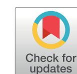
Environmental and Experimental Botany 179 (2020) 104201

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Mitigation of cadmium toxicity by zinc in juvenile cacao: Physiological, biochemical, molecular and micromorphological responses



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ARTICLE INFO

Keywords:
Theobroma cacao
Heavy metal
Photosynthesis
Oxidative stress
Gene expression
Micromorphology

ABSTRACT

Cadmium (Cd) is a trace metal without essential biological function due to its high toxicity to plants, animals and humans, even at low concentrations. On the other hand, Zn is an essential nutrient and plays important metabolic functions in plants. The study of the interaction between essential and a nonessential element may be important for understanding, analyzing and improving the defense strategies adapted by plants. The main objective of this work was to evaluate the mitigation of Cd toxicity by Zn in young plants of the CCN 51 cocoa genotype, grown in soil with different concentrations of Zn, Cd and Zn + Cd, through physiological, biochemical, molecular and micro-morphological responses. It was verified that high concentrations of Zn, Cd and Zn + Cd in the soil promoted alterations in the enzymatic and non-enzymatic antioxidative metabolism and expression of genes. This was demonstrated by increase in the activity of antioxidative enzymes, proline content and reduction in lipid peroxidation. Leaf gas exchange was affected at the highest soil Cd concentrations (0.4, 0.6 and 0.8 mmol Cd kg^{-1} soil) combined with different soil Zn concentrations (0.4, 0.8, 1.2 and 1.6 mmol kg^{-1} soil), resulting in a decrease in CO_2 fixation. The higher concentration of soil Cd (0.8 mmol kg^{-1} soil), together with the intermediate concentrations of soil Zn + Cd (0.8 + 0.4 and 0.4 + 0.6 mmol kg^{-1} soil), promoted reduction of the thickness of the leaf mesophyll and, consequently, led to decrease of the leaf gas exchange. It was observed a hormesis effect due to high photosynthetic activity in low Cd concentration. The increase in Cd concentration in the soil altered the uptake of Cd and Zn by the roots of the CCN 51 cocoa genotype. The increase of Zn concentration in the soil promoted the decrease of the Cd uptake by the root system of the plants and thereby reduced the transport of Cd to the leaves. Part of Cd uptake by the plant's root system was immobilized in roots tissues, as a tolerance strategy, preventing that it was transported to the aerial part. The increase of Zn + Cd concentration in the soil did not influence the accumulation of Zn in the leaves of the young plants of the CCN 51 cocoa genotype.

1. Introduction

Theobroma cacao is a perennial, arboreal species that produces fruits of great economic importance because its seeds are the raw material for one of the most consumed products in the world - chocolate, as well as other derivatives and by-products such as butter, liquor, cosmetics, fine drinks, jellies, ice cream and juices (Almeida and Valle, 2010). This species is grown on the tropical region of Africa, America and Asia continents, which produce approximately 76.4 %, 17.7 % and 6% of world cocoa bean production, respectively, with a forecast of 4587 thousand tons for the 2018/2019 harvest (ICCO, 2019). In the Americas, Brazil stands out as an important country in the production of cocoa beans, whose production is mainly concentrated in the southern region of the state of Bahia, with a harvest forecast of 1228 thousand tons (ICCO, 2019).

The genotype of *T. cacao* CCN 51, from the hybrid of ICS 95 x IMC 67 with an Ecuadorian cultivar called 'Canellos', is one of the most important genetic resources of Ecuador (Castro, 1981; Boza et al., 2014), whose predominant ancestors are the genetic groups Iquitos (45.4 %), Criollo (22.2 %) and Amelonado (21.5 %). In addition, the

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https://doi.org/10.1016/j.envexpbot.2020.104201
Received 14 April 2020; Received in revised form 9 July 2020; Accepted 22 July 2020
Available online 02 August 2020
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genotype CCN 51 is self-compatible, exhibits high productivity, greater resistance to witch's broom [*Moniliohthora perniciosa* (Stahel) Aime & Phillips-Mora] compared to other genotypes and has higher physical, chemical and sensory characteristics such as high concentration of fat in its beans (Boza et al., 2014). For this reason, it has been widely used by cocoa producers.

Several studies have been developed worldwide to identify the presence of heavy metals in cocoa beans and cocoa products (Dahiya et al., 2005; Aikpopodion et al., 2013; Yanus et al., 2014; Chavez et al., 2015; Vitola and Ciprovica, 2016; Argüello et al., 2019), whose levels in food have been a concern to the FAO - Food and Agriculture Organization of the United Nations - according to the health risks posed when consumed by animals and humans. Since once absorbed by the root system, heavy metals can be transported to the tissues of the aerial part, contaminating edible parts and becoming bioaccumulative in the organism (Schreck et al., 2012). Heavy metals or trace metals are elements with a density greater than 5 g cm^{-3}, persistent and cannot be naturally degraded/biodegraded in the environment (Prasad and Hagemeyer, 1999). They may occur naturally or be inserted into the cropping systems by anthropogenic and agricultural practices such as the application of pesticides and phosphate fertilizers (Zarcinas et al., 2004; Gramlich et al., 2017), irrigation water (Chavez et al., 2015) and emission from metallurgical activities (Schreck et al., 2012).

Among these metals, there is cadmium (Cd) that occurs in the earth's crust at low concentrations, usually associated with Zn in sulfite deposits. It is found in the inorganic form Cd^{2+} presenting low adsorption coefficient and with high mobility in the soil-plant system (Clemens and Ma, 2016). One of the main routes of Cd availability to plants is the soil application of phosphate fertilizers (Silva et al., 2017). It is related to the acid-soluble fraction bound to the organic matter and, therefore, its availability can be affected by the alteration of the soil pH, decomposition of organic matter (Prasad and Hagemeyer, 1999; Alloway, 2013; Chavez et al., 2016), soil cation exchange capacity and clay content (Alloway, 2013). Cd is widely distributed in the Earth’s crust at an average concentration of about 0.1 μg g^{-1} (Alloway, 1990). Cd concentration in normal plants ranges from 0.1–2.4 μg g^{-1} (Alloway, 1990), but, at higher concentration, it has been shown to affect adversely plant growth and productivity (Rizwan et al., 2018). It has no essential biological function and is characterized by its high toxicity to plants, animals and humans even in low concentrations (Ma et al., 2003; Gramlich et al., 2017) and its accumulation can cause damage to the photosynthetic machinery, antioxidative metabolism, gene expression and irreversible cell damage, affecting plant growth (Li et al., 2015; Castro et al., 2015; Araújo et al., 2017). Cd is predominantly found in fruits and vegetables due to its high rate of soil-to-plant transfer in contaminated soils (Zhu et al., 2018). The transfer of Cd from soil to the food chain depends on a number of factors, such as the plant species, soil type and pH, Zn and organic matter content of the soil, and the levels of soil Cd concentration (Yang et al., 2017; Guan et al., 2018). Food chain contamination by Cd has become a global problem and poses serious health concern all over the word. It is estimated that 40 % of the potentially arable lands worldwide are Cd polluted and the level of Cd is still increasing (Saha et al., 2017). The study of the interaction between essential nutrients and a nonessential element may be important for understanding, analyzing and improving defense strategies through various parameters (Jamali et al., 2014).

Zn occurs at high concentrations in biological systems when compared to other micronutrients (Cakmak, 2000). It plays important metabolic functions in plants, is associated with carbohydrate metabolism and plays a structural or catalytic role in several enzymes, such as the superoxide dismutase, dehydrogenase, protease, peptidase and phosphohydrolase (Kabata-Pendias, 2000; Cherif et al., 2010, 2011). In addition, it regulates gene expression, maintains the structural integrity of the ribosome, participates in phosphate metabolism (Kabata-Pendias, 2000; Cherif et al., 2010) and preserves the structural orientation of the macromolecules of the cell membranes. This maintain the integrity and the functioning of ion transport through the membranes (Cakmak, 2000; Hafeez et al., 2013), fulfilling an important role in the cellular protection against effects induced by reactive oxygen species (ROS) (Cakmak, 2000). Plants have different Zn toxic concentration thresholds depending on the species, in which the range of 100–400 mg kg^{-1} of leaf tissue is considered toxic for the growth of most plants (Kabata-Pendias, 2000). Cocoa plants have adequate leaf content ranging from 20 to 100 mg kg^{-1} dry weight (Sodré et al., 2017).

Both Cd and Zn, at high concentrations, can cause plant toxicity (Nadgórska-Socha et al., 2013; Chen et al., 2017) due to their physical and chemical similarities, considering that both belong to group II of the periodic table and are generally found associated in ores, competing with each other for several binders (Aravind and Prasad, 2005). There is the understanding that the translocation efficiency of Cd and Zn are highly correlated, suggesting the possibility of a common transport mechanism for both metals, meaning that the accumulation of Cd in plants can be modulated by the presence of Zn (Whiting et al., 2000; Hart et al., 2002; Kirkham, 2006; Xing et al., 2008; Liu et al., 2010; Mitra, 2015).

Exposure to heavy metals, such Cd, can cause oxidative stress through several mechanisms of action. Heavy metals can inactivate or activate enzymes of the antioxidante system (peroxidases, catalases and superoxide dismutase), responsible for detoxifying cells. In addition, they can disrupt metabolic pathways and result in increased formation of free radicals and ROS, that cause tissue damage by increasing senescence and lipid peroxidation processes (Bhaduri and Fulekar, 2012). When levels of ROS are very high and oxidation is at na advanced stage, damage to biomolecules can occur (Sharma et al., 2012). On the other hand, ROS also presente themselves as signaling molecules that can trigger responses of adaptation and regulation of cell death (Winterbourn, 2008; Kim et al., 2012).

To eliminate ROS and alleviate their deleterious effects, plants have developed several protection mechanisms, including enzymatic and non-enzimatic mechanisms, in order to adjust their levels (Mittler et al., 2004) and whithstand stressful conditions. The main enzymatic mechanisms comprise the enzymes of the antioxidative metabolismo, which play na important role in the antioxidante defense system, such as SOD, which is the first enzymatic barrier against oxidative stress, converting $O2^{-}$ in to H_2O_2 (Fridovich, 1995; Zhang et al., 2017). This final product must be removed in order to avoid its conversion to more reactive species such as OH-, in which the enzymes CAT and APX act by converting it to H_2O (Perl-Treves and Perl, 2002). In addition, GPX also participates in the elimination of ROS excess, transforming them into less reactive species, converting H_2O_2 into H_2O and O_2, donating electrons to guaiacol (Choudhary et al., 2017).

Among non-enzymatic antioxidants, proline is a compatible solute accumulated in response to stress, acting as a chelator, protecting enzymes from inhibition induced by Zn and Cd, forming proline-metal complexes (Sharma et al., 1998). The increase in proline content under stress conditions is explained by the reduction in electron transport activity, which would lead to the accumulation of NADH and H^{+} (Alia, 1991).

