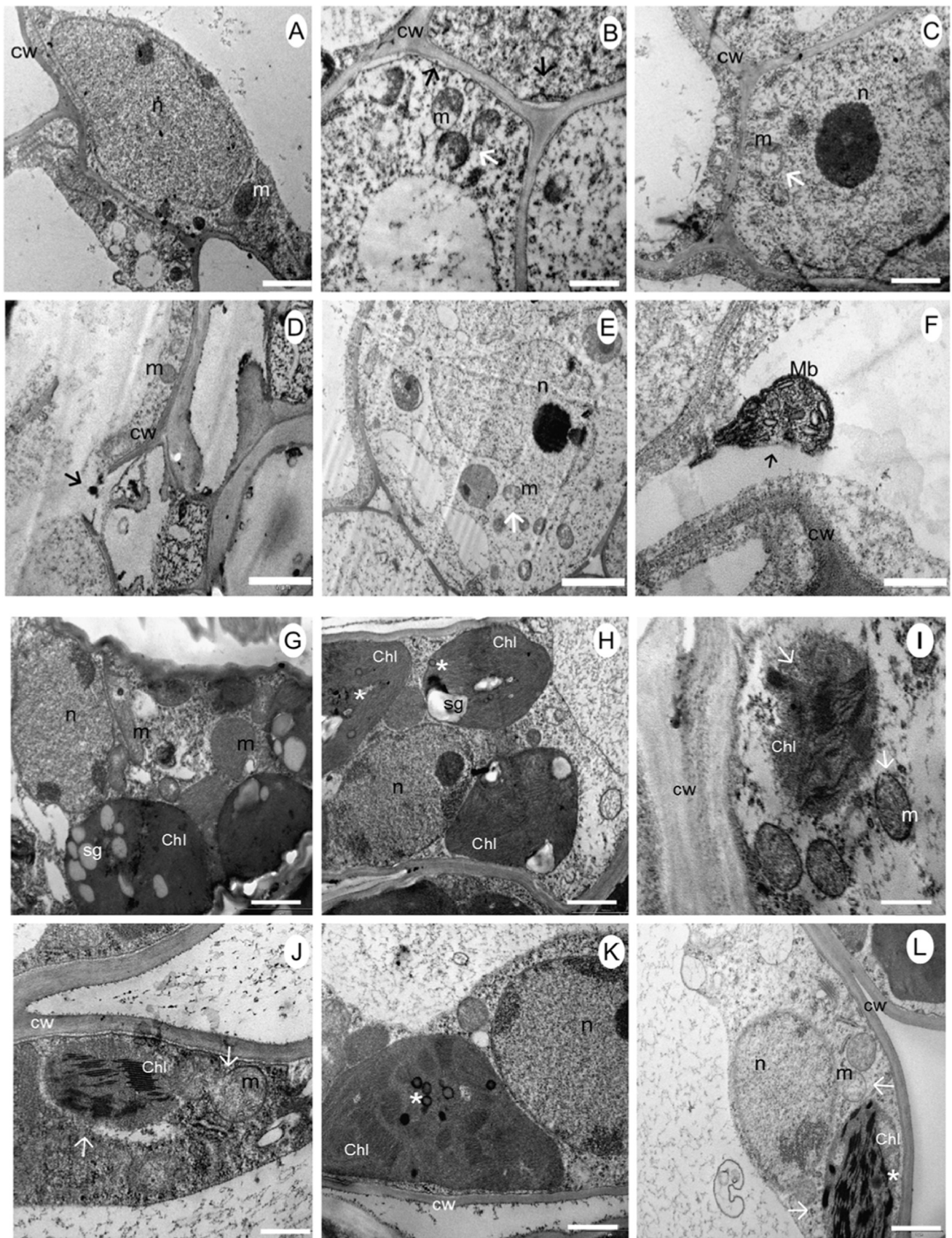
Fig. 3. TEM micrographs of root cross sections (A–F) and leaf *meso*phyll cross sections (G-L) of cacao plants exposed to concentrations of Cd in soil. Roots: Control (A), 0.05 mg Cd kg^{-1} soil (B; C) and 0.1 mg Cd kg^{-1} soil (D; E; F); Leaves: Control (G), 0.05 mg Cd kg^{-1} soil (H; I) and 0.1 mg Cd kg^{-1} soil (J; K; L). *Black arrows* indicate plasmatic membrane rupture (B), indicate deformation of the cell wall (D) and indicate disorganization of the external membrane (F); *White arrows* indicate disorganization of the organelles or nuclei (B; C; E; I; J; L). *n* nuclei, *m* mitochondria, *cw* cell wall, *Mb* Multivesicular body, *Chl* chloroplast, *sg* starch grain, * plastoglobuli. Bars = 0.5 μm (F; I; J; K), 1 μm (B; C; L), 2 μm (A; D; E; G; H.).

conditions, the toxic metal was 100% available in the solution, the pH of the solution was under control and there was little time of exposure to the metal.

