Physiological responses of sweet potato (*Ipomoea batatas* L.) plants due to different copper concentrations

Respostas fisiológicas de plantas de batata-doce (*Ipomoea batatas* L.) submetidas a diferentes concentrações de cobre

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Abstract

At low concentrations, Cu is considered as an essential micronutrient for plants and as a constituent and activator of several enzymes. However, when in excess, Cu can negatively affect plant growth and metabolism. Therefore, the aim of this study was to evaluate physiological responses of sweet potato plants at different Cu concentrations by measuring morphological parameters, antioxidant metabolism, stomatal characteristics, and mineral profile. For this purpose, sweet potato plants were grown hydroponically in complete nutrient solution for six days. Then, the plants were transferred to solutions containing different Cu concentrations, 0.041 (control), 0.082, and 0.164 mM, and maintained for nine days. The main effect of increased Cu concentration was observed in the roots. The sweet potato plants grown in 0.082 mM Cu solution showed increased activity of antioxidant enzymes and no changes in growth parameters. However, at a concentration of 0.164 mM, Cu was transported from the roots to the shoots. This concentration altered morpho-anatomical characteristics and activated the antioxidant system because of the stress generated by excess Cu. On the basis of the results, it can be concluded that the sweet potato plants were able to tolerate Cu toxicity until 0.082 mM.
Key words: Micronutrient, antioxidant metabolism, mineral content, copper sulfate, stress

Resumo

O cobre, em baixas concentrações, é considerado micronutriente essencial para as plantas por ser constituinte e ativador de diversas enzimas. Porém, quando em excesso, pode afetar negativamente o crescimento, o desenvolvimento e o metabolismo vegetal. Com base no exposto, o objetivo do trabalho foi avaliar as respostas fisiológicas de plantas de batata-doce quando submetidas a diferentes concentrações de cobre através dos parâmetros morfológicos, índice de clorofila, metabolismo antioxidante, perfil mineral e características estomáticas. Para tanto, as mesmas foram cultivadas em sistema hidropônico com solução nutritiva completa por seis dias. Após este período foram transferidas para soluções contendo diferentes concentrações de cobre: 0,041 (controle); 0,082 e 0,164 mM, onde permaneceram por nove dias. Nas raízes, o principal efeito do aumento da concentração de cobre foi

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o acúmulo do mesmo nesse órgão. As plantas de batata-doce cultivadas com 0,082 mM de cobre na solução, apresentaram aumento da atividade das enzimas antioxidantes sem nenhuma alteração na taxa de crescimento. No entanto, na concentração de 0,164 mM, o cobre foi transportado das raízes para a parte aérea. Essa concentração reduziu o crescimento, alterou características morfo-anatômicas e ativou o sistema antioxidante como forma de proteção ao estresse gerado pelo excesso de cobre. Com base nos resultados, pode-se concluir que as plantas de batata-doce foram capazes de tolerar a toxidez por Cu até a concentração de 0,082 mM.

Palavras-chave: Micronutriente, perfil mineral, sulfato de cobre, estresse

Introduction

Heavy metals occur naturally in the environment; however, their concentrations are often elevated as a result of various anthropogenic processes such as mining, smelting, application of pesticides and inorganic fertilizers, and atmospheric emissions (HARICHOVÁ et al., 2012). Environmental contamination by heavy metals has become a serious problem for plants, animals, aquatic life, and humans (HARICHOVÁ et al., 2012). Among the different toxic heavy metals in the environment, Cu is the most important and studied contaminant (CAMBROLLÉ et al., 2013).

At low concentrations, Cu is considered as an essential micronutrient for plants and as a constituent and activator of several enzymes (BURKHEAD et al., 2009). However, when in excess, Cu can negatively affect plant growth and metabolism (FIDALGO et al., 2013). Limits of Cu concentration in tissues vary among species and differentially affect metabolic requirements (BURKHEAD et al., 2009).

Excess Cu concentrations interfere directly with photosynthesis and can reduce electron transport and inhibit enzymes of the Calvin-Benson cycle or the rate of CO_2 assimilation (BURZYNSKI; ZUREK, 2007; SILVA et al., 2010). In addition to the damage to the photosynthetic process, high concentrations of Cu have been related to changes in pigment content and structure of the chloroplast (SHI-SHENG, 2007).