This present work started from the hypothesis that Zn is able to mitigate the toxic action of Cd in young cocoa plants. To test this hypothesis, this study had as main objective to evaluate the mitigation of the Cd toxicity by Zn in young plants of the CCN 51 cocoa genotype, grown in the soil with different concentrations of Zn, Cd and Zn + Cd, through physiological, biochemical, molecular and micromorphological responses.

2. Material and methods

2.1. Conditions of growth and cultivation

The experiment was conducted in a greenhouse at the Santa Cruz State University (UESC), Ilhéus, Bahia, Brazil (14° 47′ S; 39° 13′ W).

Seeds of the CCN 51 cacao genotype were obtained from eight to ten-year-old self-pollinated trees, of the Cocoa Germplasm Active Bank of the Centro de Pesquisas do Cacau (CEPEC), of the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), Bahia, Brazil, and germinated directly on the loamy sand soil, in pots with a capacity of 5 L. Soil pH was corrected with a mixture of $CaCO_3$ and $MgCO_3$, necessary to achieve the 2:1 ratio of Ca^{2+} and Mg^{2+} and increase the base saturation value to 80 %. Soil fertilization was done 15 days after germination and there after every 15 days, five times during the entire growth period. At 126 days after planting (DAP), 100 ml one of the following solutions were added in each pot, according to the treatments: 0.4, 0.8, 1.2 and 1.6 mmol Zn kg^{-1} soil and 0.2, 0.4, 0.6 and 0.8 mmol Cd kg^{-1} soil in isolated concentrations, and in combined concentrations of Zn + Cd (0.4 + 0.2, 0.4 + 0.4, 0.4 + 0.6, 0.4 + 0.8, 0.8 + 0.2, 0.8 + 0.4, 0.8 + 0.6, 0.8 + 0.8, 1.2 + 0.2, 1.2 + 0.4, 1.2 + 0.6, 1.2 + 0.8, 1.6 + 0.2, 1.6 + 0.4, 1.6 + 0.6 and 1.6 + 0.8 mmol kg^{-1} soil), together with control treatment (without addition of Zn and Cd in soil), making a total of 25 treatments, with variable number of replicates according to the parameter evaluated and one plant per experimental unit. $ZnCl_2$ and $CdCl_2$ were used as sources of Zn and Cd, respectively.

Throughout the experimental period, the plants were irrigated with rainwater, keeping the soil close to the field capacity. In addition, photosynthetically active radiation (PAR) was continuously monitored and recorded (Supplementary Fig. 1), using S-LIA-M003 radiation sensors, temperature and relative air humidity, using Hobo H8 Pro Series microprocessor sensors (Onset, USA), coupled to the climatological station Hobo Micro Station Data Logger (Onset Computer, Massachusetts, USA) installed inside the greenhouse.

2.2. Leaf gas exchange

The net photosynthetic rate per unit leaf area (A) and leaf transpiration rate (E) were measured on the second or third fully expanded leaf at 29 days after application of the treatments (AAT) between at 8 and 12 h, using a portable LI-6400 photosynthesis measurement system (Li-Color, Nebraska, USA) equipped with 6400-02B RedBlue artificial light source. The artificial light source of the system was adjusted to PAR of 800 $\mu mol\, m^{-2}\, s^{-1}$, the CO_2 flux was adjusted to maintain a concentration of 380 $\mu mol\, mol^{-1}$ in the chamber and the leaf chamber temperature was kept constant at 28 °C. The readings were recorded within the range of 2 − 3 min (coefficient of variation from 0.1 % to 0.2 %). The stomatal conductance to water vapor (gs) and intrinsic (A/gs) and instantaneous (A/E) water use efficiencies were calculated by said equipment from the values of A and E (Von Caemmerer and Farquhar, 1981).

2.3. Concentration of Cd and Zn

Cocoa seedlings were collected at the end of the experiment, at 37 days AAT, and separated into roots and leaves. The roots were subjected to successive washes in the following order: running water (2x), neutral detergent solution (0.1 %) (2x), distilled water (2x), hydrochloric acid (3% HCl, 2x), and water distilled (2x) to remove metals from root surface and that were not absorbed by the root system (Souza Júnior et al., 2011). Afterwards, the material was placed separately in an oven with forced air circulation at 70 ± 5 °C until constant mass. Subsequently, the dried plant material was milled with Willey type; model MA-340, using sieves of 20 mesh opening. Soon after, the ground plant material was subjected to nitroperchloric digestion (3:1 v: v.) (Marchiol et al., 2004). After digestion, the Zn and Cd concentrations in the different organs (root and leaf) of the plants of all treatments were determined, using inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 710-ES). White samples received the same treatment as the sample evaluated, without the addition of plant material.

2.4. Proline

Proline content was determined in leaf samples of all treatments, following the methodology proposed by Bates et al. (1973), with adaptations for reading in microcubes of quartz and volume of toluene. Was added 1 ml of 3% sulfosalicylic acid to the lyophilized leaf tissue (35 mg), macerated in liquid nitrogen, collected at 37 days AAT, followed by vigorous vortexing and centrifugation at 14,000 x g for 5 min. Subsequently, 0.2 ml aliquots of the crude extract were transferred to glass test tubes, to which 0.2 ml of acid ninhydrin (1.25 g of ninhydrin, 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid) and concentrated glacial acetic acid were added. The tubes were then hermetically sealed vortexed and incubated in a water bath at 100 °C for 60 min. immediately after, the reaction was stopped in an ice bath. Subsequently, 0.4 ml of toluene was added to the tubes, followed by vortexing again, and allowed to stand to room temperature and separation of the aqueous phase occurred. The toluene-containing chromophore (upper phase) was recovered with the aid of a glass Pasteur pipette and transferred to a quartz microcuvette. The readings were made in a spectrophotometer, in quadruplicates, in λ of 520 nm, using toluene as white.

2.5. Lipid peroxidation

The extent of lipid peroxidation was determined in leaves, in all treatments, according to the Cakmak and Horst (1991), from substances that react with thiobarbituric acid (TBARS), mainly malondialdehyde (MDA). Lyophilized leaf tissue (20 mg), of all treatments collected at 37 days AAT, was macerated in 2 ml of 0.1 % (w/v) trichloroacetic acid (TCA). The material was then centrifuged at 14,000 x g for 6 min and a 500 μL aliquot of the supernatant was added to 1.5 ml of 0.5 % thiobarbituric acid solution in 20 % TCA in glass test tubes. Subsequently, the samples were incubated at 90 °C for 20 min. Soon after, the reaction was stopped in ice bath and the supernatant was transferred to 2 ml eppendorfs and centrifuged at 14,000 x g for 4 min. The absorbance (280 μL) was read in λ of 532 nm and corrected for non-specific turbidity by absorbance subtraction at 600 nm. The TBARS concentration was calculated from its extinction coefficient of 155 mM cm^{-1}. The readings were made using SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices) UV/VIS spectrophotometer.

2.6. Antioxidative metabolism

The activity of guaiacol peroxidase (GPX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1) was performed using the second or third leaf collected from the apex of cacao young plants at 6 and 48 h AAT intervals. The plant material was immediately immersed in liquid nitrogen, stored in ultrafreezer −80 °C and subsequently lyophilized. The samples were macerated in liquid nitrogen and weighed (20 mg - SOD and GPX, 40 mg - CAT and APX), packed in eppendorf, with polyvinylpyrrolidone (PVP) in order to avoid the oxidation of the macerate. The macerated material was resuspended in 20 x extraction buffer [50 mM sodium phosphate buffer, pH 6.0 (GPX) or 50 mM potassium phosphate buffer, pH 7.0 (CAT and APX) and pH 7.8 (SOD)]. Subsequently, they were subjected to ultrasound (Ultrasonic processor Gex 130, 130 W) to break the tissues, in 8-second pulses, 10-fold intervals and 70 % amplitude, followed by centrifugation at 15,000 xg at 4 °C, for 15 min. Finally, the supernatant was collected (crude extract), transferred to 1.5 ml eppendorf, kept in ice-styrofoam, and used sequentially, according to the methodological procedure described by Kar and Mishra (1976) with adaptations made by Pirovani et al. (2008) to GPX, Havir and McHale (1987) to CAT, Nakano and Asada (1981) to APX.

2.7. Gene expression

From the Biplot analysis of the antioxidative metabolism data, four combined treatments of Zn + Cd (1.2 + 0.6, 1.2 + 0.8, 1.6 + 0.6 and 1.6 + 0.8 mmol kg^{-1} soil), were collected at 6 and 48 h AAT, plus the control treatment (without addition of Zn and Cd in soil). The same material was collected and stored for antioxidative metabolism. For RNA extraction, 20 mg of material macerated in liquid nitrogen was used, using the RNAqueous® kit (Ambion-Apllied Biosystems), following the manufacturer's recommendations. Then the samples were treated with DNase I (Thermo Scientific) and incubated at 37 °C for 30 min, followed by addition of 50 mM EDTA and incubation at 65 °C for 10 min. Finally, the RNA of the samples was quantified in spectrophotometer (*Nanodrop 2000c* UV–vis *Spectrophotometer Thermo Scientific*), at 260 and 280 nm, and expressed in μg mL^{-1}. The cDNA synthesis was performed from samples treated with DNAse I using the High Capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer's recommendation. Reactions were incubated at 37 °C for 60 min, 95 °C for 5 min and 4 °C for 1 min. The specific gene primer pairs used in the real-time quantitative PCR analysis were those related to the biosynthesis of photosystem 2 proteins (PS2) of the photochemical phase of photosynthesis (psbA and psbO) and antioxidative enzymes [Cu-Zn SOD*Cyt*, Cu -Zn SOD*Chl* and peroxidase (*per-1*)]. qPCR was performed in a "real-time PCR" system (ABI 7500 model, Applied Biosystems) using the Power SYBR® Green/PCR Master Mix kit (Applied Biosystems) according to the manufacturer's instructions. The relative expression numbers of the genes were calculated as the number of times relative to the control using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Genes involved in glyceraldehyde-3-phosphate dehydrogenase biosynthesis (gapdh), malate dehydrogenase (mdh), actin and β-tubulin were used as endogenous genes.

