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Copper modulates the biochemical and enzymatic activity and growth of tomato cultivars grown *in vitro*

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Abstract. Copper (Cu) is a micronutrient that is neglected for tomato growth. This study sought to identify the effects of exposure to Cu on the growth and biochemical activity of two tomato cultivars. Tomato seeds of 'Carolina' and 'Cereja' cultivars were disinfected and inoculated in MS medium plus copper sulfate concentrations ($CuSO_4$) (default MS, 25, 50, and 100 µm) and had their growth monitored for 30 days. It was estimated that the growth and biomass accumulation of tomato plants 'Carolina' and 'Cereja', both from the aerial part and the roots, were benefited by 25 e 50 µm of $CuSO_4$. However, it was observed that these concentrations were inefficient in controlling hyperhydricity and leaf deformation. There was a reduction of these phenomena in the treatment with 100 µm, in both cultivars. Tomato of 'Carolina' cultivar subjected to 100 µm showed an increase in anthocyanins and superoxide dismutase (SOD) activity in the root system. There was a reduction of catalase (CAT) activity in shoots exposed to Cu. 'Cereja' tomatoes subjected to 100 µm showed an increase in CAT and SOD activity in shoots and roots, respectively. It was concluded that the 'Carolina' and 'Cereja' tomatoes have their growth impaired when exposed to 100 µm $CuSO_4$. Concentrations higher than 50 µm of $CuSO_4$ cause an increase in the antioxidant activity in the shoot of tomato plants from the 'Carolina' cultivar. Concentrations higher than 50 µm $CuSO_4$ increase SOD activity in the root system of tomato plants from the 'Cereja' cultivar.

Key words: *Solanum lycopersicum* L., antioxidant, hyperhydricity, anthocyanins, SOD.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a popular fruit vegetable around the world. Brazil allocated about 57,717 acres to produce this crop and produced 4,004.991 tons of this fruit in the 2019 harvest (IBGE, 2019). However, it has not been quantified how much of this production was directed to grape tomatoes. This fruit class has high added value, as it is used as a culinary ornament and has a large amount of sugar (Maia et al., 2013). The importance of this fruit is mainly due to its nutritional value, which has polyphenols, flavonoids, tannins, vitamin C, and anthocyanins as functional compounds (Butt et al., 2008; Maia et al., 2013). This, combined with the rusticity of the plants,

which may show determined or indeterminate growth, makes it a great option for family farming (Brasil, 2018).

In this case, the Cereja and Carolina cultivars have little cultivation information. The Carolina cultivar has indeterminate growth, moderate resistance to diseases, tasty and sweet fruits. The Cereja cultivar, on the other hand, shows vigorous and determined growth, and it is indicated for planting in pots or the garden. Its fruits are sweet. Studies indicate that this fruit consumption can reduce the risk of some diseases whose reactive oxygen species (ROS) are the causative agents. This is due to the high concentration of antioxidant compounds present in this fruit (Arredondo et al., 2016).

Several factors limit the yield of tomatoes; among them, the excessive use of chemical products stands out, with around 36 applications per crop (Dossa & Fuchs, 2017), and a large number of infectious diseases (Lopes & De Ávila, 2005). Another factor is nutritional neglect, especially regarding micronutrients (Da Silva et al., 2006). The absence of a specific fertilization recommendation for tomatoes does not exploit its maximum production potential. Although required in smaller quantities, micronutrients are essential for the proper development of plants, and their imbalance can cause disturbances in plant growth and development (Graham et al., 2001).

Copper (Cu) is an essential micronutrient for cellular processes such as photosynthesis, mitochondrial respiration, and a role in the metabolism of carbon and nitrogen (Hänsch & Mendel, 2009). It is also a protein constituent and is required by more than 30 enzymes, most of which are catalysts for redox reactions (Yruela, 2005; Epstein & Bloom, 2006). The deficiency of this ion can alter the plant architecture, impairing the growth of the plant or giving rise to malformed plants (Yruela, 2005; Yruela, 2009). On the other hand, high concentrations of Cu can also cause reduced growth and impair plant development, as it is a metal with toxic potential (Adrees et al., 2015).

Cupric toxicity causes the formation of reactive oxygen species (ROS) (Nagajyoti et al., 2010), and, consequently, the plant tends to increase the activity of antioxidant enzymes to avoid oxidative damage and maintain cell balance (Wang et al., 2004). Studies that test the tolerance of species to exposure to Cu have been carried out over the years (Drazkiewicz et al., 2004; Xu et al., 2005; Benimeli et al., 2010; İşeri et al., 2011; Azmat & Riaz, 2012; Barbosa et al., 2013), and have found that besides the species, cultivars also have different levels of metal tolerance. Although studies on *in vitro* cultivation have been concerned with defining the interaction between nutrients and plant growth, reports on the nutritional influence on the biochemical activity of tomato plants in this condition are still lacking. Once the functions of Cu are highlighted, it would be interesting to determine the biochemical changes due the exposure to this metal cause in plants.