Excess Cu concentrations have been related to oxidative stress generation (SHI SHENG, 2007) and connected to the production of reactive oxygen species (ROS) in Fenton-like redox reactions (FIDALGO et al., 2013). ROS are generated as natural products of photosynthetic and aerobic metabolisms in plants. At low concentrations, ROS can serve as signaling molecules in the redox signal transduction pathway in plants (SILVA et al., 2010). However, plants are better able to protect themselves against homeostatic disturbance and cellular damage by inducing antioxidant enzymes as a general response to the toxic effects of heavy metals (BOOJAR; GOODARZI, 2007).

Changes in stomatal characteristics may result in tolerance to different environmental conditions such as drought, flooding, and stress due to metals such as Cu (PANOU-FILOTHEOU et al., 2001; RIBEIRO et al., 2012), promoting changes mainly in the stomata. Stomata are sensitive and important for plant growth and development. They have a prominent role in regulating both transpiration flow to the atmosphere and CO_2 flow into leaves (SILVA et al., 2005). Stomata may vary in shape, size, and quantity on the basis of abiotic factors that cause changes in photosynthetic conditions (ADAMSKI et al., 2012).

Plants that grow in environments with high Cu concentrations can develop defense mechanisms against toxicity (CAMBROLLÉ et al., 2013). The extent of such tolerance and the degree of adaptation are highly variable, and efficiency and capacity of detoxification mechanisms play an important role (BOOJAR; GOODARZI, 2007). In addition, a network of sequestration activities and immobilization functions regulate the uptake,

distribution, and detoxification of excess metal ions in plants (JANAS et al., 2010).

Sweet potato [*Ipomoea batatas* (L.) Lam.] is a vegetable known for its ease of cultivation, hardiness, adaptation to different soil types and climate, high tolerance to drought, high biomass, and low production cost (LOW et al., 2007; ANDRADE JÚNIOR et al., 2012). In addition to these characteristics, sweet potato has been used as a model plant in studies of plant nutrition and metabolism because of the ease of propagation and fast growth (ADAMSKI et al., 2011, 2012; FOLONI et al., 2013).

The objective of this study was to evaluate the physiological responses of sweet potato plants to different Cu concentrations by measuring growth parameters, chlorophyll content, antioxidant metabolism, mineral profile, and stomatal characteristics.

Materials and Methods

Sweet potato (*Ipomoea batatas* L. 'ILS19') plants were obtained from apical branches that were approximately 8 cm in length and had four leaves per branch of plants rooted for five days in distilled water in a greenhouse. After rooting, the plants were placed in a continuous-flow root-floating hydroponic system and were cultured using the Hoagland and Arnon (1938) complete nutrient solution for six days. The plants were then cultured in a nutrient solution for nine days at different Cu concentrations: 0.041, 0.082, and 0.164 mM in the form of $CuSO_4 \cdot 5H_2O$. Treatments were determined after preliminary experiments, which showed that cultivation in concentrations lower than those mentioned above did not cause changes in growth parameters, and the control concentration was set at 0.041 mM Cu. Every three days, the solution was renewed, and the pH was adjusted to ±5.8. At the end of the stress period, the plants were collected and evaluated for growth parameters, chlorophyll

content, antioxidant metabolism, mineral profile, and stomatal characteristics.

The following growth parameters were evaluated: branch length, determined with a metric ruler (cm); leaf number; fresh weight of shoot and root (mg); dry weight of shoot and root, measured with an analytical scale after drying the material in a forced ventilation oven at 70 °C ± 2 °C until a constant weight was achieved (mg); and leaf area, measured using a leaf area meter (LI-3000, LI-COR Inc., Lincoln, NE, USA) ($cm^2 \cdot plant^{-1}$). Chlorophyll content was estimated using a portable chlorophyll meter (CL-01, Hansatech, King's Lynn, Norfolk, UK), and the results were expressed using the chlorophyll index (CASSOL et al., 2008).