2.8. Micromorphological analysis

Micromorphological analysis using scanning electron microscopy (SEM) was performed in cross sections of the leaf mesophyll of cacao young plants submitted to additions of Zn (0.8 and 1.6 mmol kg^{-1} soil), Cd (0.4 and 0.8 mmol kg^{-1} soil), combination of Zn + Cd (1.2 + 0.2 mmol kg^{-1} soil, 0.8 + 0.4 mmol *kg*$^{-1}$ soil and 0.4 + 0.6 mmol kg^{-1} soil), in the control treatment (without metal addition). The second or third mature leaf was collected from the apex of the plant at 37 days AAT. The middle region of the mature leaves was sectioned using hand sections and embedded in 2.5 % glutaraldehyde, prepared in 0.1 M sodium cacodylate buffer, pH 7.2. Subsequently, sections of the leaves were washed three times for 10 min each wash in the same sodium cacodylate buffer and dehydrated in increasing series of acetone (50, 60, 70, 80, 90 % and three times at 100 % for 10 min). Immediately after dehydration, the material was brought to the critical point (model CPD 030, BAL-TEC), which allowed the removal of much of the water from the plant tissue. Afterwards, the metallization of the samples was carried out, which consisted of covering the plant material with a thin layer of gold of 30 nm of thickness, using SPUTTER COATER, model SCD 050, brand BAL-TEC, followed by observation in SEM, model Quanta 250 (FEI Company). Afterwards, the thickness of the palisadic (PP) and spongy (SP) parenchymas, epidermis on the adaxial (EAd) and abaxial (EAb) faces of leaf, leaf mesophyll (LM) and leaf total (LT) were measured in three different locations of three leaves per tratment by the xT Microscope Control program. In addition, were measurements of the transverse (TOS) and longitudinal (LOS) openings of the stomatal pore and calculated stomatal density (SD) in area of 291.4 × 252.1 μm in three different locations of three leaves per treatment by the xT Microscope Control program.

2.9. Programmed cell death (PCD)

To detect DNA fragmentation, as described by Souza et al. (2011), the Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) technique was used. Samples of cocoa mature leaves were dehydrated in an ethanolic series and infiltrated with methacrylate (Historesin Leica Microsystem). Ultra-thin transversal sections with a thickness of 5 μm were obtained using the aid of rotary microtome (RM 2235, Leica Deerfiel, IL, USA) and mounted on glass blades and subjected to the TUNEL reaction. The detection of DNA fragments was performed using the "In situ cell death detection fluorescein" kit (Roche, Mannheim, Germany), and the nucleus, observed and photographed under a fluorescence microscope Leica DM 2500, equipped with a digital camera Leica DFC 295. The images were captured with the filter D (450–490 nm of excitation).

2.10. Statistical analysis

The experimental design was a completely randomized design, with 25 treatments, (20 repetitions per treatment, totaling 500 plants), represented by isolated concentrations of Zn (0.4, 0.8, 1.2, 1.6 mmol kg^{-1}) and Cd (0.2, 0.4; 0.6, 0.8 mmol kg^{-1}) and combined of Zn + Cd (0.4 + 0.2, 0.4 + 0.4, 0.4 + 0.6, 0.4 + 0.8, 0.8 + 0.2, 0.8 + 0.4, 0.8 + 0.6, 0.8 + 0.8, 1.2 + 0.2, 1.2 + 0.4, 1.2 + 0.6, 1.2 + 0.8, 1.6 + 0.2, 1.6 + 0.4, 1.6 + 0.6 and 1.6 + 0.8 mmol kg^{-1} soil) and control (without addition of Zn and Cd in soil), with variable number of replicates according to the parameter evaluated and one plant per experimental unit. Four replicates were used for leaf gas exchange, antioxidative metabolism enzymes, Cd and Zn concentrations, proline content and lipid peroxidation and three replicates for gene expression and leaf micromorphology. For the micromorphological analysis, only eight treatments were selected and five treatments and two plant material collect times were selected for gene expression. Data were submitted to ANOVA and means were compared by the Scott-Knott test ($p < 0.05$).

3. Results

3.1. Leaf gas exchange

The net photosynthetic rate (*A*) of the young plants of the CCN 51 cacao genotype at 29 days AAT showed significant differences ($p < 0.05$) between the Zn, Cd and Zn + Cd treatments, whose mean values varied between 1.09 and 3.38 μmol (CO_2) m^{-2} s^{-1}. The highest values of *A* were presented by the control treatment (without addition of Zn and Cd to soil), in the two lowest concentrations of Zn applied alone (0.4 and 0.8 mmol Zn kg^{-1} soil), in plants subjected to the lowest concentration of soil Cd (0.2 mmol kg^{-1} soil) alone or combined with different concentrations of Zn (0.4, 0.8, 1.2 and 1.6 mmol kg^{-1} soil) and the concentration of 0.6 mmol Cd kg^{-1} soil applied alone (Fig. 1). Concentrations ≥ 0.4 mmol Cd kg^{-1} soil (except for the last treatment mentioned) showed the lowest values and did not present significant differences ($p < 0.05$), independent of the Zn concentration, as well as the two highest concentrations of this element applied alone (Fig. 1).

Stomatal conductance to water vapor (*gs*) differed significantly ($p < 0.05$) between treatments at 29 days AAT. The control treatment presented higher mean values of *gs* than the other treatments (Supplementary Fig. 2A). In addition, treatments with addition ≥ 0.4 mmol Cd kg^{-1} soil, alone or combined with any Zn concentration, showed the lowest values and did not present significant differences ($p < 0.05$) for the mean values of *gs* (Supplementary Fig. 2A). Leaf transpiration (*E*) differed significantly ($p < 0.05$) among the treatments, whose plant responses to the different treatments of Zn, Cd and Zn + Cd applied to the soil were similar to those observed for *gs* (Supplementary Fig. 2B). On the other hand, the intrinsic efficiency of water use (*A/gs*) and instantaneous efficiency of water use (*A/E*) presented significant differences ($p < 0.05$) between the treatments, although the mean values of the treatments were very close, except in the two highest concentrations of Zn applied alone (Supplementary Fig. 2C-

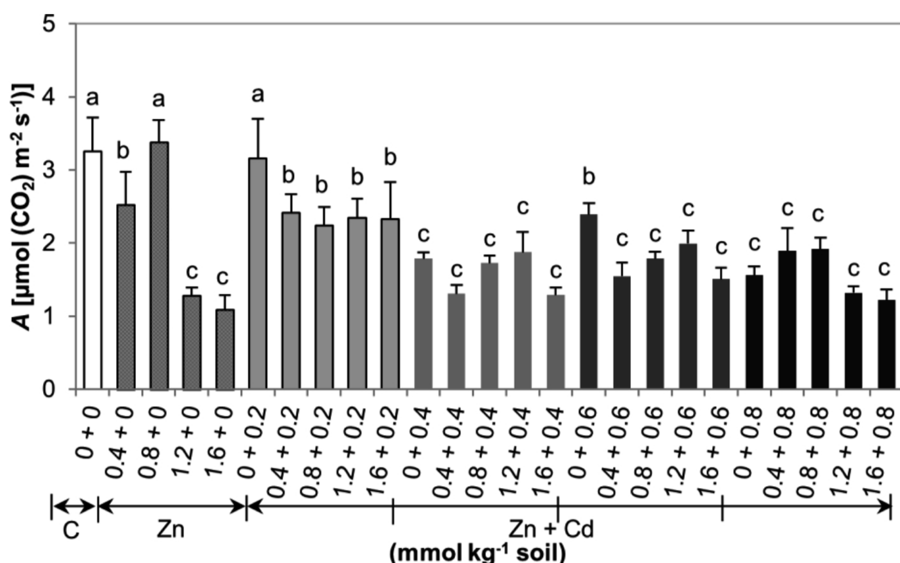

Fig. 1. Net photosynthetic rate per unit area of leaf area (A) in young plants of the CCN 51 cacao genotype, submitted to different concentrations of soil Zn, Cd and Zn + Cd in the soil at 29 days after application of the treatments. Mean values of four replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$). C - control treatment.

D).

3.2. Cd and Zn concentrations

Soil application of various levels of Zn, Cd and Zn + Cd affected concentrations of Zn and Cd differently in leaves and roots, and differed significantly ($p < 0.05$) between treatments at 37 days AAT. Zn concentrations in the roots (Fig. 2C) was higher in relation to the Zn concentrations in the leaves (Fig. 2A), including the control treatment. Zn additions ≥ 0.8 mmol Zn kg^{-1} led to significantly higher leaf Zn concentrations (average of 180 mg kg^{-1}) compared to lower concentration (0,4 mmol Zn kg^{-1} soil) and the control, and where not affected by additions Cd in the soil. The different Cd concentrations (0.2, 0.4, 0.6 and 0.8 mmol Cd kg^{-1} soil), when applied alone, showed a similar response to the control. Such levels were the lowest, as expected, and represented on average 70 % lower compared to the highest values (Fig. 2A). The highest concentration of Zn the roots occurred mainly in the treatments in which Zn was applied alone at all concentrations, without addition of Cd, as well as in those in which it was applied in combination with the lowest Cd concentration (0.2 mmol kg^{-1} soil)

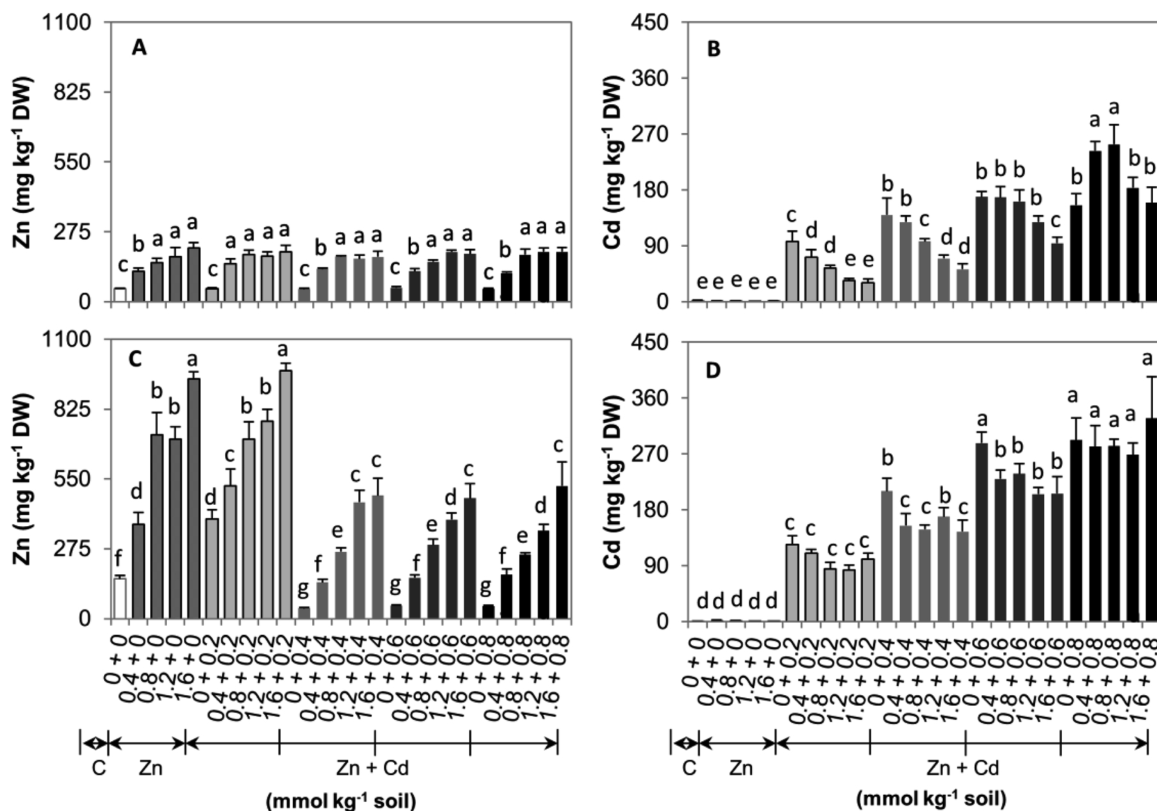

Fig. 2. Concentration of soil Zn and Cd in leaves (A and B) and roots (C and D) of young plants of the CCN 51 cocoa genotype, submitted to different concentrations of soil Zn, Cd and Zn + Cd in soil at 37 days after the application of treatments. Mean values of 4 replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$). C - control treatment.