The determination of biochemical variations caused by the exposure of tomato cultivars to copper will assist in the nutritional process, allowing the plant to reach its maximum growth potential. It is assumed that minimal Cu concentrations will benefit plant growth (Hristozkova et al., 2006). Still, that excess Cu will hinder shoot growth and mass accumulation in general (Adrees et al., 2015). It is also assumed that Cu will cause oxidative stress in the shoots, which will increase the antioxidant activity, either by the activity of enzymes with antioxidant potentials, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) or by the synthesis of phenolic compounds (Nagajyoti et al., 2010).

Therefore, this study sought to determine the morphological, biochemical, and enzymatic changes in tomato cultivars grown *in vitro* under $CuSO_4$ concentrations.

MATERIAL AND METHODS

Plant material and cultivation conditions

Seeds of the cultivar 'Cereja' originated from the United States of America were purchased commercially (Hortiseres® seeds), lot no. 18000211, with 88% germination and 99% purity. 'Carolina' tomato seeds were purchased commercially (Feltrin® seeds) lot no. 0069701610000280, with 99% germination and 99.9% purity. The seeds were subjected to an asepsis process in 2% sodium hypochlorite solution for 10 minutes, with agitation, and subsequently, washed four times in a row with sterile distilled water.

The Murashige & Skoog (1962) MS culture medium (100%) was supplemented with 30 g L^{-1} of sucrose, 6.5 g L^{-1} of agar (Kasvi®), and pH adjusted to 5.8. Copper sulfate ($CuSO_4$) was added to the medium in three concentrations. The control treatment was composed by the MS medium (default) in its full strength (0.1 µm of $CuSO_4$), and the subsequent treatments were composed by the MS medium in its full strength plus 25, 50, and 100 µm of $CuSO_4$. Subsequently, 50 mL of medium was poured into glass flasks with a capacity of 350 mL. These flasks were autoclaved for 20 minutes at 121 °C.

After asepsis, each flask was inoculated with two seeds of the same cultivar. The flasks were sealed with a plastic cover and polyvinylpyrrolidone film (PVP) and kept in a controlled growth chamber for 30 days, with a 24-hour light period through the use of light emitter diodes (LEDs) Blumenau®, LED T8 10W 6,000K, 100-240V-50/60Hz, power factor ≥ 0.92 (High PF), at a temperature of 25 °C ± 2 °C and light intensity of 72.02 µmol m^{-2} s^{-1}. A completely randomized design was used in the trial; 20 replications per treatment were used, totaling 80 plots per cultivar.

Measurement of plant growth

At 30 days after inoculation, the following variables were measured: length of the shoot (cm) and root (mm), the fresh weight of shoot (g) and roots (g), dry weight of shoot (g) and roots (g), and the number of leaves. Evaluations were made with the aid of a digital caliper and analytical balance to measure the average among all shoots of each treatment.

Physiological disorders observed during *in vitro* culture

During the period in which the plants remained in the growth chamber, three evaluations were carried out at 10-day intervals to measure the percentage of the presence of shoots with deformed leaves and hyperhydric.

Total chlorophylls, flavonoids, and anthocyanins

The total chlorophyll, flavonoid, and anthocyanin indexes were measured in fresh leaves and obtained with the aid of the DUALEX SCIENTIFIC+™ equipment, Quick Start model (FORCE A®, France, Paris) according to the manufacturer's instructions, with four replicates per treatment, on the first expanded leaves.

Total phenolic compounds measured in leaves

Fresh leaves (100 mg) were weighed in falcon tubes to obtain the extract, obtained according to Waterhouse (2002). The determination of the total phenolic content was

carried out by the method proposed by Kuskoski et al. (2005). The readings were performed on a 700 plus - Femto spectrophotometer with a wavelength of 750 nm, and the results were expressed in mg of gallic acid equivalents per 100 g of the sample (mg AGE per 100 g). Triplicates were performed with three biological repetitions, and the calculation was performed using the calibration curve of gallic acid $y = 0.0004x + 0.0003$, whose $R^2 = 0.9327$.

Antioxidant activity measured in leaves - DPPH

From the extract obtained to determine total phenolic compounds, antioxidant activity was determined based on the extinction of the absorption of the radical 2.2-diphenyl-1-picryl hydrazine (DPPH 60 μM), proposed by Rufino et al. (2009). The readings were taken on the 700 plus - Femto spectrophotometer with a wavelength of 515 nm. They were monitored every 30 minutes, in a total of three readings, in which the reduction in absorbance was observed until its stabilization. Samples were evaluated in triplicate, with three biological replicates. The results were expressed as a percentage of free radical sequestration (% FRS), according to the equation:

$$\%FRS = \frac{(CA + SA)}{SA} \times 100 \quad (1)$$

where CA = Control Absorbance; SA = Sample Absorbance.