Antioxidant enzyme activities in young leaves and roots were determined. Approximately 200 mg of fresh leaf tissue from each sample was ground in liquid N_2 with 20% polyvinylpolypyrrolidone and homogenized in 1.8 mL of an extraction medium (100 mM potassium phosphate buffer, pH 7.8; 0.1 mM EDTA; and 20 mM ascorbic acid). The homogenate was centrifuged at 13,000 × *g* for 20 min (4 °C), and the supernatant was collected to determine enzymatic activity and quantify proteins by using the Bradford method (BRADFORD, 1976).

Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined on the basis of the ability of SOD to inhibit the photoreduction of nitroblue tetrazolium (NBT) (GIANNOPOLITIS; RIES, 1977) in a reaction medium (100 mM potassium phosphate, pH 7.8; 14 mM methionine; 0.1 μM EDTA; 75 μM NBT; and 2 μM riboflavin). One unit of SOD was considered as the amount of enzyme able to inhibit 50% NBT photoreduction under test conditions. The results were expressed in U mg^{-1} protein.

Ascorbate peroxidase activity (APX, EC 1.11.1.11) was evaluated according to the method described by Nakano and Asada (1981), that is, by monitoring the oxidation rate of ascorbate (ASA) at 290 nm. The incubation buffer was composed

of 100 mM potassium phosphate (pH 7), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and enzyme extract. The decrease in absorbance was monitored over a period of 1.5 min, and the results were expressed in µmol ASA min^{-1} mg^{-1} protein.

Catalase activity (CAT, EC 1.11.1.6) was determined as described by Azevedo et al. (1998). The enzyme extract was added to a reaction medium (100 mM potassium phosphate buffer, pH 7, and 12.5 mM H_2O_2), and the activity was indicated by decreased absorbance at 240 nm over a period of 1.5 min. The results were expressed in µmol H_2O_2 min^{-1}mg^{-1} protein.

The levels of macronutrients (N, Ca, and Mg) and micronutrients (Cu and Fe) were determined from the dry weights of leaves and roots, according to the methods reported by Tedesco et al. (1995). Nutrient levels were evaluated in leaves that had already been established before the Cu treatments (called "old leaves"), leaves that developed after the Cu treatments (called "young leaves"), and roots.

For stomatal evaluation, fully expanded young leaves from the second or third branch nodes were collected and fixed in Karnosky solution (RUZIN, 1999). Paradermal cuts were manually made in the middle third of the leaves and cleared in 5% sodium hypochlorite solution. Then, each section was stained with 0.05% toluidine blue solution in 0.1 M phosphate buffer (KRAUS; ARDUIN, 1997) and mounted on microscope slides in 50% glycerin. The sections were observed and photographed using a Zeiss Axiostar Plus optical microscope (Carl Zeiss, Light Microscopy, Gottingen, Germany) and a Sony digital camera, (model EX MPEG Movie, 3.3 megapixels). The images were digitized at 20× magnification. According Adamski et al. (2012), stomatal index (SI, percentage of stomata in relation to total epidermal cells) of the abaxial (Ab) and adaxial (Ad) epidermis in two areas for five plants per treatment was observed. For polar diameter (SPD, length of guard cells) and equatorial diameter (SED, width of guard cells), five plants

per treatment were evaluated and five stomata per plant were measured. To measure the dimensions of the stomata, Zeiss-Axio Vision 3.1 software was used. SI was calculated using the Cutter equation (CUTTER, 1986): SI = [SN/(ECN + SN)] × 100, where SI = stomatal index; SN = stomatal number; and ECN = epidermal cell numbers.

The experiment was performed using a completely randomized design that consisted of three doses of Cu (0.041, 0.082, and 0.164 mM) and five replicates (five plants per replicate). The results were subjected to analysis of variance, and the means were compared using Tukey's test at 5% significance with Winstat software (MACHADO; CONCEIÇÃO, 2003).