(Fig. 2C).

In addition, it was observed that the higher the Zn concentration in soil, more Zn is accumulated in roots, and that the higher Cd concentrations in soil decreasing Zn concentrations in roots, when the Cd concentration was greater than 0.2 mmol kg^{-1} soil. On the other hand, it was also verified that the plants cultivated at the lowest concentration of Zn (0.4 mmol kg^{-1} soil) presented the same response for the lowest concentration of Cd (0.2 mmol kg^{-1} soil), when applied in isolation (Fig. 2C).

Similar to Zn, Cd was more concentrated in the roots of the plants in relation to the leaves, the uptake of which increased with increasing Cd addition (Fig. 2B and D). However, Cd concentration in leaves decreased with increasing concentration Zn in soil (Fig. 2B) and in roots this concentration reduced with the increase of the Zn concentration in soil compared to the Cd applied alone or in combination with the Zn, mainly in the concentrations of 0.4 and 0.6 mmol Cd kg^{-1} soil (Fig. 2D).

Cd concentrations in the leaves increased compared to the control with increasing concentrations of Cd in the soil, alone or in combination with any concentrations of Zn. In addition, with increasing Zn concentration in the soil, mainly in the treatments with 1.2 and 1.6 mmol Zn kg^{-1} soil, combined with any of Cd, there was a reduction in the uptake of Cd by the roots and transport to the leaves of 49, 46, 33 and 31 %, respectively, in the concentrations of 0.2, 0.4, 0.6 and 0.8 mmol Cd kg^{-1} soil, considering the mean values of the two smaller ones, in relation to the two higher concentrations of Zn applied (Fig. 2B).

3.3. Proline

The proline content in the leaves, determined at 37 days AAT, differed significantly ($p < 0.05$) between the treatments (Fig. 3). The highest levels of leaf proline were observed in the plants subjected to the highest concentration of Zn (1.6 mmol kg^{-1} soil) combined with higher Cd concentrations (0.4, 0.6 and 0.8 mmol kg^{-1} soil). On the other hand, the lowest levels of proline were found in the leaves of the control plants and of the plants subjected to the lowest concentrations of Zn (0.4 and 0.8 mmol kg^{-1} soil) without addition of Cd in the soil. Proline content in the leaves of plants grown in soil with the highest Zn + Cd concentrations (1.6 + 0.8 mmol kg^{-1} soil) was of 43.4 μmol g^{-1} DW (Fig. 3), representing a 4000 % increase in the proline content in relation to content found in leaves of control plants (0.9 μmol g^{-1} DW). In addition, plants grown at the two lowest concentrations of Cd (0.2 and 0.4 mmol kg^{-1} soil) applied and in the combination of each of these concentrations with the lowest concentration of Zn (0.4 mmol kg^{-1} soil) presented similar responses regarding the proline content at leaf level.

3.4. Lipid peroxidation

There were significant differences ($p < 0.05$) between treatments of Zn, Cd and Zn + Cd applied to the soil and leaf content of thiobarbituric acid reactive substances (TBARS). The highest TBARS content (171.3 nm g^{-1} DW) and, consequently, the higher lipid peroxidation of cell membranes, was observed in the treatment resulting from the combination of the lowest concentration of Zn (0.4 mmol kg^{-1} soil) with the highest concentration of Cd (0.8 mmol kg^{-1} soil) (Fig. 4).

3.5. Antioxidant metabolism

There were significant differences ($p < 0.05$) between treatments for CAT, APX and GPX activity in mature leaves, at two times evaluated AAT (Fig. 5). The fact that drew the most attention was the lower activities of CAT, APX and GPX observed in the treatments with the two highest concentrations of Zn (1.2 and 1.6 mmol kg^{-1} soil) combined with the highest concentration of Cd (0.8 mmol kg^{-1} soil), which still were smaller than the control, at 6 and 48 h AAT (Fig. 5A, B, C, D, E and F).

It was also found increase in CAT activity at the highest concentration of Cd (0.8 mmol kg^{-1} soil) and in the combinations of Zn + Cd (1.2 + 0.4 mmol kg^{-1} soil) at 6 h AAT (Fig. 5A). The same fact occurred at 48 h AAT for the concentration of 0.4 mmol Cd kg^{-1} soil (Fig. 5B). The highest activity of APX was found in the concentration of 0.6 mmol Cd kg^{-1} soil combined with 1.6 mmol Zn kg^{-1} soil (Fig. 5C). In this combination, at 6 and 48 h AAT, APX activity was 8 and 5 times, respectively, higher in relation to the control (Fig. 5C and D). The highest activities of GPX were observed in the treatment 0.4 mmol Zn + 0.8 mmol Cd kg^{-1} soil at 6 h AAT (Fig. 5E) and 1.2 mmol Zn + 0.6 mmol Cd kg^{-1} soil at 48 h AAT (Fig. 5F).

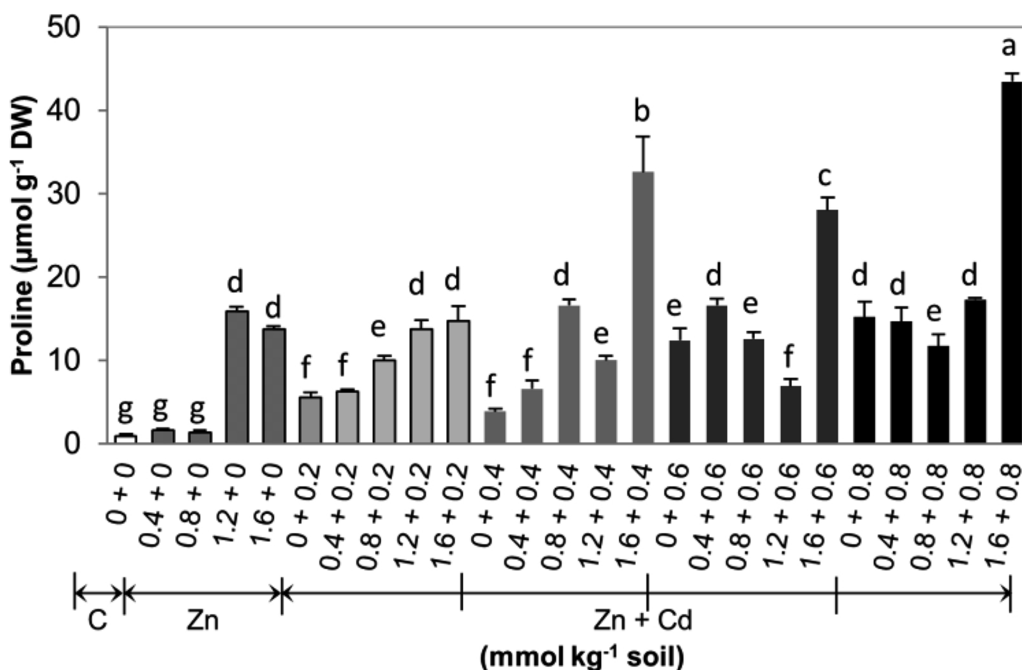

Fig. 3. Proline content in leaves of young plants of the CCN 51 cacao genotype, submitted to different concentrations of soil Zn, Cd and Zn + Cd in the soil at 37 days after application of the treatments. Mean values of four replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$). C - control treatment.

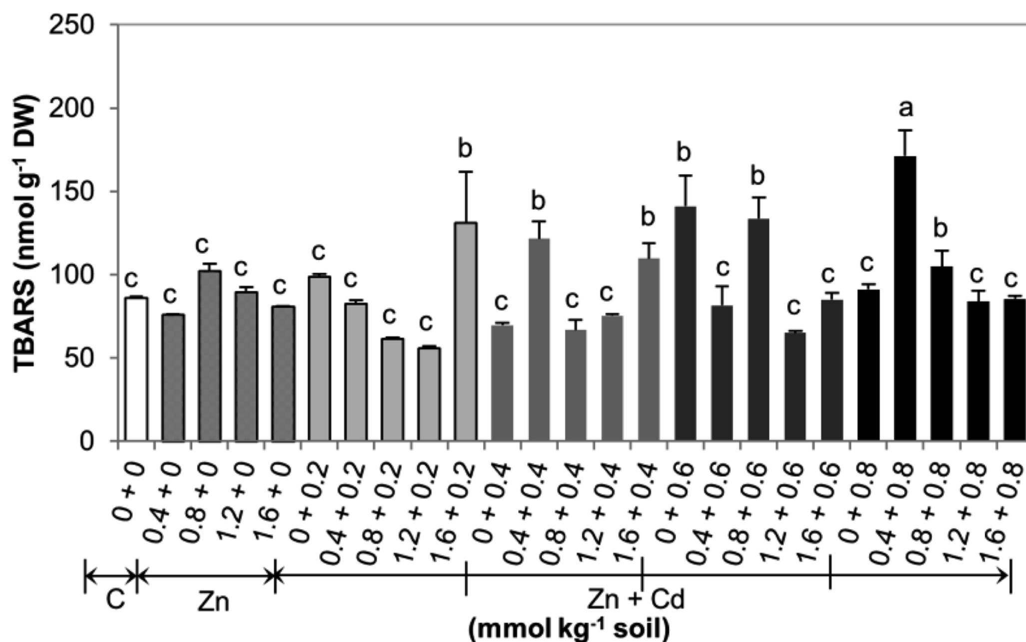

Fig. 4. Thiobarbituric acid reactive substances (TBARS) in mature leaves of young plants of the CCN 51 cacao genotype, submitted to different concentrations of soil Zn, Cd and Zn + Cd in the soil at 37 days after application of the treatments. Mean values of four replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$). C - control treatment.