Enzymatic evaluation

The enzymatic extract was obtained (Bonacina et al., 2017) from fresh leaves and roots. All enzymes were evaluated using 96-well flat-bottomed Elisa plates. Biochemical tests were performed in three biological replicates. The equipment used was the UV-VIS spectrophotometer, Spectramax Plus, with SoftMax Pro 6.5.1 software.

SOD (EC 1.15.1.1)

The activity of superoxide dismutase (SOD) was measured by its ability to inhibit nitroblue tetrazolium (NBT) photoreduction, as described by Giannopolitis & Ries (1977). The reading was performed at 560 nm, in which a unit of SOD activity (U) was defined as the amount of enzyme needed to inhibit 50% of reduction in NBT. SOD activity was expressed in U SOD mg^{-1} FM min^{-1}.

CAT (EC 1.11.1.6)

The activity of catalase (CAT) was carried out according to the methodology proposed by Havir & McHale (1987). The degradation of H_2O_2 determined it in 3 minutes at 260 nm. The enzymatic activity was quantified using the molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al., 1995) and expressed in mmol H_2O_2 g^{-1} FM min^{-1}.

APX (EC 1.11.1.11)

The activity of ascorbate peroxidase (APX) was carried out as proposed by Nakano & Asada (1981). The degradation of H_2O_2 determined the activity of this enzyme in 3 minutes at 290 nm. The enzymatic activity was quantified, using the molar extinction coefficient of 2.8 mm^{-1} cm^{-1} (Nakano & Asada, 1981) and expressed in mmol ascorbic acid g^{-1} FM min^{-1}.

Statistical analysis

The data from the growth characteristics were submitted to polynomial regression using the SISVAR 5.6 software (Ferreira, 2011). The maximum values of the cubic equations generated by the polynomial regression were calculated using the equation proposed by Ferreira (2011). When the quadratic model was adopted, the maximum values were obtained through the derivative of the equation.

The data of total chlorophylls, anthocyanins, flavonoids, antioxidant activity, phenolic compounds, and activity of the enzymes SOD, CAT, and APX were submitted to analysis of normality by the Shapiro Wilk test ($p \leq 0.05$) and variance (ANOVA) at $p \leq 0.05$. The means were compared using the *Tukey test* ($p \leq 0.05$), using the SISVAR 5.6 software (Ferreira, 2011). In periodic evaluations, data were plotted in Microsoft Excel, and graphs were constructed with the percentage of variables evaluated at 10, 20, and 30 days of *in vitro* culture. The data were transformed in $ArcoSeno\left[\sqrt{\frac{x}{100}}\right]$.

RESULTS AND DISCUSSION

Copper in initial plant growth

In the 'Carolina' cultivar, the highest average shoot length occurred in the treatment with 25 µm (14.91 cm), which corresponded to a 21.8% increase compared to the control treatment. The lowest average of this variable was observed in the treatment with 100 µm (6.1 cm), where there was a 59.1% reduction compared to the highest average obtained. The averages obtained in the treatment with 50 µm did not differ statistically from the control and 25 µm treatments (Fig. 1, A). There were no significant differences between the control, 25, and 50 µm treatments for the length of the root system, whose average was 102.17 mm. However, in the treatment with 100 µm, there was a reduction of 69.85% in the root length compared to the average of the treatments described previously (Fig. 1, B).

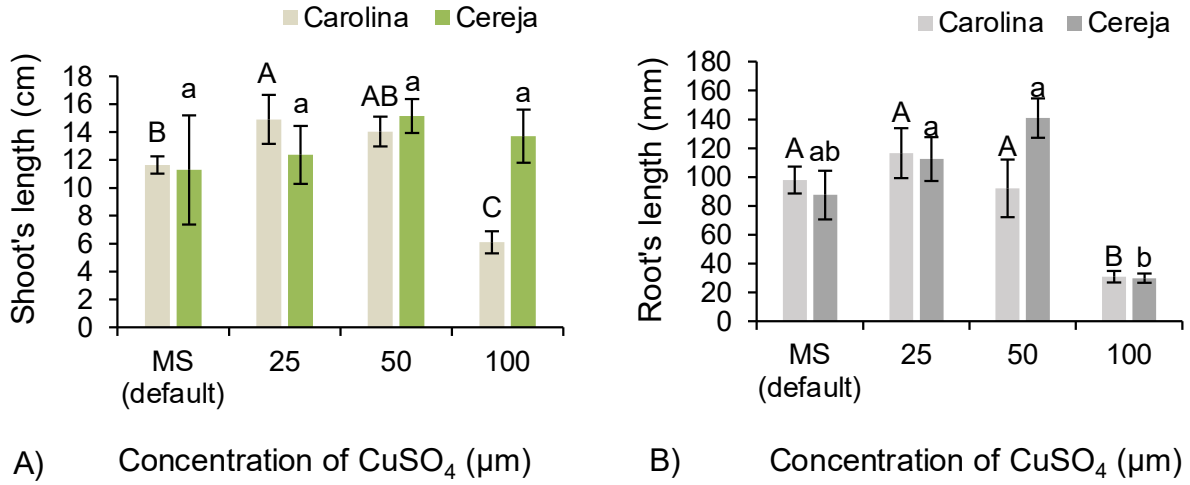

Figure 1. Length of shoots (A) and roots (B) of *Solanum lycopersicum* L. 'Carolina' and 'Cereja' exposed to $CuSO_4$ concentrations; (n = 20), *Bar* means ± standard deviation followed by the same uppercase letter and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