Results and Discussion

Although the sweet potato plants treated with different Cu concentrations survived the experiment, they showed some typical visible characteristics of Cu-induced toxicity. According to Cambrollé et al. (2012), chlorosis and growth inhibition are described as common symptoms in most plants grown at high Cu concentrations. In this study, plants grown at 0.041 and 0.082 mM Cu concentrations showed no difference in growth parameters; however, exposure to 0.164 mM Cu concentration caused a drastic decrease in the analyzed variables ($p < 0.05$; Figure 1). Branch length ($p = 0.0002$), leaf number ($p = 0.0005$), and leaf area ($p = 0.003$) of plants cultivated at 0.164 mM Cu concentration were 29, 56, and 21%, respectively, of the control values. Similarly, for fresh shoot weight ($p = 0.002$) and free root weight ($p = 0.01$) and dry shoot weight ($p = 0.02$) and dry root weight ($p = 0.005$), plants cultivated using the highest Cu concentration showed 19, 46, 24, and 40%, respectively, of the values observed for the control plants. These results show that the strategy of plants under stress due to high metal concentrations is to use tolerance mechanisms, limiting other processes such as growth (POSMYK et al., 2009; LARRÉ et al., 2014). Furthermore,

recent studies have shown a relationship between the biochemical phase of photosynthesis, which is highly sensitive to the effects of heavy metals, and the decrease in growth (SHI-SHENG, 2007).

Figure 1. Effect of copper (Cu) in sweet potato plants (*Ipomoea batatas* L.) on the following growth parameters: (A) Shoot length (cm); (B) Leaf number; (C) Leaf area (cm^2 $plant^{-1}$); (D) Chlorophyll index; (E) Shoot fresh weight (mg); (F) Root fresh weight (mg); (G) Shoot dry weight (mg); (H) Root dry weight (mg). Means followed by the same letter do not differ according to Tukey's test (p > 0.05).
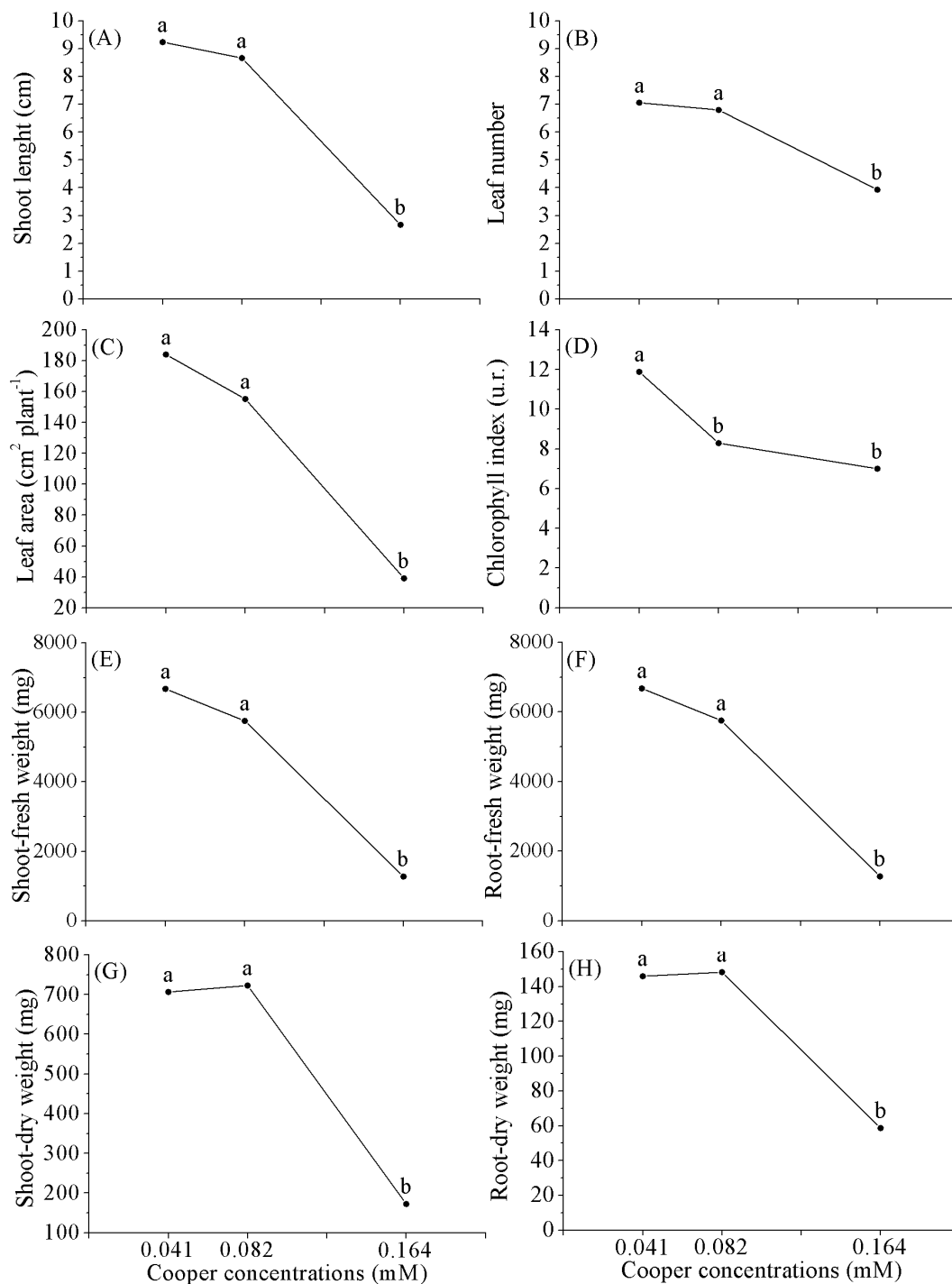
Leaf chlorosis was not visibly observed in the sweet potato plants; however, at the intracellular level, there was a decrease in the content of photosynthetic pigments; this effect may be associated with Cu toxicity (LIU et al., 2004; MOURATO et al., 2009). The highest Cu concentrations (0.082 and 0.164 mM) caused a significant decrease of 69% in chlorophyll content (p = 0.0001; Figure 1D). According to Xiong et al. (2006) and Adamski et al. (2011), chlorophyll biosynthesis is dependent on the supply of assimilated N and Fe, and substitution of the central Mg ion by Cu in the chlorophyll molecule causes damage to the chlorophyll-synthesizing system (CAMBROLLÉ et al., 2013). The mineral profile of the sweet potato plants (Figure 2) suggests that higher concentrations of Cu (0.082 and 0.164 mM) (1) considerably reduced the foliar concentration of some macronutrients, such as Mg, Ca, and N; (2) caused Fe immobilization in the roots and this micronutrient was probably not translocated to chlorophyll synthesis system in the leaves; and (3) led to the degradation of chlorophyll molecules (MATEOS-NARANJO et al., 2008).

