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Physiological, ultrastructural, biochemical and molecular responses of young cocoa plants to the toxicity of Cr (III) in soil

Junea Leandro do Nascimento[a], Alex-Alan Furtado de Almeida[a,*], Joedson P. Barroso[a], Pedro A.O. Mangabeira[a], Dário Ahnert[a], Artur G.R. Sousa[a], José Vitor S. Silva[a], Virupax C. Baligar[b]

[a] State University of Santa Cruz, Department of Biological Sciences, Rodovia Jorge Amado, km 16, 45662-900 Ilhéus, BA, Brazil
[b] USDA-ARS-Beltsville Agricultural Research Center, Beltsville, MD, USA

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ABSTRACT

The objective of this study was to evaluate Cr toxicity in young plants of the CCN 51 *Theobroma cacao* genotype at different concentrations of Cr^{3+} in the soil (0, 100, 200, 400 and 600 mg kg^{-1}) through physiological, ultrastructural, antioxidant and molecular changes. Doses of 400 and 600 mg Cr^{3+} kg^{-1} soil severely affected foliar gas exchange, promoted by damages in photosynthetic machinery evidenced by the decrease in CO_2 fixation. Decreased expression of *psbA* and *psbO* genes, changes in enzymatic activity and lipid peroxidation also affected leaf gas exchange. A hormesis effect was observed at 100 mg Cr^{3+} kg^{-1} soil for the photosynthetic activity. As a metal exclusion response, the roots of the cocoa plants immobilized, on average, 75% of the total Cr absorbed. Ultrastructural changes in leaf mesophyll and roots, with destruction of mitochondria, plasmolysis and formation of vesicles, were related to the oxidative stress promoted by excess ROS. The activity of the antioxidant enzymes SOD, APX, GPX and CAT and the amino acid proline coincided with the greater expression of the *sod cyt* gene demonstrating synchronicity in the elimination of ROS. It was concluded, therefore, that the tolerance of the cocoa plants to the toxicity of Cr^{3+} depends on the concentration and time of exposure to the metal. Higher doses of Cr^{3+} in the soil promoted irreversible damage to the photosynthetic machinery and the cellular ultrastructure, interfering in the enzymatic and non-enzymatic systems related to oxidative stress and gene expression. However, the low mobility of the metal to the leaf is presented as a strategy of tolerance to Cr^{3+}.

1. Introduction

Theobroma cacao L. is a tropical crop of high importance, mainly due to the commercial value of its beans. The beans are ground for the preparation of products such as cocoa butter, jellies, liqueurs, cosmetics, chocolate, etc. (Almeida et al., 2014; Almeida and Valle, 2009). Due to the high concentration of fats, carbohydrates, polyphenols and antioxidants, in addition to benefits provided to human health, cocoa by-products are much appreciated and even are considered as a luxury items (Bertoldi et al., 2016). The CCN 51 (Colección Castro Naranjal) cultivar has become the preferred one among the producers due to its high productivity, tolerance to climatic variations and pathogens (Boza et al., 2014; Herrmann et al., 2015). Recent studies have reported the presence of traces of various heavy metals, including chromium (Cr) in beans and cocoa by-products such as chocolate (Arévalo-Gardini et al., 2017; Bertoldi et al., 2016; Yanus et al., 2014), with a positive linear correlation between Cr concentration in the beans and by-products (Yanus et al., 2014).

The most common and stable forms of Cr on the Earth's surface are Cr^{3+} (trivalent) and Cr^{6+} (hexavalent), which differ from each other in terms of soil mobility, bioavailability and toxicity to plants (Ashraf et al., 2017; Panda and Choudhury, 2005). In the soil solution, Cr is made available to plants by weathering the original rock and is around 10 e 100 mg kg^{-1} soil (Kabata-Pendias, 2011). However, the increase of its concentration in soil and availability also occurs by anthropogenic actions, such as the production of metallic alloys, leather, electroplating and pigments (Gomes et al., 2017; Hasan et al., 2017), as well as chemical fertilizers and sewage sludge (Hayat et al., 2012; Srivastava et al., 2017).