There was no significant difference in the shoot length of the 'Cereja' cultivar (Fig. 1, A). There was no significant difference in control, 25, and 50 µm treatments in the root system, whose average was 113.61 mm. There were also no significant differences between the control and 100 µm treatments, with an average of 58.69 mm. However, a 78.7% reduction in root length was observed when the averages obtained at 50 and 100 µm were compared (Fig. 1, B).

The increase in plant growth variables corroborated the hypothesis that Cu acts as a micronutrient. This ion is a constituent of proteins, required for the normal functioning of more than 30 enzymes (Epstein & Bloom, 2006), such as enzymes that catalyze redox reactions (Yruela, 2005) and enzymes involved in the initial processes of nitrate assimilation (Hristozkova et al., 2006). Its lack can impair the use of the nitrogen (N) absorbed and, consequently, disturb the plant growth (Yruela, 2005; Yruela, 2009). Drazkiewicz et al. (2004) concluded that *Arabidopsis thaliana* tends to accumulate higher amounts of Cu in the root system and lower amounts in the aerial part. Wójcik & Tukiendorf (2003) stated that this phenomenon functioned as a plant defense system, which seeks to delay or even prevent the metal from damaging the shoot. Therefore, the roots suffer more severe damage, both in terms of length and accumulation of mass.

Cu toxicity can reduce the growth of the shoot and roots, preventing cell elongation and/or decreasing the absorption of other minerals (Adrees, 2015). This may result from a variation in the synthesis of some plant hormones, such as abscisic acid (ABA) and jasmonic acid (JA). According to Wilkinson & Davies (2010), the increase in ABA concentration causes stomatal closure, thus reducing the nutrient transition flow. JA, in turn, is considered a molecular sign of the activation of the plant defense apparatus, causing growth retardation (Maksymiec & Krupa, 2007). In their study with hydroponic maize, Reckova et al. (2019) observed an increase in the hormones previously described due to the increase in the concentration of $CuSO_4$ in the nutrient solution.

The comparison of means revealed no significant differences in the fresh weight of 'Carolina' shoots among control, 25, and 50 µm treatments. There were also no significant differences among 25, 50, and 100 µm treatments. However, comparing the average obtained in control (1.41 g) with that obtained at 100 µm (0.19 g), there is a reduction of 86.5% in the accumulation of the fresh weight of shoots (Fig. 2, A). In the root system of 'Carolina', there were no significant differences between treatments with 25 and 50 µm, whose average was 1.27 g. A reduction of 78.7% was observed in this variable when we compared the average previously described with that obtained in the treatment with 100 µm (0.02 g). The control and 100 µm treatments had no significant differences (Fig. 2, B).

In the shoots of 'Cereja', there was a 79.1% reduction in fresh weight comparing the control (1.34 g) and the treatment with 100 µm (0.28 g) (Fig. 2, A). The comparison of means indicated no significant differences between treatments with 25 and 50 µm (1.04 g) for root fresh weight. However, a reduction of 96.2% in the fresh weight of the roots was observed when we compared the average previously described with that obtained in the treatment with 100 µm (0.04 g) (Fig. 2, B).

For the dry weight of shoots, the comparison of means showed no significant differences in the 'Carolina' cultivar according to Cu concentration (Fig. 2, C). For the dry weight of the root system, there were no significant differences among control, 25, and 50 µm treatments, whose average was 0.033 g. However, comparing this average to

that obtained at 100 µm, there was a reduction of 99.4% in the accumulation of the root dry weight (Fig. 2, D).