Figure 2. Effect of copper (Cu) in sweet potato plants (*Ipomoea batatas* L.) on the following nutrients: (A) Nitrogen content (N); (B) Calcium content (Ca); (C) Magnesium content (Mg); (D) Copper content (Cu); (E) Iron content (Fe).
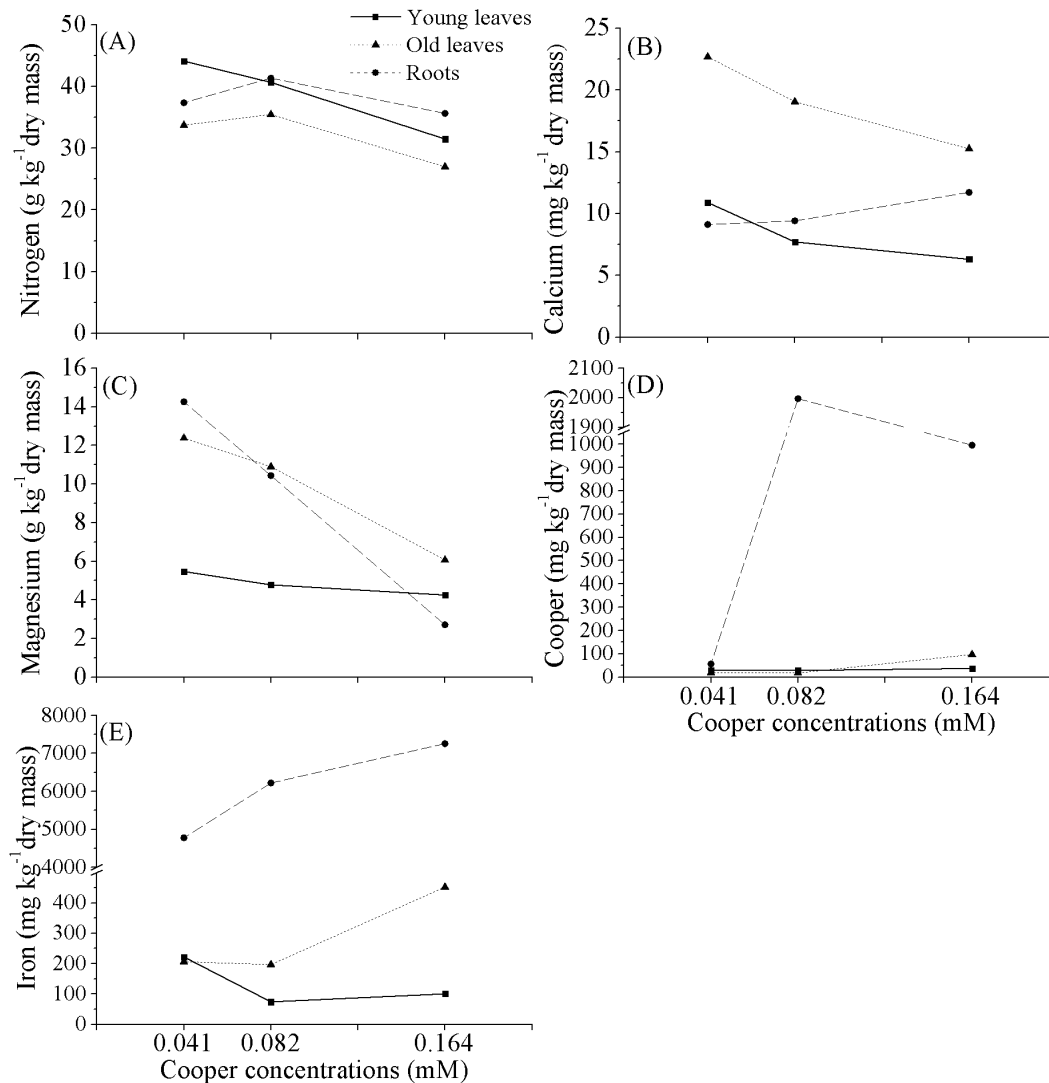
SI is a parameter that provides information on cell differentiation in the epidermal leaf surface (TICHÁ, 1982), and diameter of the stomata is directly related to the size of these structures; environmental conditions can influence these characteristics (RIBEIRO et al., 2012). Sweet potato leaves are characteristically amphistomatic and have paracytic stomata (GLÓRIA; GUERREIRO, 2006). In the adaxial epidermis,

