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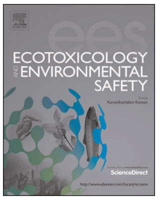

Morphological, biochemical, molecular and ultrastructural changes induced by Cd toxicity in seedlings of *Theobroma cacao* L.

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ABSTRACT

Seeds from *Theobroma cacao* progenies derived from the self-pollination of ‘*Catongo*’ × ‘*Catongo*’ and the crossing between CCN-10 × SCA-6 were immersed for 24 h in different Cd solutions (2; 4; 8; 16 and 32 mg L^{-1}) along with the control treatment (without Cd). Shortly after, the seeds were sown in plastic tubes containing organic substrate and were grown in a greenhouse for 60 days. The treatment with Cd was observed to cause morphological, biochemical, molecular and ultrastructural changes in both progenies of *T. cacao*. There has been deformation in chloroplasts, nuclear chromatin condensation, and reduction in thickness of the mesophyll. As for ‘*Catongo*’ × ‘*Catongo*’, a decrease in thickness of the epidermis was noted on the abaxial face. There has been increased guaiacol peroxidase activity in the roots of CCN-10 × SCA-6, as well as in the"*Catongo*’ × ‘*Catongo*’ leaves. In the presence of Cd, CCN-10 × SCA-6 showed increased expression of the genes associated with the biosynthesis of phytochelatin (PCS-1) and class III peroxidases (PER-1) in leaves, and metallothionein (MT2b), in roots. In ‘*Catongo*’ × ‘*Catongo*’, there has been an increase in the expression of genes associated with the biosynthesis of PER-1 and cytosolic superoxide dismutase dependent on copper and zinc (Cu–Zn SOD_{Cyt}) in leaves and from MT2b and PCS-1 and roots. There was higher accumulation of Cd in the aerial parts of seedlings from both progenies, whereas the most pronounced accumulation was seen in"*Catongo*’ × ‘*Catongo*’. The increase in Cd concentration has led to lower Zn and Fe levels in both progenies. Hence, one may conclude that the different survival strategies used by CCN-10 × SCA-6 made such progeny more tolerant to Cd stress when compared to"*Catongo*’ × ‘*Catongo*’.

1. Introduction

Cd is considered one of the most toxic heavy metals that exist in nature (Al-Kedhairy et al., 2001) and that can contaminate plants, animals and humans (Almeida et al., 2007). The bioavailability of heavy metals in the soil, including Cd, is regulated by physical, chemical and biological processes and their interactions (Ernest, 1996). Small variations in pH can greatly alter the availability of metals (Pierangeli et al., 2001). During weathering, Cd is easily transferred to the soil solution. Despite its occurrence as Cd^{2+} is known, it can form many complex ions such as $CdCl^{+}$, $CdOH^{+}$, $CdHCO^{3+}$, $CdCl_4{}^{2-}$, $CdCl^{3-}$, $CdCl_4{}^{2-}$, $Cd(OH)^{3-}$, $Cd(OH)_4{}^{2-}$ and organic chelates (Kabata-Pendias and Pendias, 2001). In the soil, Cd is chiefly seen in the available form, which is exchangeable and can be easily absorbed by plants and cause phytotoxicity (Soares et al. 2005). The phosphate rocks used in fertilizer production are the major sources of Cd contamination in agricultural soils (Mortvedt and Beaton, 1995). In recent decades, it has been observed a significant increase of Cd in the environment, mainly as a result of industrial activities such as mining, smelting and refining of zinc, manufacture and use of phosphate fertilizers and fungicides (Al-Kedhairy et al., 2001; Arduini et al., 2004; Benavides et al., 2005). Plants normally absorb Cd present naturally in the soil or from atmospheric depositions, or from that present in organic or phosphate fertilizers (Gallego et al., 2012). Once inside the plant, Cd accumulation promotes morphological and ultrastructural changes, and alterations in physiological, biochemical and molecular processes, modifying metabolic activities (Almeida et al., 2013).