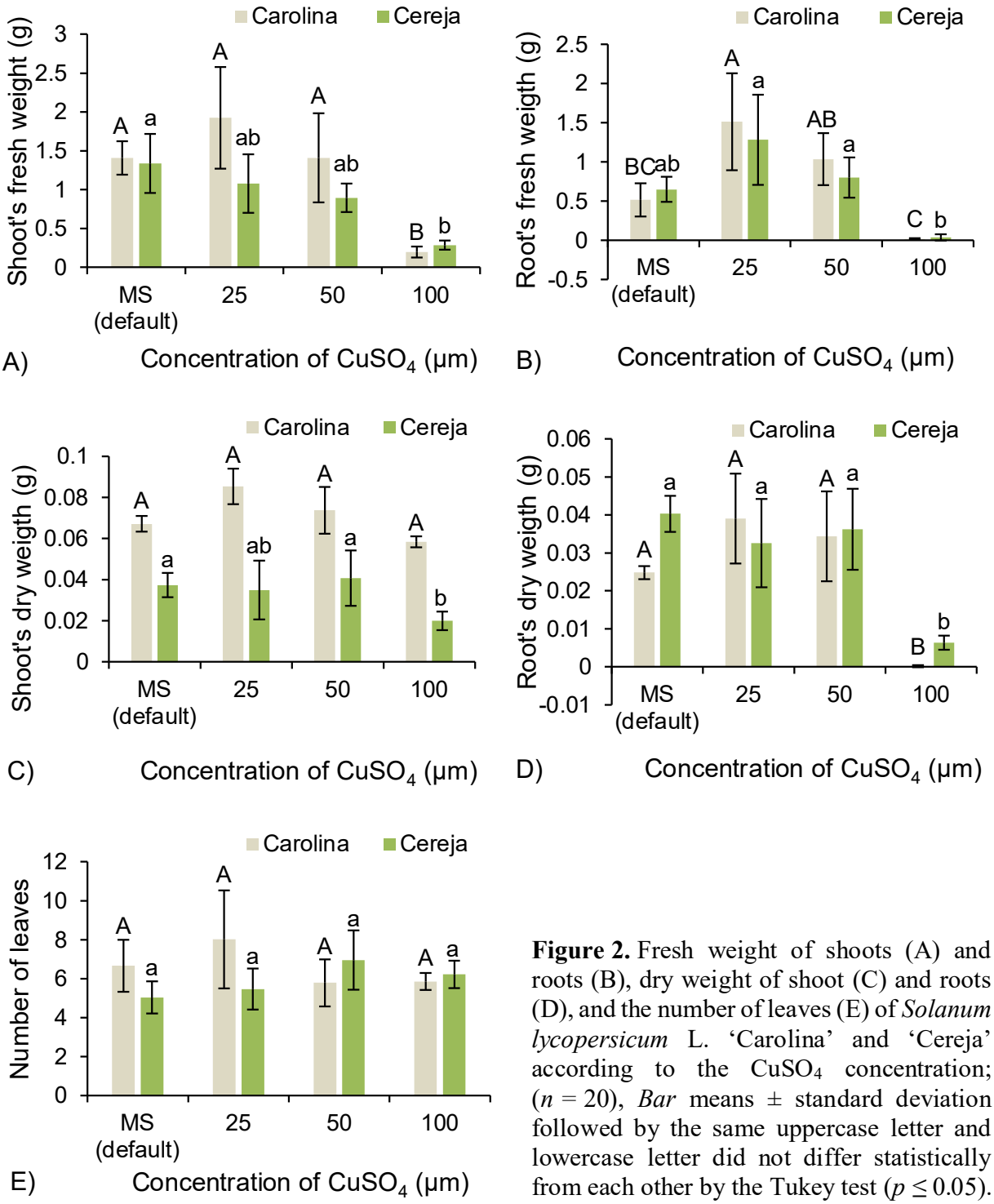

Figure 2. Fresh weight of shoots (A) and roots (B), dry weight of shoot (C) and roots (D), and the number of leaves (E) of *Solanum lycopersicum* L. 'Carolina' and 'Cereja' according to the $CuSO_4$ concentration; (n = 20), *Bar* means ± standard deviation followed by the same uppercase letter and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

In the 'Cereja' cultivar, the highest average of the dry weight of shoots was observed at 50 µm (0.04 g), not differing statistically from the control and 25 µm. However, this average was 50% higher than the average obtained in 100 µm (Fig. 2, C). There were no statistical differences among control, 25, and 50 µm treatments for the root dry weight, whose average was 0.04 g. The average observed at 100 µm was 85% less than previously described, with 0.006 g (Fig. 2, D).

The reduction of biomass is one of the dominant effects of excess Cu (Adrees, 2015). The negative influence of Cu on the accumulation of dry weight was observed in 'Cereja' tomato, both in the shoot and roots; only the roots of 'Carolina' cultivar suffered this effect.

The data obtained in this work corroborate this statement and the study by Benimeli et al. (2010), in which the application of Cu concentrations greater than 10 µm in the maize nutrient solution (Cargil 350 Hybrid) decreased both fresh and dry mass. Dresler et al. (2014) and Azooz et al. (2012) observed that the excess of this metal decreased the fresh weight of roots and shoots of corn and wheat shoots, respectively. This reduction in biomass accumulation may be related to decreased nutrient absorption caused by cupric stress (Adrees, 2015).

There were no significant changes in the number of leaves of shoots from the 'Carolina' and 'Cereja' tomato cultivars due to the increase in $CuSO_4$ concentration (Fig. 2, E).