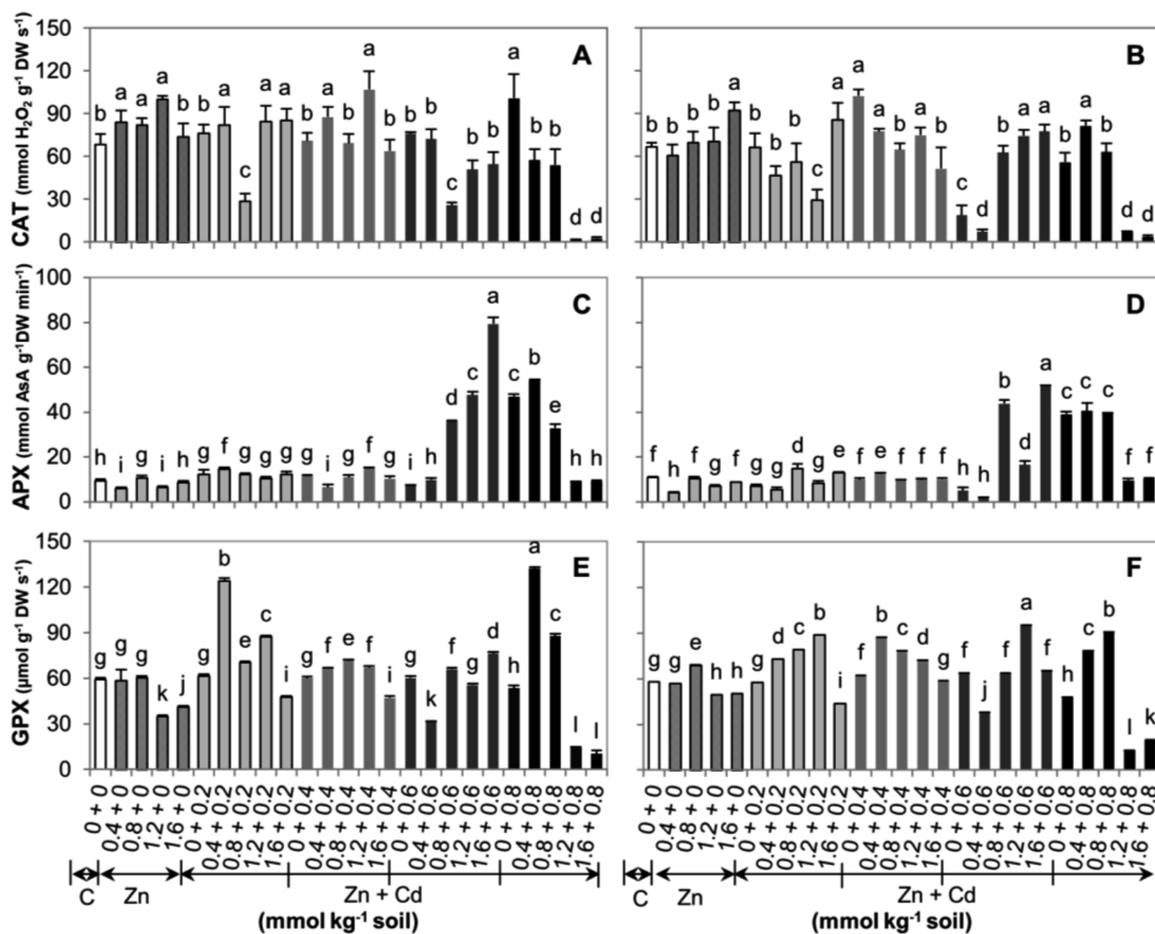

Fig. 5. Activity of catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) in mature leaves of young plants of the CCN 51 cacao genotype, submitted to different concentrations of soil Zn, Cd and Zn + Cd in the soil at 6 h (A, C and E) and 48 h (B, D and F) after application of the treatments. Mean values of four replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$). C - control treatment.

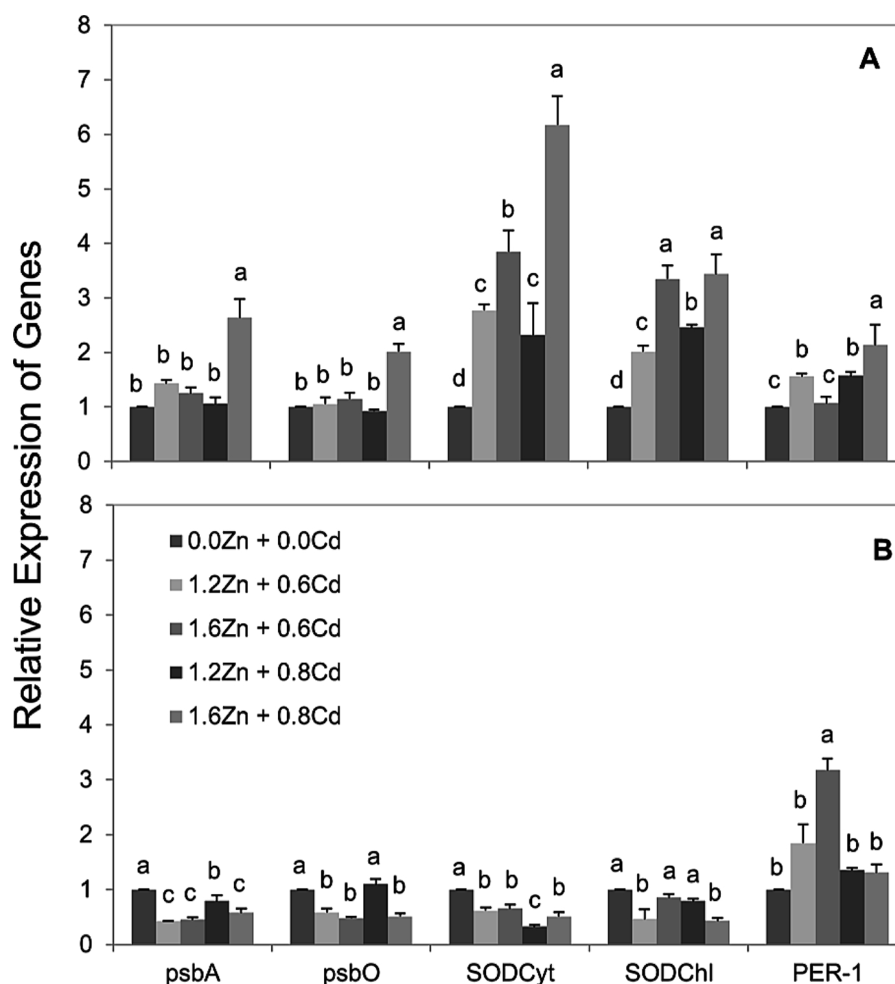

Fig. 6. Relative expression of genes related to intrinsic (*PsbA*) and extrinsic (*PsbO*) proteins biosynthesis to photosystem 2 of the photochemical phase of photosynthesis and of antioxidative metabolism enzymes (*SODCyt*, *SODChl* and *per-1*) in mature leaves of young plants of the CCN 51 cacao genotype, submitted to different concentrations of Zn + Cd (mmol kg^{-1} soil) in the soil, together with the control treatment, at 6 (A) and 48 h (B) after the application of the treatments. Mean values of three replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$).

3.6. Gene expression

Significant differences ($p < 0.05$) in the relative expression of the *psbA* and *psbO*, Cu-Zn *SODCyt* and Cu-Zn *SODChl* and *per-1* genes were observed in mature leaves of young plants of the CCN 51 cocoa genotype submitted to concentrations Zn, Cd and Zn + Cd applied to the soil (Fig. 6A and B). There was induction of relative expression, at 6 h AAT, in the leaves of plants submitted to the highest combined concentrations of Zn + Cd (1.6 + 0.8 mmol kg^{-1} soil) for all the evaluated genes. Expressions were more significant for genes Cu-Zn *SODCyt* and Cu-Zn *SODChl*, whose combined concentration of 1.6 mmol Zn kg^{-1} soil + 0.6 mmol Cd kg^{-1} soil also showed induction of the expression of said genes at the same time (Fig. 6A). The same fact occurred with the combined concentrations of Zn + Cd (1.2 + 0.6 and 1.2 + 0.8 mmol kg^{-1} soil) for Cu-Zn *SODCyt*, Cu-Zn *SODChl* and per-1 genes, whose expressions were significantly different ($p < 0.05$) from the control treatment. In addition, there was an increase in relative expression of the *per-1* gene at the combined concentrations of Zn + Cd (1.6 + 0.6 mmol kg^{-1} soil) 48 h AAT (Fig. 6B). For the other cases there was suppression of genes.

3.7. Leaf micromorphology

The cacao mature leaf is characterized by the presence of mesophyll composed of a layer of palisadic parenchyma and two or three layers of cells forming the spongy parenchyma, with stomata present only on the abaxial side of the leaf (Fig. 7A-H). Significant differences ($p < 0.05$) were observed between treatments for PP, SP, LM, LT, SD and LOS parameters (Table 1). PP thickness was higher in the control treatment and in the combinations of intermediate concentrations of Zn + Cd (0.8 + 0.4 and 0.4 + 0.6 mmol kg^{-1} soil). On the other hand, SP thickness was higher and significantly different ($p < 0.05$) from the control in the combination of 1.2 mmol Zn kg^{-1} soil + 0.2 mmol Cd kg^{-1} soil. Similar results were also verified for the LT thickness at the concentration of 0.8 mmol Zn kg^{-1} soil. Among all the parameters, what attracted more attention was the LM thickness, whose control differed and was superior to all other treatments. In addition, treatments with the highest concentration of Cd (0.8 mmol kg^{-1} soil) and with intermediate concentrations of Zn + Cd (0.8 + 0.4 and 0.4 + 0.6 mmol kg^{-1} soil) presented the lowest LM values. The highest value of SD was observed for the combination of 1.2 mmol Zn kg^{-1} soil + 0.2 mmol Cd kg^{-1} soil, whereas the lowest value was found for the combination 0.4 mmol Zn kg^{-1} soil + 0.6 mmol Cd kg^{-1} soil. The longitudinal opening of the stomatal pore (LOS) was superior in all treatments involving the Zn + Cd combinations in relation to the other evaluated ones.