There are no reports that Cr^{3+} has any essential function in plant metabolism or on its specific mechanisms of absorption (Oliveira, 2012). Recently its essentiality in human and animal health has been

* Corresponding author.
E-mail address: alexalan.uesc@gmail.com (A.-A.F. de Almeida).

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questioned (Vicent, 2017). However, at low concentrations beneficial effects have been reported in some plant species such as *Allium cepa, Mentha citrata* and *Solanum nigrum* (Patnaik et al., 2013; Prasad et al., 2010; UdDin et al., 2015). At high soil concentrations, Cr can accumulate in plant tissues, preferably in the roots (Shanker et al., 2005), promoting toxic effects and thus establishing a bi-phasic dose-response process called hormesis. According to hormesis concepts a contaminating substance applied at low doses may exert a beneficial or stimulatory action, whereas, applied at high doses, it will exert a deleterious and inhibitory action (Calabrese and Blain, 2009; Calabrese, 2015; Poschenrieder et al., 2013).

High dosages of Cr^{3+} in plant tissues may promote molecular, biochemical and ultrastructural changes. As a result of the damage to cell membranes, alteration of chloroplasts, reductions in photosynthetic activity, degradation of chloroplastidic pigments and induction oxidative stress with excessive formation of reactive oxygen species (ROS) are observed, which can lead to senescence (Afshan et al., 2015; Scoccianti et al., 2016). Overproduction of ROS may result in imbalance in cellular homeostasis, inducing the formation of antioxidative enzymes, preferably at their sites of production (Hossain et al., 2012), as a mechanism of ROS elimination and cellular detoxification (Gomes et al., 2017). In addition, may promote lipid peroxidation (Shahid et al., 2017) and in some cases autophagy (Farah et al., 2016). In autophagy, autophagosomes (specialized organelles) sequester cytoplasmic components including metals, forming vesicles, which fuse with the vacuole (Hasan et al., 2017). However, if induced in large quantities autophagosomes can act as initiator or executor of programmed cell death (Minina et al., 2014). In addition to enzymes, increases in the synthesis and accumulation of non-enzymatic osmolytes, such as proline, have also been reported in the elimination of ROS induced by heavy metals (Tripathi et al., 2013) and also by Cr^{3+} (Anjum et al., 2017; Tang et al., 2012). Proline has multiple functions, such as free radical scavenger, enzyme, membrane and protein structure stabilizer and helps to maintain the cytosolic pH and the equilibrium of the redox reactions of the cells (Aslam et al., 2017).

Another important mechanism of detoxification and tolerance of plants against Cr and other heavy metals stress is chelation of the metal, with peptides such as phytochelatins (*phyt*) and metallothioneins (*Mt2b*) (Hasan et al., 2017; Srivastava et al., 2017). The biosynthesis of both peptides is stimulated as a function of the free metal concentration in the cells, however phytochelatin synthesis occurs more rapidly in response to stress. Both acts in the Cr sequester, inactivating the metal in the cytoplasm and transporting the Cr-protein complexes to the vacuoles, where they are compartmentalized, making them incapable of damaging the plants (Gomes et al., 2017; Hasan et al., 2017). Phytochelatins are synthesized by the enzyme *phytochelatin synthase* using reduced glutathione (GSH) as a substrate (Taiz et al., 2017). Metallothioneins are low-molecular-weight, cysteine-rich proteins, which give them high affinity for metal ions (Hasan et al., 2017; Shahid et al., 2017).

The purpose of this work was to describe the main defense mechanisms to stress of Cr^{3+} in clonal CCN 51 plants grown in soil as substrate. Contribute to elucidate the intoxication process, aiming to reduce health risks due to the accumulation of Cr in the plants of *T. cacao* and consequent contamination of the food chain by this metal. We examined (i) the physiological changes in plants after exposure to different Cr concentrations, (ii) explored Cr uptake and translocation, (iii) changes in cell ultrastructure in leaves and roots, (iv) the role of cellular antioxidant activities (enzymatic and non-enzymatic) in protecting the plants from Cr-toxicity, (v) the expression of genes associated with the biosynthesis of proteins involved in antioxidative metabolism and in metal chelation. The results may be used in future programs of genetic enhancement.