Cu did not cause any stomatal changes ($p < 0.05$; Table 1). However, in the abaxial epidermis, SI was increased by 34% with an increase in Cu concentration ($p = 0.0003$). The increase in Cu concentration caused a decrease in polar and equatorial diameters of abaxial epidermis stomata ($p > 0.05$), reducing the polar diameter by about 19% at higher Cu concentrations and the equatorial diameter by 9% ($p = 0.01$).

Table 1. Stomatal index and diameter (mean ± SEM) of sweet potato leaves (*Ipomoea batatas* L.) cultivated at different copper (Cu) concentrations.

Characteristics	Cooper concentrations (mM)		
	ADAXIAL EPIDERMIS		
	0.041	0.082	0.164
SIAd [ns]	4.67 ± 0.39 a	5.56 ± 0.40 a	4.74 ± 0.38 a
SPDAd [ns]	19.74 ± 0.27 a	18.59 ± 0.38 a	18.31 ± 0.46 a
SEDAd [ns]	13.65 ± 0.13 a	13.45 ± 0.20 a	13.25 ± 0.35 a
	Cooper concentrations (mM)		
	ABAXIAL EPIDERMIS		
Characteristics	0.041	0.082	0.164
SIAb [**]	19.73 ± 1.52 b	29.32 ± 0.71 a	26 51± 1.10 a
SPDAb [**]	19.40 ± 0.30 a	16.95 ± 0.20 b	16.30 ± 0.42 b
SEDAb [**]	13.87 ± 0.24 a	13.34 ± 0.32 ab	12.67 ± 0.39 b

SIAd = stomatal index in the adaxial epidermis; SPDAd = stomatal polar diameter in the adaxial epidermis; SEDAd = stomatal equatorial diameter in the adaxial epidermis; SIAb = stomatal index in the abaxial epidermis; SPDAb = stomatal polar diameter in the abaxial epidermis; SEDAb = stomatal equatorial diameter in the abaxial epidermis; **, significant at 5% significance level. [ns] not significant.