3.8. Programmed cell death (PCD)

The TUNEL reaction, on transversal sections of mature leaves of

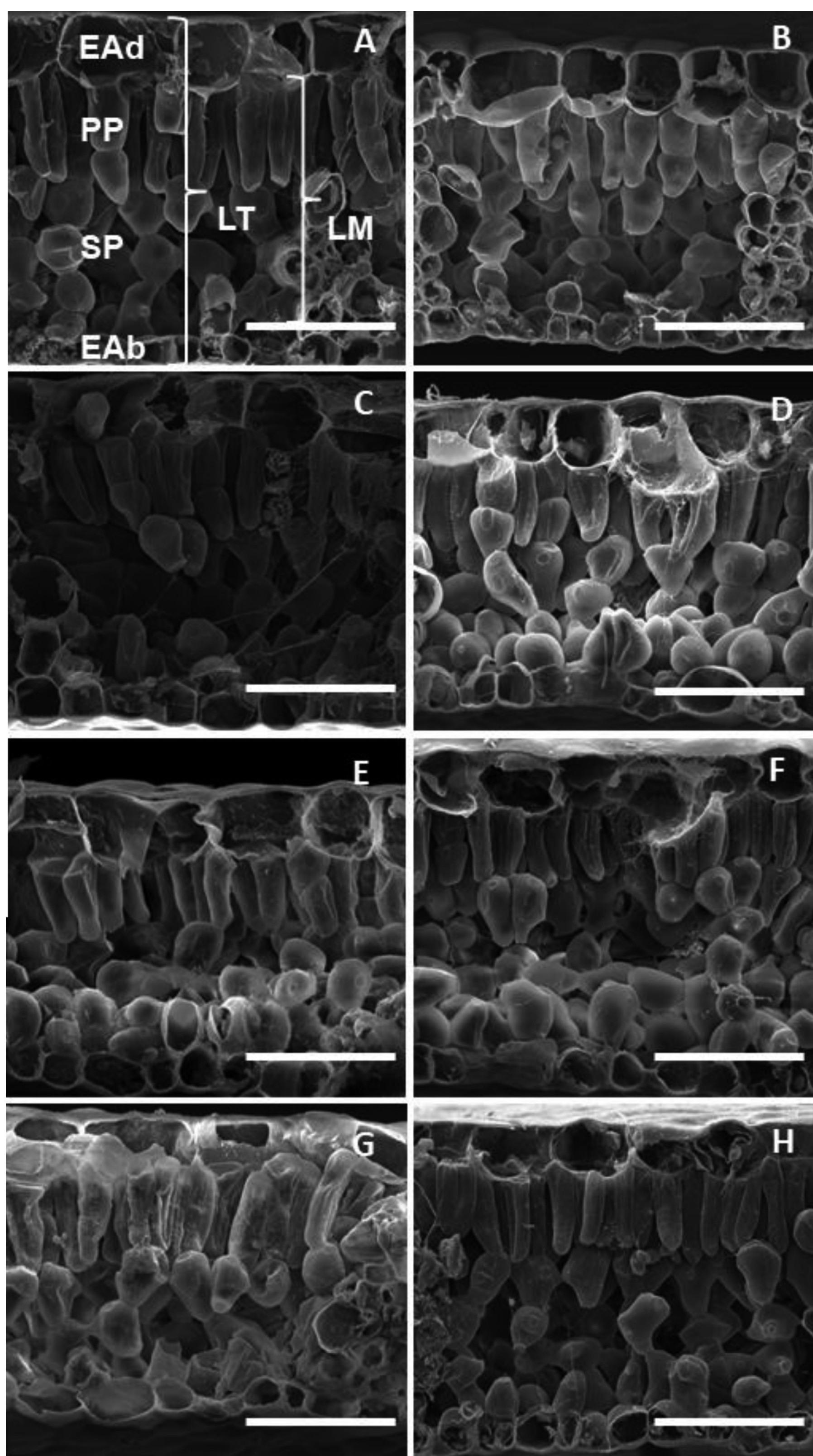

Fig. 7. Micromorphology of transverse sections of the leaf mesophyll of young plants of the CCN 51 cacao genotype, submitted to different concentrations of Zn, Cd and Zn + Cd in the soil at 37 days after application of the treatments, using scanning electron microscope (SEM). (A) Control treatment, (B) Zn (0.8 mmol kg^{-1} soil), (C) Zn (1.6 mmol kg^{-1} soil), (D) Cd (0.4 mmol kg^{-1} soil), (E) Cd (0.8 mmol kg^{-1} soil), (F) Zn + Cd (1.2 + 0.2 mmol kg^{-1} soil), (G) Zn + Cd (0.8 + 0.4 mmol kg^{-1} soil) and (H) Zn + Cd (0.4 + 0.6 mmol kg^{-1} soil). EAd: Epidermis on the adaxial face; EAb: Epidermis on the abaxial face; PP: Palisadic parenchyma; SP: Spongy parenchyma; LM: Leaf mesophyll; LT: Leaf total thickness. Bar: 40 μm.

cocoa young plant subjected to different concentrations of Zn, Cd and Zn + Cd, showed the presence of TUNEL-positive nucleus, showing a bright red fluorescence color (Fig. 8). The number of TUNEL-positive cells increased as the concentration of Zn and Cd increased in the leaf tissue (Fig. 8B-E), as well as the interaction between these elements (Fig. 8F-H). Isolated Zn concentrations did not demonstrate a large number of TUNEL-positive cells, unlike the isolated Cd concentrations, which demonstrated a large number of TUNEL-positive cells, mainly in the 0.8 mmol Cd kg^{-1} soil treatment (Fig. 8E), showing that the Cd significantly affected leaf tissue cells. When Cd was placed in interaction with Zn, it was observed that the number of TUNEL-positive cells decreased in relation to Cd alone (Fig. 8F-H) showing that Zn was able

Table 1
Thickness measurements of tissues and stomatal pore in cross section of the mature leaf median region of young plants of the CCN 51 cacao genotype, submitted to different concentrations of Zn, Cd and Zn + Cd in the soil at 37 days after application of the treatments, using scanning electron microscope (SEM). Mean values of three replicates (± SE). Lowercase letters indicate comparisons between treatments by Scott-Knott test ($p < 0.05$). ns: not significative.

Treatments (mmol kg^{-1})	Ead (μm)	Eab (μm)	PP (μm)	SP (μm)	LM (μm)	LT (μm)	SD (μm)	TOS (μm)	LOS (μm)	TOS × LOS (μm^2)
0 Zn + 0 Cd	16.6 ± 0.6 ns	10.3 ± 0.7 ns	25.6 ± 0.3 a	43.3 ± 4.2 a	68.4 ± 2.1 a	91.4 ± 2.1 a	671 ± 27.4 b	1.5 ± 0.4 ns	3.4 ± 0.4 b	5.3 ± 1.9 ns
0,8 Zn + 0 Cd	17.3 ± 1.1 ns	9.0 ± 1.0 ns	21.5 ± 0.7 b	34.2 ± 1.4 b	57.5 ± 3.6 b	85.1 ± 3.6 a	689 ± 9.1 b	1.8 ± 0.2 ns	3.7 ± 0.3 b	6.9 ± 0.9 ns
1,6 Zn + 0 Cd	13.4 ± 1.3 ns	8.2 ± 2.0 ns	21.1 ± 2.3 b	35.0 ± 1.8 b	54.5 ± 4.0 b	76.3 ± 4.0 b	621 ± 24.2 b	2.2 ± 0.3 ns	3.9 ± 0.0 b	8.8 ± 0.9 ns
1,2 Zn + 0,2 Cd	15.0 ± 1.0 ns	11.9 ± 1.6 ns	20.5 ± 2.1 b	41.4 ± 3.0 a	57.7 ± 3.7 b	83.0 ± 3.7 a	872 ± 36.5 a	1.8 ± 0.2 ns	4.6 ± 0.6 a	8.5 ± 1.9 ns
0 Zn + 0,4 Cd	15.6 ± 0.8 ns	10.1 ± 0.3 ns	21.9 ± 0.9 b	30.4 ± 1.0 b	53.2 ± 2.1 b	76.0 ± 2.1 b	699 ± 23.7 b	2.3 ± 0.1 ns	3.6 ± 0.4 b	8.2 ± 1.1 ns
0,8 Zn + 0,4 Cd	12.4 ± 1.8 ns	8.0 ± 1.1 ns	25.6 ± 0.7 a	24.8 ± 1.3 b	45.7 ± 0.5 c	71.0 ± 0.5 b	648 ± 22.8 b	2.2 ± 0.3 ns	4.9 ± 0.6 a	11.2 ± 2.6 ns
0,4 Zn + 0,6 Cd	14.2 ± 1.1 ns	11.1 ± 0.3 ns	24.4 ± 1.6 a	25.6 ± 2.0 b	47.7 ± 0.4 c	72.2 ± 0.4 b	539 ± 45.7 c	2.4 ± 0.1 ns	4.7 ± 0.2 a	11.5 ± 1.1ns
0 Zn + 0,8 Cd	17.6 ± 1.2ns	9.2 ± 0.9ns	20.8 ± 0.8b	25.9 ± 1.0b	44.3 ± 0.9c	68.9 ± 0.9b	658 ± 76.3b	1.8 ± 0.2ns	3.7 ± 0.3b	6.9 ± 1.4 ns

EAd: Epidermis on the adaxial face; EAb: Epidermis on the abaxial face; PP: Palisadic parenchyma; SP: Spongy parenchyma; LM: Leaf mesophyll; LT: Leaf total thickness; SD: Stomatal density; TOS: Transverse opening of the stomatal pore; LOS: Longitudinal opening of the stomatal pore.

to mitigate Cd toxicity and decrease cell death in leaves of cocoa, mainly in the Zn + Cd treatment (0.8 + 0.4 mmol kg^{-1} soil) (Fig. 8G).

4. Discussion

In the present study, Zn and Cd accumulated mainly in the roots of the young plants of the CCN 51 cocoa genotype compared to the aerial part, whose accumulation was gradual with the increase of the isolated or combined concentrations of Zn and Cd metals in the soil (Fig. 2A). The same fact was observed by Castro et al. (2015) and Llatance et al. (2018) in *T. cacao* and by Luo et al. (2018) when evaluating the toxicity of Cd in the woody species *Arundo donax, Broussonetia papyrifera, Robinia pseudoacacia* and *Cryptomeria fortunei*. Much of the Cd taken up by plants is retained in the roots, but a portion of it is translocated to the aerial parts of the plant and into the seed (Tran and Popova, 2013). As Cd presents high mobility in the plant and high solubility in water, it ends up being transported to the aerial part through the transpiration current, and, in certain plant species, its accumulation occurs mainly in the leaves (Sekara et al., 2004).