The assimilation of CO_2 depends on the photochemical and biochemical phases of photosynthesis (P_N), losses in the electronic excitation of chlorophyll molecules, low values of *Fv/Fm* and reduction of *gs* and *E* contribute to the reduction of P_N values. The reduction of *gs* and *E* in the presence of Cd may also be associated with root necrosis and death, which contribute to the reduction of soil water absorption and transport to the aerial part of the plant. Moreover, with the increase of ions in the guard cells, a greater influx of water occurs and leads to the loss of cell distention, resulting in closure of the stomata (DalCorso et al., 2008). On the other hand, Cd^{+2} can replace Ca^{+2} and cause disturbances in the control of stomata opening and closure (Perfus-Barbeoch et al., 2002). Similar data were presented in *Gossypium hirsutum* plants subjected to increasing concentrations of Cd in nutrient solution (Ibrahim et al., 2015).

4.3. Cd, chloroplastidic pigments and antioxidative

The content of chloroplastidic pigments was another indicator of the toxicity of Cd in cacao plants. The effect caused by Cd activates the oxidative stress, increasing the production of reactive oxygen species (ROS) that affect the integrity of some biomolecules, such as lipids and proteins (Gill and Tuteja, 2010). The chloroplasts, along with mitochondria and peroxisomes, are the main organelles that produce ROS. Thus, when the enzymatic and non-enzymatic system cannot eliminate all ROS produced at the cellular level, the remaining ROS promote oxidative stress, which results in greater damage to the thylakoid membranes, justifying the decrease in chloroplastidic pigment content (Choudhury et al., 2016). The observed increase for *Car*/Chl ($a + b$) ratio can be explained by the photoprotective role of the carotenoids. Under stress conditions, with the destruction of complex light collectors, energy quenching can cause greater damage to the photosynthetic machinery, a process known as photoinhibition, the moment when the carotenoids act, through the xanthophyll cycle, (Gururani et al., 2015).

As a mechanism of tolerance to oxidative stress, antioxidant enzymes act by reducing the levels of ROS. The enzyme SOD has the superoxide as substrate and converts it into H_2O_2, which, in turn, are detoxified by peroxidases, such as APX and GPX (Demidchik, 2015). In the present study, the toxicity of Cd promoted the reduction of the activities of SOD and APX in the root. This metal element may inactivate and/or disintegrate proteins by high affinity with cysteine residues, in addition to compromising the transcription and translation of the respective genes (DalCorso et al., 2013). With less time of exposure to the metal, Khan et al. (2013) obtained different results, from those found for cacao, in the roots of *Gossypium hirsutum* plants, because, in the presence of Cd, they verified that there was an increase in SOD activity and a reduction in POD activity. Besides, they certified that GPX activity increased in the root. Similar results were found in cacao genotypes in the presence of Cd (Castro et al., 2015). The activity of the antioxidative enzymes, presented in our study in the presence of Cd was similar to the activity evaluated in *G. hirsutum* (Khan et al., 2013; Ibrahim et al., 2015) and *Populus* sp. (Jakovljević et al., 2014).

4.4. Cd and gene expression

The relative expression of the *Cu/Zn sod cyt* gene in the roots and leaves of cacao coincided with the response of the activity of this enzyme in these organs. The repression of this gene in the roots is in agreement with the results found in cacao seedlings, which had their seeds exposed to Cd before germination, by Castro et al. (2015) and by Souza et al. (2014) when evaluating Cu toxicity on cacao leaves.

The overexpression of *phyt* and *met2b* genes in roots and cacao leaves at the concentration of 0.05 g Cd kg^{-1} soil is a mechanism of tolerance to Cd toxicity. Phytochelatins are non-protein metal chelators

synthesized via enzyme, which use glutathione as a substrate, whose phytochelatin synthetase genes are constitutively expressed, but the enzymatic activation is dependent on the presence of the metal (Sunitha et al., 2012). On the other hand, metallothioneins are polypeptides genetically encoded and expressed under abiotic stress conditions (DalCorso et al., 2013). The phytochelatins and metallothioneins act in the sequestration of Cd, thereby inactivating it in the cytoplasm or transferring it to the vacuole (Sunitha et al., 2012). In addition, the role of phytochelatins in transporting Cd to the aerial part in *Citrus* plants has also been demonstrated (Lopez-Climent et al., 2014).