High Cu concentrations resulted in a reduction in the size of stomata; conversely, the number of stomata increased. The opposite response was observed in leaves treated with 0.041 mM Cu. Studies have shown that the number of stomata is inversely proportional to the size of the guard cells, reflecting better control of the transpiration rate (PANOU-FILOTHEOU et al., 2001). According to Ribeiro et al. (2012), this response may be associated with increased stomatal functionality because the stomata become more elliptical. This behavior is often observed at high light intensity and low water availability. Thus, these results also indicate the presence of heavy metals during stomatal initiation,

development, and function (RIBEIRO et al., 2012).

Excess Cu concentrations can catalyze the generation of harmful reactive oxygen species (ROS) such as superoxide anion (O_2^{*-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^*), and they can damage biological molecules (DNA, RNA, and proteins) and cellular membranes. Ability of these antioxidases, SOD, CAT, and APX, to remove ROS, depending on the relationship between these enzymes, is the key factor in determining the resistance of plant cells to environmental stresses (SHI-SHENG, 2007). SOD, which is considered as the first line of antioxidant defense (MITTLER, 2002), has the function of dismutating O_2^{*-} in H_2O_2

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(MELONI et al., 2003). H_2O_2 is highly toxic and needs to be degraded by CAT and APX (MITTLER, 2002).

In the leaf tissues, activities of SOD and APX showed no significant difference between treatments (p > 0.05; Figure 3A and 3B). However, CAT enzyme activity at 0.164 mM Cu was 2.6 times higher than that at other Cu concentrations (p = 0.005; Figure 3C). CAT can eliminate H_2O_2 from plants by degradation to water and O_2, and it is mainly located in the peroxisomes. It is produced under oxidative stress conditions in the presence of heavy metals and metalloids (MITTLER, 2002; THOUNAOJAM et al., 2012). CAT activity can be related to the photorespiration cycle, which increases H_2O_2 content in peroxisomes, and this could justify an increase in the activity of this enzyme in the leaves. Photorespiration is considered as an alternative sink to help in consuming the reduction power (NADPH) generated by the electron transport chain in the chloroplast (WINGLER et al., 1999).

Figure 3. Effect of copper (Cu) in sweet potato plants (*Ipomoea batatas* L.) on antioxidant metabolism: (A) Superoxide dismutase (SOD); (B) Ascorbate peroxidase (APX); (C) Catalase (CAT). Means followed by the same lowercase letters do not differ according to Tukey's test at 5% error probability. Means followed by the same uppercase letters do not differ according to Tukey's test at 5% error probability.
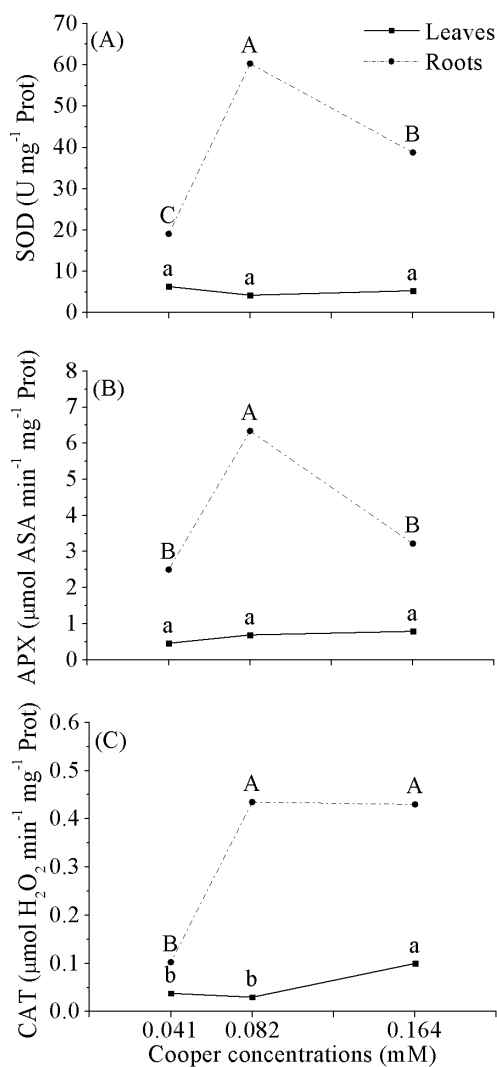
In the this study, activity of the antioxidant system was more pronounced in the root systems of the sweet potato plants than in the shoots (Figure 3), and this difference was mainly due to the low amounts of Cu found in the leaves when compared to that in the roots (Figure 2D). In the roots, SOD activity was 2.1 and 1.03 times greater at 0.082 mM and 0.164 mM Cu concentrations, respectively, than at 0.041 mM Cu (p = 0.0006; Figure 3A). APX activity at 0.082 mM Cu was 1.5 times greater than that at the lowest concentration observed, whereas plants grown at 0.164 mM Cu showed reduced APX activity, showing values equal to that observed in the control treatment (p = 0.001; Figure 3B). CAT activity in the roots grown at 0.082 and 0.164 mM Cu was increased 3.2 times (p = 0.01; Figure 3C).