2. Material and methods

The experiment was conducted in a greenhouse at the State University of Santa Cruz (UESC), Ilhéus, Bahia, Brazil (14° 47 ′S, 39° 13′ W) in the period from 24 July 2015–14 January 2016. Clonal plants of the CCN 51 cacao cultivar were grown on sandy soil as substrate in pots with a capacity of 15 L. The soil was previously liming (pH 5.6) and the plants were fertilizer during planting and biweekly coverage after planting. Treatments without addition of Cr^{3+} (control) and with addition of Cr^{3+} were applied in the form of chromium chloride hexahydrate ($CrCl_3{\cdot}6H_2O$), in the concentrations of 100, 200, 400 and 600 mg Cr^{3+} kg^{-1} soil, (Han et al., 2004; Tang et al., 2012). The plants were obtained by rooting stem cuttings of plagiotropic branches. The stem cuttings were collected from plants with 5–10 years of age, at the Biofábrica de Cacau Institute (IBC, Banco do Pedro, Ilhéus, BA). The treatments with concentrations of Cr^{3+} (100, 200, 400 and 600 mg kg^{-1} soil) were applied in solution (100 mL per pot) on 14/12/2015 in one-year-old *T. cacao* plants. During the acclimation period [24/07/2015–13/12/2015] and during the period of exposure to treatments with Cr^{3+} [14/12/2015–13/01/2016], the plants were irrigated with rainwater, aiming to keep the soil close to field capacity. During the experimental period, photosynthetically active radiation (PAR) was recorded using Hobo S-LIA-M003 light radiation sensors coupled to the Hobo Micro Station Data Logger (Onset Computer, Bourne, MA, USA). Air temperature (T °C) and relative humidity (RH %) were recorded by means of Hobo H8 Pro sensors (Onset, Computer, Bourne, MA, USA) for characterization of cropping environments (Fig. S1). Soil characterization and recommended fertilization were described in Table S1.

2.1. Foliar gas exchanges

During the experimental period, the net photosynthetic rate per unit leaf area (P_N), stomatal conductance to water vapor (*gs*) and leaf transpiration (*E*) were monitored at 1, 4, 8, 15, 21 and 28 days after application of the treatments (AAT) in a completely expanded and mature leaf. Four plants were evaluated per treatment, between 7 and 11 h, using a portable LI-6400 photosynthetic measurement system (Li-Cor, Nebraska, USA) equipped with a 6400-02B RedBlue artificial light source. During the measurements the PAR value was set at 800 μmol photons m^{-2} s^{-1}, above the saturation irradiance without photoinhibition, and the leaf temperature at 26 °C (Rehem et al., 2011a, 2011b). The readings were recorded in the range of 2–3 min (coefficient of variation < 0.3%). The values of P_N, *gs* and *E* were used to calculate the intrinsic water use efficiency (*WUEi*) and instantaneous water use efficiency (*WUE*).

2.2. Photosynthetic pigments

Mature leaves (2nd or 3rd from the apex) of the plants of the various treatments were collected 96 h AAT. Immediately after, they were immersed in liquid nitrogen, stored in ultrafreezer − 80 °C and later lyophilized. Subsequently, the leaves were macerated in liquid nitrogen, 50 mg of leaf tissue was weighed into eppendorf tubes and 2 mL of 80% acetone was added for the extraction of the photosynthetic pigments, according to methodology described by Torres et al. (2006). The absorbances of the extracts were determined using a microplate spectrophotometer (SpectraMax Paradigm Multi-Mode Microplate Reader, Molecular Device, USA), at wavelengths corresponding to 646 nm, 663 nm and 470 nm, for the determination of the concentrations of *Chl a*, *Chl b* and *Car*, respectively, using the equations described by Lichtenthaler and Wellburn (1983) for 80% acetone extracts.

2.3. Total Cr determination

At 30 days AAT, the total Cr concentration was determined in the