In plant cells, Cd triggers a sequence of metabolic reactions and promotes a number of changes in plants, such as induction of differential gene expression; increased activity of antioxidant enzymes, like for example class III peroxidases and superoxide

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dismutase (SOD); induction of phytochelatin production (PCs); and morphological and ultrastructural changes (Almeida et al., 2010; Pietrini et al., 2010; Callahan et al., 2006; Alscher et al., 2002). Class III peroxidases are located in vacuoles and cellular walls and belong to a family of multigenes involved in various physiological processes. Moreover, they act on a wide variety of substrates and show a moderate specificity for phenols (Almagro et al., 2009). SOD enzymes are classified into three groups: Fe SOD, Mn SOD and Cu–Zn SOD, which are located in different cellular compartments (Alscher et al., 2002). Cu–Zn SOD enzymes are divided into two classes of isoforms, one located in the cytoplasm and another located in the chloroplasts (Kurepa et al., 1997). PCs are enzymatically produced from the precursor of GSH tri-peptide (Glu-Cys) *n*-Gly; where $n=2–11$ (Inouhe, 2005). Due to the presence of cysteine thiol groups, PCs chelate Cd and form complexes with various molecular weights, thus protecting the harmful effects of free Cd ions and eventually remove Cd in the vacuole (Clemens, 2006; Cobbett, 2000; Ernst et al., 2008; Schat et al., 2002). The accumulation of PC has been observed as a response to stress by heavy metals (Lee and Korban, 2002; Noctor et al., 1998; Rauser, 1999).

For living in environments contaminated with toxic metals, some plant species have developed survival strategies such as the exclusion of metals through the selective absorption of mineral elements; chelation of toxic metal ions by means of a specific high affinity ligand (Patra et al., 2004); accumulation of phytochelatins and metallothioneins ([Pietrini et al., 2010,Schützendübel and Polle, 2002]); retention of toxic metals in the roots, preventing its translocation to the aerial part; compartmentalization of toxic metals for vacuoles, and adsorption to the cell wall; activation of antioxidant enzymes; differential expression of proteins (Gomes et al., 2012); exclusion and accumulation of large amounts of toxic metals in their tissues; and cellular repair mechanisms (Almeida et al., 2010).

Theobroma cacao is a tropical evergreen tree species that is Eudicotyledons and diploid ($2n=20$) (Figueira et al., 1992), preferentially allogamous, and belongs to the Malvaceae family; the geographic location of the species, in turn, is South America (Almeida and Valle, 2007). This is one of the most important perennial crops worldwide, with an estimated production exceeding 400,000 ton in 2012–2013 (ICCO, 2013). This species is chiefly exploited for the production of chocolate; but it can also be used in cosmetics, beverages, jellies, creams and juices (Almeida and Valle, 2007). Cd concentrations were observed in cocoa powders and related products (beans, liquor, butter) of different geographical origins (Mounicou et al., 2003).

With regard to its morphological and physiological characteristics, *T. cacao* displays great genetic variability (Daymond et al., 2002). The 'Catongo' genotype is self-compatible, midsize, and medium to high productivity. This genotype is the result of a natural mutation for anthocyanins in *T. cacao* variety 'Common'. Logo is devoid of anthocyanins, and water-soluble vacuolar pigments reddish in color. In normal plants, these pigments are typically more concentrated in young leaf tissue cells as well as young fruits, and seeds ripe. The main characteristic of 'Catongo' is the absence of anthocyanins in these organs, although there is fruit seeds showing ocher by cross pollination effect (Bartley, 2005). It is highly susceptible to *Moniliophtora perniciosa* and has moderate resistance to *Phytophthora palmivora*. Moreover, SCA-6 is considered as a wild type genotype, low size, self-incompatible, low productivity; with moderate resistance to high to *M. perniciosa*, moderate to *P. palmivora*; and susceptible to *Moniliophtora roreri* (Cervantes-Martinez et al., 2006); presents with leaves young, fruits and seeds slightly red by the presence of anthocyanins (Bartley, 2005). Another study in order to evaluate the effects of flooding on growth and mineral nutrition of six *T. cacao* clones, the authors observed that the CCN-10 genotype reached an intermediate position in relation the flooding tolerance compared to other clones evaluated (Rehem et al., 2009). Therefore, it is expected that the F1 progenies resulting from the crossing of these genotypes (CCN-10 × SCA-6) are genetic materials of high resistance to various biotic and abiotic stress, by show a high degree of heterozygosity. On the other hand, the F1 progenies, resulting from self-pollination of 'Catongo' × 'Catongo', are thus more homozygous and intolerants to biotic and abiotic stresses, contrast with the CCN-10 × SCA-6.