A high amount of accumulated Cu in the roots induced oxidative stress, which may be proved by the increased activity of the antioxidant enzymes until 0.082 mM Cu. However, at higher concentrations (0.164 mM), SOD and APX activities (Figure 3A and 3B) were reduced and CAT activity (Figure 3C) remained constant. These results suggest that 0.082 mM can be considered as a toxic Cu concentration because the roots induced combined activity of antioxidant enzymes. Plants that received the highest dose of Cu (0.164 mM) possibly developed exclusion and accumulation mechanisms (JANAS et al., 2010), as suggested by the high Cu content found in old leaves (Figure 2D). According to Kabata-Pendias and Pendias (2001), Cu at adequate concentrations has low mobility in plants, and most of this metal appears to remain in the roots and old leaf tissues until senescence. Only small amounts of this metal may move to young organs. However, the high supply of Cu may increase transport to senescent leaves and remove excess of this micronutrient. The decrease in SOD and APX activities also suggests that the increase in Cu availability (0.164 mM) may have inhibited the expression of the enzymes involved in the antioxidant metabolism on a molecular level (MOURATO et al., 2009; POSMYK et al., 2009).

According to the results of this study, the roots were the main organ affected by the increased Cu availability in the solution. Root tissue, the first organ in contact with the metal, showed increased antioxidant enzyme activity. The roots were capable of carrying excess Cu to the shoots, probably because of increased Cu availability (0.164 mM). In the old leaves, there was exclusion of excess Cu. In the new leaves (Figure 3D), the nutrient caused a decrease in the chlorophyll index, changing the size of stomata and generating oxidative stress. The increase in CAT activity in the leaves occurred because of the formation of free H_2O_2 from the photorespiration process (Figure 3C). This alternative pathway consumes the reduction power (NADPH) generated by the electron transport chain and not used in carbon metabolism (BUSSOTTI et al., 2011). On the basis of impairment of the biochemical phase of photosynthesis, it can be assumed that excess Cu in the leaves would cause NADPH accumulation because of a (1) decrease in the photosynthetic carbon assimilation cycle because high Cu concentrations caused a decrease in growth (Figure 1) and, possibly, inhibition of enzyme activity in the Calvin-Benson cycle, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglyceric acid kinase (BURZYNSKI; ZUREK, 2007); (2) reduction and/or inhibition of Rubisco synthesis and activities of most enzymes related to nitrogen metabolism (such as nitrate reductase) because of the decrease in foliar N levels (CAMBROLLÉ et al., 2012); and (3) changes in the activity and structural maintenance of the photosynthetic apparatus as a result of a decrease in Mg and Ca content in the leaves (CAMBROLLÉ et al., 2013).

Conclusion

The sweet potato plants were able to tolerate Cu toxicity up to a specific concentration (0.082 mM). Beyond this concentration, excess Cu was transported from the roots to the shoots, causing changes in morphological and anatomical

characteristics and activating the antioxidant system because of the stress generated by excess Cu.

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