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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 ($O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, $^1O_2$) 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_2O_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 $(\alpha\text{-Glu-Cys})_n$-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 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 μmol m^{-2} 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 μmol (CO_2) 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 m^{-2} s^{-1}, wavelength of 650 nm, 1 s) to obtain the minimal fluorescence (F_0). A saturating white-light pulse (20 kHz; 6000 μmol m^{-2} 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