Increasing Zn concentration in the soil provided a reduction in Cd absorption in the leaves of the young plants of the CCN 51 cacao genotype (Fig. 2B), demonstrating that the mitigation of the toxicity of Cd by Zn in cocoa plants is dependent on the concentration of Zn in the soil. A fact also observed by Hussain et al. (2018) in *Triticum aestivum* plants when evaluating the mitigation of Cd toxicity by Zn. Moreover, the lower accumulation of Cd in the leaves may be a strategy to protect the photosynthetic functions of the oxidative stress induced by this metal (Dixit et al., 2001), since the differences in the pattern of Cd allocation in different parts of the plant may vary according to the species (Barraza et al., 2017) and the cultivar in question (Gramlich et al., 2017).

Increase in combined concentration of Zn and Cd in the soil did not influence the accumulation of Zn in leaves of cocoa, because the increase in the soil Cd concentration with any concentration of soil Zn presented similar responses (Fig. 2A). On the other hand, there was an increase of the Zn concentration in the cocoa plants roots, despite of Cd addition in soil (Fig. 2C). Fact also observed in the lowest concentration of Cd applied to soil (0.2 mmol Cd kg^{-1} soil). This indicates the occurrence of a possible plant defense mechanism by immobilization or chelation of Zn and Cd in the roots (Luo et al., 2018). According to Pereira et al. (2015), the main mechanism of tolerance of *Schinus molle* tree to Cd was the compartmentalization in the roots and the reduction of the translocation to the aerial part, through the formation of apoplastics barriers. The root epidermis and the cortex also represent a physical barrier against the uptake of excess Zn via xylem flow (Di Baccio et al., 2009). The accumulation of toxic metals in the roots can contribute to the tolerance of the plants for toxic metals (Marques and Nascimento, 2014), depending on the mechanism that immobilizes the heavy metals in the roots (Luo et al., 2018). In addition, the roots may promote the secretion of exudates, such as low molecular weight organic acids, which play an important role in detoxification of heavy metals, such as Cd in the rhizosphere (Zhu et al., 2011). Tian et al. (2012) showed in *S. alfredii* plants that Cd accumulated preferentially in the parenchyma tissues (pith, cortex, and mesophyll), all of which consist of large vacuolar cells, supporting the contention that vacuoles may serve as major storage sites for Cd in this plant species.

The lower Cd concentration in the leaves of cocoa in the treatments with higher concentrations of Zn in the soil (Fig. 2B) also suggests that Zn protects the plant from the Cd toxicity by competition with Cd in the plant cell metabolism. This, in turn, confirms the hypothesis that one element can be modulated by the presence of the other because of its chemical similarity. In the present study, a higher tendency of Cd uptake by the root system of the cocoa plants was observed when the Zn concentrations in the soil were less. Part of the absorbed Cd, due to its high mobility in the plant, may have been accumulated in older and senescent leaves, mainly in the treatments with higher concentration of

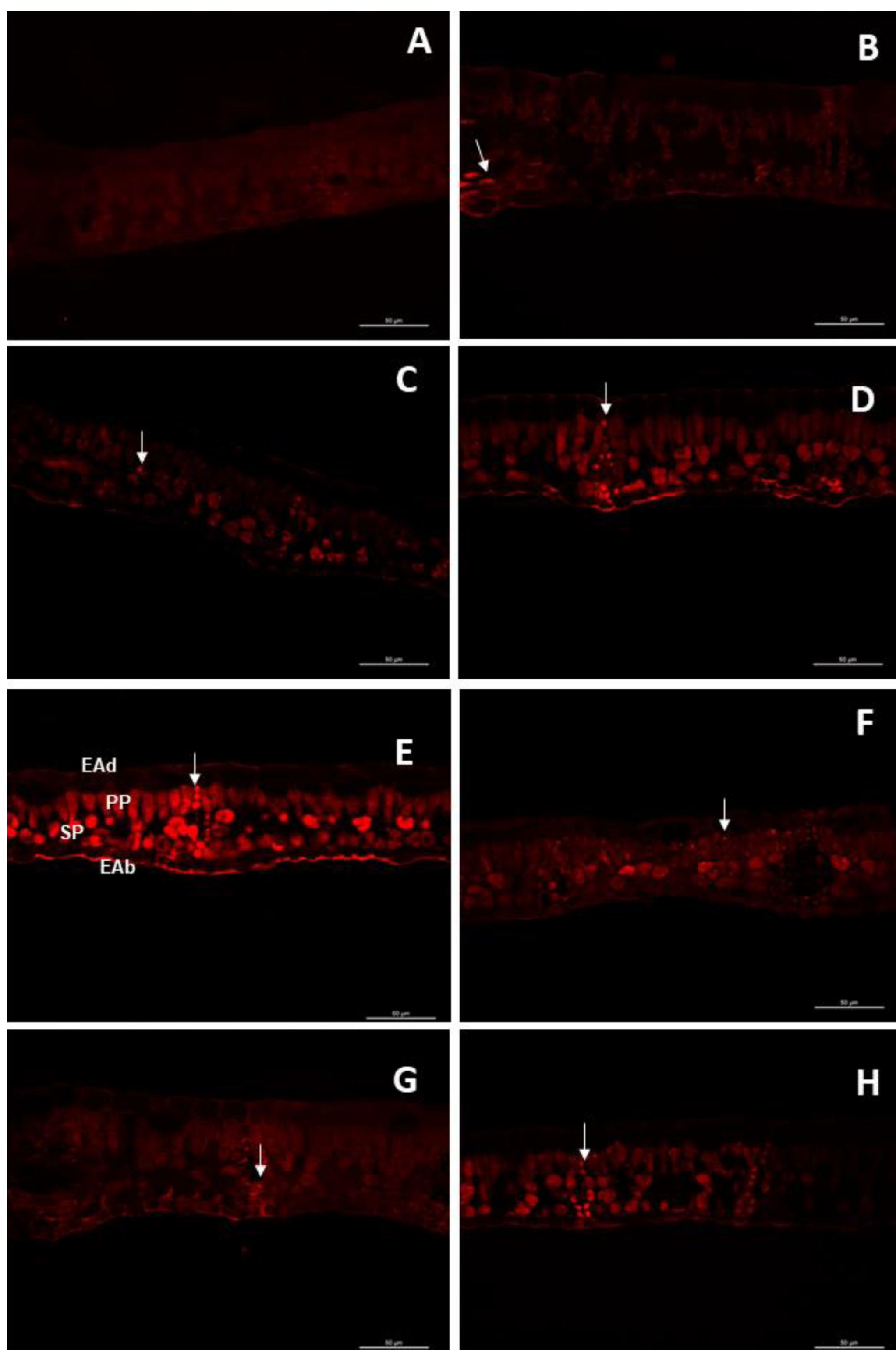

Fig. 8. Programmed cell death in leaf mesophyll of young plants of the CCN 51 cacao genotype, submitted to different concentrations of Zn, Cd and Zn + Cd in the soil at 37 days after application of the treatments. (A) Control treatment, (B) Zn (0.8 mmol kg^{-1} soil), (C) Zn (1.6 mmol kg^{-1} soil), (D) Cd (0.4 mmol kg^{-1} soil), (E) Cd (0.8 mmol kg^{-1} soil), (F) Zn + Cd (1.2 + 0.2 mmol kg^{-1} soil), (G) Zn + Cd (0.8 + 0.4 mmol kg^{-1} soil) and (H) Zn + Cd (0.4 + 0.6 mmol kg^{-1} soil). Arrows indicate TUNEL-positive nucleus. EAd: Epidermis on the adaxial face; EAb: Epidermis on the abaxial face; PP: Palisadic parenchyma; SP: Spongy parenchyma. Bar: 40 μm.

Zn combined with the two higher concentrations of Cd in the soil, as observed by Godzik (1993) in other plant species.

At the highest concentrations of soil Zn and Cd, isolated or combined, it was possible to infer that Zn and Cd have an effect on leaf gas exchange of young plants of the CCN 51 cacao genotype, when in high concentrations, evidenced by the reduction of the net photosynthetic rate (*A*) (Fig. 1), especially for Cd when its concentration was higher than 0.2 mmol Cd kg^{-1} soil. However, there was an increase in the photosynthetic rate at the Cd concentration corresponding to 0.2 mmol kg^{-1} soil, combined with any concentrations of Zn. Mateos-Naranjo et al. (2008) consider that the exposure of plants to excess Zn can inhibit the transport of PS2 electrons, influencing the fixation and assimilation of CO_2, triggered by the decline of Rubisco activity. On the other hand, the increase of the net photosynthesis may be the result of the increase of the Rubisco concentration, in low soil Cd concentrations, evidencing its positive effect (Jia et al., 2014).

Reduction of *A* values, with the increase of soil Zn and Cd concentrations, may be associated with the reduction of *gs*, due to the

partial closure of the stomata and resistance to diffusion of CO_2 to the sub-stomatal camera, and, consequently, reduction of water loss by means of transpiration (Fig. 1 and Sup. Fig. 2 and 3). Jia et al. (2014); Singh and Prasad (2015); Liu et al. (2016) and Araújo et al. (2017) also observed increase of *A*, *gs* and *E* in low soil Cd concentrations, with subsequent decline of these parameters with increasing soil Cd concentrations. This mechanism is known as the hormesis effect, which is a phenomenon that is characterized by concentration-response when there is a stimulus at low concentrations and inhibition at high concentrations (Jia et al., 2014). The reduction of *A* values can be explained by a perturbation in the leaf photosynthetic system, such as the inhibition of the electron transport chain of the chloroplast (Myśliwa-Kurdziel et al., 2004), inhibition of enzymes from the Calvin cycle (Aravind and Prasad, 2003; Myśliwa-Kurdziel et al., 2004; Lösch, 2004) or reduction of chlorophyll concentration (Myśliwa-Kurdziel et al., 2004; Küpper et al., 2007).

H_2O_2 can be removed by CAT, GPX and APX in order to avoid further its conversion into more ROS such as OH^- (Perl-Treves and Perl, 2002). Lower CAT activity in leaves of young plants of the CCN 51 cacao genotype subject to two largest Cd concentrations in soil (Fig. 5A e B), may be due to the action of Cd in the substitution of Fe in CAT active sites and the Fe inadequate uptake from soil (Benavides et al., 2005). In addition, Cd may also lead to enzymatic inhibition interacting with thiol groups in its structure (Ulusu et al., 2017), by binding to cysteine residues at their active sites (D'alessandro et al., 2013). The same fact was also observed for (Fig. 5C and D) and GPX (Fig. 5E and F) at all times evaluated. According to Kurdziel and Prasad (2004) and Lösch (2004) the action of metals like Cd falls on the enzymes and proteins that contain sulfhydryl groups (SH) linked together by disulfide bonds, oxidizing these groups, which, reacting with S, destroys the disulfide bonds, denaturing the protein, resulting in the reduction of the enzymatic activity. Thus, Cd, due to its chemical similarity to Zn as a divalent cation, can substitute it in these enzymes, resulting in a change in the enzymatic activity (Shaw et al., 2004). CAT (Fig. 5A and B) and APX (Fig. 5C and D) activities, in many treatments with combined concentrations of soil Zn and Cd, it also showed similarities to the control treatment, showing once again that Zn was able to mitigate the Cd toxicity in cocoa plants.