In the present work, it was verified that the toxicity of Cd in cacao promoted the reduction of the *Fv/Fm* values, an indicator of damages at the PS2 level. This was confirmed by overexpression of the *psba* gene at the levels of Cd corresponding to 0.05 g kg^{-1} soil. The *psba* gene is involved in the coding of protein D1, the main intrinsic protein of PS2, which is affected by the oxidative mechanisms induced by Cd (Møller et al., 2007). In addition, the PsbO protein, extrinsic to PS2 and encoded by the *psbo* gene, which was repressed at the Cd levels of 0.1 g kg^{-1} soil, is associated with the protection of the surface of the D1 protein to the ROS attack and prevents the agglomeration of PS2 damaged proteins (Nickelsen and Rengstl, 2013). On the other hand, the level of 0.1 g Cd kg^{-1} soil provoked gene repression for most of the genes evaluated in roots (*Cu/Zn sod cyt* and *phyt*) and leaves (*Cu/Zn sod chl, phyt, met2b* and *psbo*). Probably, degradation of DNA and/or transcripts by direct or indirect action of this metallic element occurred by the production of ROS. (Demidchik, 2015).

4.5. Cd and ultrastructure

The toxicity of Cd caused ultra-structural drift in levels of roots and leaves of cacao. Plasma membrane disruption in root cells, the disorganization of thylakoid membranes in foliar *meso*phyll cells and the destruction of mitochondria in root and leaf cells are the result of oxidative stress promoted by excess ROS, which damage lipids and structural proteins (Anjum et al., 2015). On the other hand, the appearance and increase of plastoglobules in the chloroplast stroma of foliar *meso*phyll cells is another indication of the stress promoted by the toxicity of Cd. Increase of plastoglobules is considered a symptom of foliar senescence, since these globules are lipid droplets of degraded thylakoid membranes (Esposito et al., 2012). The breakdown of thylakoid membranes was also observed in cacao plants from seeds treated with Cd (Castro, 2015). Damaged chloroplasts and increased plastoglobules were also evidenced in *Populus* (Jiao et al., 2015) and *G. hirsutum* (Ibrahim et al., 2015) subjected to stress by Cd.

The soil level of 0.1 g Cd kg^{-1} soil caused greater damage to the root ultrastructure. Deformations on the cell wall of root cells may be associated with the reduction of Ca^{2+} content and the effects of ROS that can disrupt the structure of glycoproteins and flavonoids (Tenhaken, 2015; Zhu, 2016). Cell wall disturbances greatly affect stress resistance in plants (Tenhaken, 2015). The body of vesicles evidenced in the cells of cacao roots is related to autophagy, which is responsible for the elimination of toxic cellular waste, reusing the available energy (Michaeli and Galili, 2014). There are two types of autophagy in plants called macroautophagia and microautophagia. The autophagy involved in our study demonstrates the occurrence of macroautophagia, which is mediated by a structure known as auto phagosome, which sequesters damaged structures in the cytoplasm through vesicles. These structures are transported to the vacuole, where degradation will occur by vacuolar hydrolases, observed through the double-membrane (Avila-Ospina et al., 2014). Studies with *Arabidopsis*, lacking carbohydrates, demonstrated the appearance of autophagic bodies by means of electromicrographs, indicating that there is a relationship with stress (Rose et al., 2006). There are also records of autophagosomes in unicellular algae submitted to Cd toxicity (Andosch et al., 2012). The main function of autophagy is to maintain longevity of life and homeostasis; however, it can promote cell death under

extreme conditions (Avila-Ospina et al., 2014).

5. Conclusions

Toxic levels of Cd in the soil caused damage to the photosynthetic machinery, antioxidative metabolism, gene expression and irreversible damage to the cell and root ultrastructure and thereby affected the growth and development of the CCN 51 cacao plants. The intensity of the damage in cacao plants depended on the concentrations and time of exposure to the Cd metal.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.06.006.

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Supplementary material

Table 1. Physical and chemical characteristics of the substrate for growth of CCN 51 cacao plants. P, Na, K, Fe, Zn, Mn, Cu (extracted by Mehlich 1), Ca, Mg, Al (extracted by KCl, 1 M) H + Al (extracted by Ca-acetate 0.5 M, pH 7.0), B (extracted by hot water). SB, sum of bases; t, effective cation exchange capacity; T, cation exchange capacity (pH 7.0); V, base saturation; m, Al saturation; NaSI, Na saturation index; OM, organic matter= Org C. × 1.724; TS, total sand.

pH	(H$_2$O)	4.7
P	mg dm^{-3}	-
K	mg dm^{-3}	36
Na	mg dm^{-3}	17
Ca^{2+}	cmol	0.6
Mg^{2+}	cmol	0.3
Al^{3+}	cmol	1.2
H + Al	cmol	8.4
SB	cmol	1
(t)	cmol	2.2
(T)	cmol	9.4
Zn	mg dm^{-3}	1.6
Fe	mg dm^{-3}	183
Mn	mg dm^{-3}	46
Cu	mg dm^{-3}	0.8
B	mg dm^{-3}	0.6
V	%	10.6
m	%	55
NaSI	%	0.7
OM	dag kg^{-1}	2.6
TS	dag kg^{-1}	74.1
Silt	dag kg^{-1}	12.6
Clay	dag kg^{-1}	13.3