Development of deformed leaves during *in vitro* cultivation 'Carolina'

In the first evaluation, carried out at ten days after inoculation, we observed that 10% of shoots in the control treatment (default MS) had twisted and thin leaves, with a translucent aspect (Fig. 3). The treatments with 25 and 50 µm $CuSO_4$ did not show shoots with this disorder, and the treatment with 100 µm $CuSO_4$ had 5% of shoots with deformed leaves (Fig. 3). At 20 days of cultivation, in the control treatment, 15% of shoots had deformed leaves; in treatments with 25 and 50 µm of $CuSO_4$, 25% and 20% of shoots had this disorder, respectively. Treatment with 100 µm continued with 5% of shoots with deformed leaves (Fig. 3).

At 30 days of cultivation, 45% of shoots in the control treatment (MS default) showed deformed leaves. Treatments with 25 and 50 µm $CuSO_4$ had 40% and 35% of the shoots with deformed leaves, respectively, followed by the treatment with 100 µm of $CuSO_4$, in which only 5% of the shoots presented such deformity (Fig. 3).

Based on the Tukey test ($p \leq 0.05$), there were no significant differences in the percentage of shoots with deformed leaves according to the $CuSO_4$ concentration in any of the evaluations performed (Fig. 3).

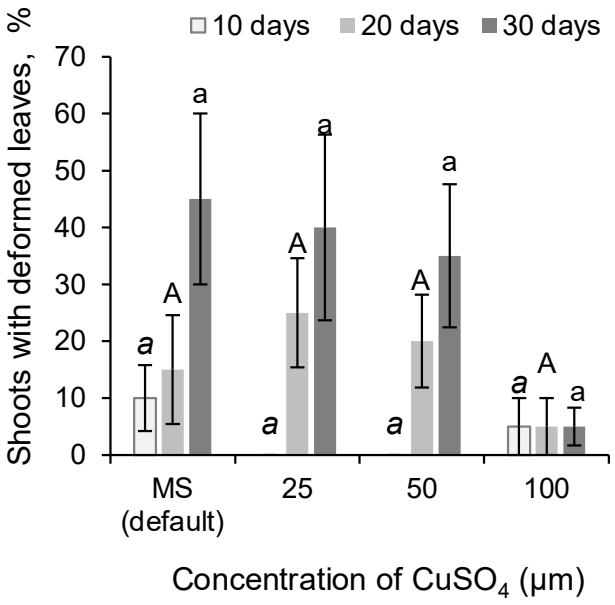

Figure 3. Percentage of *Solanum lycopersicum* L. 'Carolina' with deformed leaves according to the in vitro cultivation days and $CuSO_4$ concentration; (n = 20). *Bar* means ± standard error followed by the same italic letter, uppercase letter, and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

'Cereja'

In the first ten days of cultivation, 20% and 30% of the shoots from the control treatment and 50 µm $CuSO_4$, respectively, presented twisted and pointed leaves with and vitreous aspect. Shoots grown in medium with 25 µm of $CuSO_4$ did not present leaf deformation in the first ten days (Fig. 4). However, at 20 days of cultivation, this treatment resulted in 40% of shoots with deformed leaves, and control treatment and 50 µm $CuSO_4$ both presented 60% of the shoots with this disorder (Fig. 4).

At 30 days of cultivation, the control treatment and the concentrations of 25 and 50 µm of $CuSO_4$ presented 70%, 65%, and 70% of the shoots with deformed leaves, respectively (Fig. 4). Shoots exposed to 100 µm $CuSO_4$, in turn, were slow to present this symptom, which occurred in only 5% of them, at 30 days of cultivation (Fig. 4).

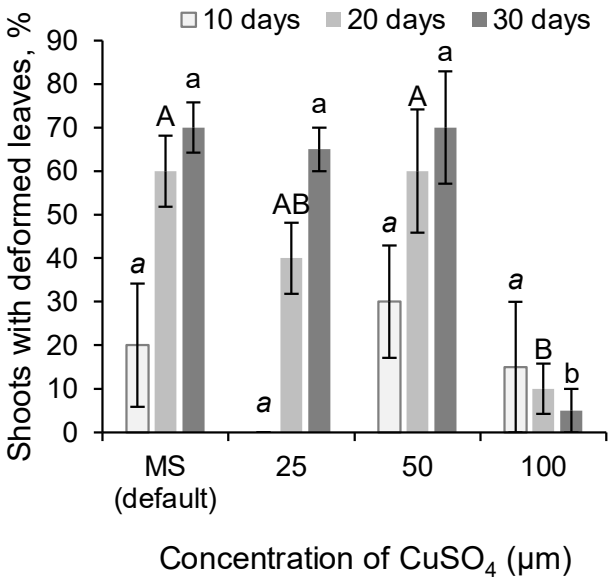

Figure 4. Percentage of *Solanum lycopersicum* L. 'Cereja' with deformed leaves at 10, 20, and 30 days of in vitro cultivation; (n = 20). Bar means ± standard error followed by the same italic letter, uppercase letter, and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

According to the comparison of means, there were no significant changes in the percentage of shoots with deformed leaves at 10 days after inoculation. At 20 days, it was found that treatments with 25 and 100 µm did not show significant differences between them. However, the treatment with 100 µm showed significantly fewer seeds with deformed leaves than control and 50 µm treatments. At 30 days after inoculation, it was found that the treatment with 100 µm obtained, statistically, a lower percentage of shoots with deformed leaves (Fig. 4).