Induction of the relative expression of all the evaluated genes (*psbA*, *psbO*, *SODCyt*, *SODChl* and *per-1*), mainly 6 h AAT, in the highest concentrations of Zn + Cd applied to soil reveals the ability of young plants of the genotype of cocoa CCN 51 to tolerate high Zn and Cd concentrations (Fig. 6). Thus, plants respond to abiotic stresses by increasing the expression of genes encoding proteins involved in the stress response (Hasan et al., 2017). Uptake of Zn and Cd, at high concentrations, by cocoa young plants, caused damage to the photosynthetic apparatus (Fig. 1), due in part to the suppression of *psbA* and *psbO* expression, 48 h AAT, except in the treatment 1.2 mmol Zn kg^{-1} soil + 0.8 mmol Cd kg^{-1} soil (Fig. 6). Reduction of photosynthetic activity in cocoa young plants with increasing Cd concentration and, consequently, reduction in *psbA* and *psbO* expression was also verified by Araújo et al. (2017). According to Araújo et al. (2017) suppression of the *psbO* gene is a protection mechanism of the D1 protein, in the sense of avoiding damage to PS2. Such gene is mainly responsible for the coding of PsbO protein, extrinsic to PS2 (Nickelsen and Rengstl, 2013). The *psbA* gene encodes protein D1, the main intrinsic PS2 protein, which is affected by Cd-induced oxidative stress (Møller et al., 2007).

The expression induction of Cu-Zn *SODCyt* and Cu-Zn *SODChl* genes at 6 h AAT and subsequent expression reduction at 48 h AAT (Fig. 6) reveal the efficiency of the SOD protection mechanism in the ROS detoxification. In this case, the oxidative damages were influenced by the concentration and time of exposure to Zn and, or Cd. Similar results on expression of Cu-Zn *SODChl* gene were obtained by Araújo et al. (2017) and Castro et al. (2015) when evaluating the Cd toxicity in young plants of the CCN 51 cocoa genotype and of the cocoa progeny resulting from the crossing of CCN 10 x SCA 6. On the other hand, GPX activity is associated with the *per-1* gene, which was overexpressed in the combination of 1.6 mmol Zn kg^{-1} soil + 0.8 mmol Cd kg^{-1} soil at 6 h AAT and 1.6 mmol Zn kg^{-1} soil + 0.6 mmol Cd kg^{-1} soil only at 48 h AAT. This mechanism is presented as a possible defense against excess ROS caused by high Zn and Cd concentrations. A similar fact was also observed by Castro et al. (2015) when evaluating the toxicity of Cd in cocoa young plants. Expression reduction of most of evaluated genes (*psbA*, *psbO*, *SODCyt* and *SODChl*) at 48 h ATT (Fig. 6B), in relation to the control, shows that Zn, when combined with Cd, is able to mitigate the toxicity of Cd in cocoa plants, since these genes are related to stress.The highest proline content observed in the leaves of the young plants of the CCN 51 cacao genotype submitted to the highest concentration of Zn (1.6 mmol kg^{-1} soil), combined with the highest concentrations of Cd (0.4, 0.6 and 0.8 mmol kg^{-1} soil), and in the highest concentrations of Zn + Cd (1.6 + 0.8 mmol kg^{-1} soil) (Fig. 3), indicate the effectiveness of the non-enzymatic metabolism in the control of ROS production. This mechanism of ROS removal is due to the easy reaction between proline and singlet oxygen ($^1O_2$), which contributes to the stabilization of proteins, DNA and membranes (Matysik et al., 2002), as well as to play an important role in reducing lipid peroxidation of cell membranes. Increase of the proline content, with the increase of the Cd concentration, was also reported in other plant species by Yilmaz and Parlak (2011) and Singh and Prasad (2014).

According to Shetty (2004), there are relationships between proline content and the glucose-6-phosphate dehydrogenase (G6PDH) activity of the pentose phosphate route, when, in response to stress, phenolic biosynthesis is stimulated and proline, through proline dehydrogenase reactions, can enter the mitochondria, contribute to oxidative phosphorylation, rather than NADH, and synthesis of ATP. Reduction of pyrroline-5-carboxylase (P5C) provides $NADP+$, a cofactor for G6PDH, an enzyme that catalyzes the rate-limiting step of the pentose phosphate pathway. Sharma et al. (1998) observed that proline protect the G6PDH enzyme from toxic levels of Zn and Cd, the latter with fewer efficacies, suggesting that this was due to the reduction of free Cd^{2+} metal ion activity, due to the formation of proline-metal complexes.

Cd can induce the accumulation of ROS and, consequently, promote a lipid peroxidation of cell membranes (Dey et al., 2007). However, in the present work, there was no lipid peroxidation of cell membranes of leaf and root tissues at the highest concentrations of soil Zn and Cd, except for the lower concentration of soil Zn, associated with the higher concentration of soil Cd (Fig. 4). A non-enzymatic mechanism is an important indicator of stress, since the free radicals react with polyunsaturated fatty acids from the membranes, oxidizing the lipids (Ayala et al., 2014). According to Ulusu et al. (2017), the increase in lipid peroxidation with increasing Cd concentration in *Petroselinum hortense* plants is due to the detrimental effect of ROS derivatives. Similarly, Liu et al. (2016) observed increased malondialdehyde content resulting from lipid peroxidation with increasing exposure time and Cd concentration.

The exposure of the young plants of the CCN 51 cacao genotype to high concentrations of soil Zn, Cd and Zn + Cd caused a reduction in the thickness of the leaf mesophyll (Table 1). Castro et al. (2015) also observed a reduction in leaf thickness of young plants of the cocoa progenies, resulting from 'Catongo x Catongo and 'CCN 10 x SCA 6' crosses, submitted to soil concentrations of 8 and 32 mg L^{-1} Cd, due to the reduction in thickening of the palisadic and spongy parenchymas. According to Chugh and Sawhney (1999), the compression of the leaf tissue can lead to the decrease of photosynthetic capacity in the presence of Cd. In fact, in the present work, the lower photosynthetic rates were observed in the treatments where there was a reduction in the thickness of the leaf mesophyll, due to the increase of the concentration of soil Zn, Cd and Zn + Cd.

The higher leaf stomatal density of young plants of the CCN 51 cocoa genotype, at the combined concentration of soil Zn + Cd (1.2 + 0.2 mmol kg^{-1} soil) (Table 1), promoted an increase in photosynthetic rates at 29 days AAT. According to Taiz and Zeiger (2017), the

increase in stomatal density should follow the increase of the conversion of light energy into chemical energy. In addition, this same treatment, along with control treatment, presented a greater thickness of the spongy parenchyma (Table 1). Di Baccio et al. (2009) observed increased leaf thickness in *Populus* sp. with increasing Zn concentration. According to Pereira et al. (2015), the anatomy of leaves of *S. molle* trees, submitted to small concentrations of solution Cd (up to 20 μM), showed beneficial characteristics, such as thickening of the mesophyll. According to these authors, the increase in the concentration of solution Cd (> 50 μM) promotes a decrease in the thickness of the mesophyll, a decrease in the size and density of stomata, and also reinforce the occurrence of the hormesis effect verified in this work.

Exposure to various stresses generally leads to at least some degree of DNA damage resulting in various lesions such as thymine dimerization, alkylation of bases, single stranded nicks, and double-stranded breaks (Bray and West, 2005; Manova and Gruszka, 2015). DNA fragmentation is of particular concern during stress conditions, which may either be a direct effect of the stress or an indirect effect or may even be acumulative consequence of both (Bray and West, 2005; Kapoor et al., 2015). Through the TUNEL reaction we can observe that the greater the plant's exposure to Cd, the greater the DNA fragmentation (Fig. 8). TUNEL positivity correlates with double strand DNA breaks in general, but it can detect single-stranded DNA breaks as well and it is known to be a marker of eukaryotic and particularly plant PCD (Thomas et al., 2015). PCD is a functional process, which occurs as a defensive strategy to remove mutated, infected or damaged cells during development or under environmental stress (Vardar and Ünal, 2011; Wang et al., 2011). In Cd treatments showed high DNA fragmentation and cell death (Fig. 8D-E). When Cd and Zn interacted, showed less DNA fragmentation and less cell death (Fig. 8F-H). Nuclear changes and DNA fragmentations are the most widely evaluated and sensitive parameters of PCD (Vardar et al., 2016).

5. Conclusions

High concentrations of Zn, Cd and Zn + Cd in the soil promoted alterations in enzymatic and non-enzymatic antioxidative metabolism, expression of genes involved in photosynthesis and antioxidative metabolism in young plants of the CCN 51 cacao genotype.

Leaf gas exchange were affected at the highest concentrations of soil Cd (0.4, 0.6 and 0.8 mmol Cd kg^{-1} soil), combined with different concentrations of soil Zn (0.4, 0.8, 1.2 and 1.6 mmol kg^{-1} soil), resulting in a decrease in CO_2 fixation and assimilation.

Low soil Cd concentration (0.2 mmol kg^{-1} soil), combined with different concentrations of soil Zn (0.4, 0.8, 1.2 and 1.6 mmol kg^{-1} soil), promoted the decrease of the photosynthetic rate in young plants of the CCN 51 cacao genotype.

The higher soil Cd concentration (0.8 mmol kg^{-1} soil) together with the intermediate concentrations of soil Zn + Cd (0.8 + 0.4 and 0.4 + 0.6 mmol kg^{-1} soil) promoted thickness reduction of the leaf mesophyll.

The increase in the concentration of Zn in the soil promoted a decrease in the Cd uptake by the root system of plants and reduced the transport of Cd to the leaves in the Cd concentrations of 0.4 mmol and 0.6 mmol kg^{-1} of soil.

Roots immobilized much of the Cd in their tissues, as a tolerance strategy, thereby avoiding part of absorbed Cd transported to aerial part.

Increase of Zn + Cd concentration in the soil did not influence the accumulation of Zn in the leaves of the young plants of the CCN 51 cocoa genotype. Combinations of Zn + Cd applied in the soil reduced oxidative stress and programmed cell death, showing that Zn was able to mitigate the toxicity of Cd in young plants of CCN 51 cacao genotype.

Author contributions

All authors contributed equally to this work.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

The second author gratefully acknowledges the National Council for Scientific and Technological Development (CNPq), Brazil, for the grant of a fellowship of scientific productivity. This study was funded by a grant from USDA-ARS, in an international cooperative agreement with UESC.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2020.104201.

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