This study aimed to describe the main defense mechanisms to stress cadmium used by two progenies of *T. cacao*. The results may be used in future programs of genetic enhancement.

2. Material and methods

2.1. Plant material and growing conditions

The experiment was conducted under greenhouse conditions at Universidade Estadual de Santa Cruz-UESC, Ilhéus, BA, Brazil. The assay has used two *T. cacao* progenies contrasting for the tolerance to various types of stresses, like flooding, salinity, drought, disease, among others (Silva et al., 2012; Bertolde et al., 2010). The progenies were derived from the crossing between CCN-10 × SCA-6 and the self-pollination of 'Catongo' [('*Catongo*' × '*Catongo*')]. CCN-10 × SCA-6 and '*Catongo*' × '*Catongo*' were obtained via controlled pollination at the Active Germplasm Bank (BAG) from the *Centro de Pesquisas do Cacau* (CEPEC) of the *Comissão Executiva do Plano da Lavoura cacaueira* (CEPLAC). SCA-6 is a wild genotype native to Peru, has alleles with resistance to fungal diseases (witches' broom) caused by the basidiomycete *M. perniciosa* and by black pod rot, which is caused by *Phytophthora spp*. Furthermore, its fruit and seeds are small (<1 g) and vary in number between fruit (Bartley, 2005). CCN-10 is a genotype native to Ecuador, also exhibits high resistance to *M. pernicisa* and has large fruit and seeds (>1 g) (Silva et al., 2010). On the other hand, '*Catongo*' is a genotype native from Bahia, Brazil, and derives from the '*Cacau Comum*", is susceptible to *M. perniciosa* and has medium size fruit and seeds (~ 1 g); aside from being a natural mutant for anthocyanins, it soon exhibits leaves and young, light-green fruit that will become yellow when ripe, and white seeds (Neto et al., 2005).

Seeds from physiologically ripe fruit deriving from both progenies were previously cleaned by rubbing with sawdust to remove the mucilage, followed by removal of seed coat in such a way as to make tissues in contact with the metal when in solution. After that, the seeds were fully immersed in various solutions containing different concentrations of Cd (2, 4, 8, 16 and 32 mg L^{-1} Cd), with the same volume (500 mL), provided in the form of $CdCl_2 \cdot 5/2H_2O$ (Sigma, USA) along with the control (deionized H_2O). The seeds were soaked in solution for 24 h and, at the moment the root was seen in protrusion (about 2 mm long), seeds were grown in black plastic tubes of 235 cm^3 containing *Pinus* bark and turf+triturated coconut fiber (1:1) as organic substrate. During the experimental period, the seedlings were daily watered and weekly fertilized with 4 g of $NH_4H_2PO_4$ (Sigma, USA) 3 g of $(NH_2)_2CO$ (Sigma, USA) and 3 g of KNO_3(Sigma, USA) for each liter of water; 5 mL of this mixture were placed on each tube.

2.2. Collection of plant material for analysis

Sixty days after seedlings emerged-same period when cotyledon reserves were totally depleted and cotyledons started to fall-primary roots, stem and 2nd or 3rd mature leaves were collected from the apex of the orthotropic axis of seedlings from both *T.*