According to Broadley (2012), Cu deficiency can alter the leaf construction, giving rise to pointed and twisted leaves, besides reducing the leaf area. Yruela (2005) stated that the Cu deficit impairs leaf construction, possibly due to less lignin biosynthesis, as described by Liu et al. (2018). The results obtained in this work corroborate the statements of Broadley (2012) and Yruela (2005; 2009) since the highest occurrence of deformed leaves occurred in the treatments with the three lowest concentrations of Cu. Lignin synthesis may have been compromised in these treatments. In treatment with 100 µm $CuSO_4$, only 5% of the shoots developed deformed leaves. Our results provide evidence that an increase in lignin synthesis may have occurred, which has reduced the occurrence of deformed leaves.

Hyperhydricity observed during the *in vitro* cultivation 'Carolina'

In the first ten days of cultivation, the control and 100 µm $CuSO_4$ treatments showed 15% and 5% of hyperhydric shoots, respectively (Fig. 5). This disturbance was only observed at 20 days after inoculation in the concentrations of 25 and 50 µm $CuSO_4$. In this situation, 20% of the shoots had this symptom. Even at 20 days after inoculation,

45% of the control shoots were hyperhydric, and, interestingly, the treatment with 100 µm of Cu did not have shoots with this disorder (Fig. 5).

At 30 days of cultivation, shoots submitted to 100 µm of $CuSO_4$ did not present this disorder. The control treatment showed 55% of hyperhydric shoots. Treatments with 25 and 50 µm $CuSO_4$ had 40% and 35% of hyperhydric shoots, respectively (Fig. 5).

According to the means comparison test, at ten days after inoculation, the treatment with 100 µm obtained a significantly lower percentage of hyperhydric shoots than the control treatment. At 20 and 30 days after inoculation, this same treatment showed a statistically lower percentage of shoots with this disorder than other treatments (Fig. 5).

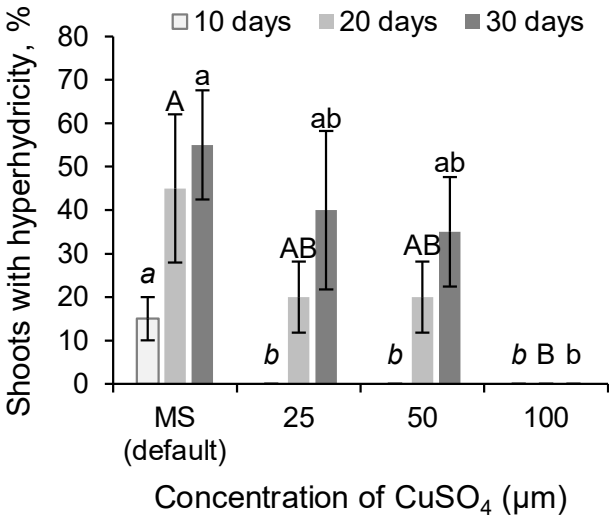

Figure 5. Percentage of hyperhydric shoots of *Solanum lycopersicum* L. 'Carolina' over 30 days of in vitro cultivation according to the $CuSO_4$ concentration; (n = 20) Bar means ± standard error followed by the same italic letter, uppercase letter, and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

'Cereja'

At ten days of cultivation, it was possible to observe in the treatment with 50 µm of $CuSO_4$ that 25% of the shoots had a translucent green stem. However, in other treatments, this phenomenon had not yet occurred (Fig. 6). When the shoots completed 20 days of cultivation, there was a large increase in the number of hyperhydric shoots. It was evaluated that 60% of shoots in the control treatment, 25% of the shoots in the treatment 25 µm, 45% in the treatment 50 µm, and 5% of shoots in the treatment 100 µm presented this disorder (Fig. 6).

At 30 days of cultivation, we observed that the control, 25, and 50 µm of $CuSO_4$ had 70%, 65%, and 75% of the shoots with hyperhydricity, respectively. Treatment with 100 µm of $CuSO_4$ maintained the percentage of hyperhydric shoots; that is, there was no development of this disorder between 20 and 30 days of cultivation (Fig. 6).

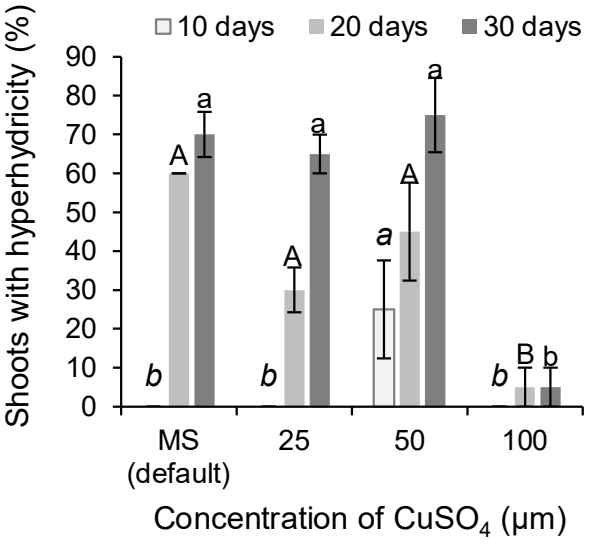

Figure 6. Percentage of hyperhydric shoots of *Solanum lycopersicum* L. 'Cereja' over the days of *in vitro* cultivation; (n = 20). Bar means ± standard error followed by the same italic letter, uppercase letter, and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

According to the Tukey test ($p \leq 0.05$), the treatment with 50 µm showed a higher percentage of hyperhydric shoots than the other treatments ten days after inoculation. At 20 and 30 days after inoculation, treatment with 100 µm showed a significantly lower percentage of shoots with such disorder (Fig. 6).

Hyperhydricity is a metabolic disorder common in *in vitro* culture. The conditions of this type of cultivation can trigger this disturbance, such as, for example, the concentration of salts in the medium, the constant temperature, the high relative humidity in the cultivation container, among others (De Vasconcelos et al., 2012). Among the characteristics of hyperhydric plants, there is the excessive accumulation of water in the tissues, the swelling of the tissues of the shoot, such as the stem and leaves, vitreous aspect, low lignin rate in the cell wall, and low accumulation of biomass (Kevers et al., 2004).

In the treatment with 100 µm of $CuSO_4$, smaller shoots with better architecture were observed in both genotypes. According to Zeng et al. (2016), hydrogen peroxide (H_2O_2) formed by cupric stress is involved in the polymerization of lignin and its accumulation in the cell wall. This may have justified the drop in the percentage of hyperhydric shoots due to the increase in the concentration of $CuSO_4$ and leads us to believe that in Cu concentrations below 100 µm, there was not enough lignin synthesis to control hyperhydricity.

Physiological changes due to copper

In 'Carolina' plants, the indexes of total chlorophylls (Fig. 7, A) and flavonoids (Fig. 7, B) were constant in all concentrations of $CuSO_4$, with averages of 7.29 and 0.22, respectively. Shoots exposed to 100 µm of $CuSO_4$ showed an increase of anthocyanins of 49.4% compared to the control treatment; 39.28% in the treatment with 25 µm $CuSO_4$ and 46.42% in the treatment with 50 µm $CuSO_4$ (Fig. 7, C).

In 'Cereja', the total chlorophylls, flavonoids, and anthocyanins were not affected by the $CuSO_4$ concentration. Average levels of 12.75 (Fig. 7, A), 0.226 (Fig. 7, B), and 0.162 (Fig. 7, C) were observed, respectively.

Anthocyanins and total phenolic compounds are part of the metabolism responsible for reducing ROS, mostly serving as a substrate for potential redox enzymes (Juadjur et al., 2015). In the 'Cereja' cultivar, there was no change in the concentration of these compounds due to an increase in the Cu concentration to which shoots were exposed against the initial hypothesis that oxidative damage caused by Cu would intensify the antioxidant activity of the plant. Drazkiewicz et al. (2004) stated that, under oxidative stress conditions, not all the antioxidant apparatus is activated. Thus, there may be an overload of a certain metabolism and, consequently, a reduction in the intensity of other metabolic routes. For example, the increase in the intensity of the route responsible for the enzymatic activity can occur simultaneously with reduced synthesis of anthocyanins and phenolic compounds.

The 'Carolina' tomato increased the synthesis of anthocyanins when the shoots were exposed to 100 µm of $CuSO_4$. According to Juadjur et al. (2015), anthocyanins have redox potential in *in vitro* conditions. More than any other phenolic compound, they contribute to the degradation of H_2O_2 (Silva et al., 2010), as they are substrates for the peroxidases existing inside the vacuole. Thus, it is assumed that shoots exposed to 100 µm $CuSO_4$ increased the production of anthocyanins to control the H_2O_2 molecules generated by copper toxicity.

Biochemical variations due to copper

'Carolina' lowest average of phenolic compounds was obtained in shoots exposed to 50 µm $CuSO_4$, with 12,534.09 mg AGE per 100 g. The rest of the treatments did not differ statistically, with an average of 14,033.58 mg AGE per 100 g (Fig. 7, D). 'Cereja' synthesis of phenolic compounds did not show sensitivity to the Cu concentrations studied here, maintaining an average of 16,747.35 mg AGE per 100 g (Fig. 7, D).

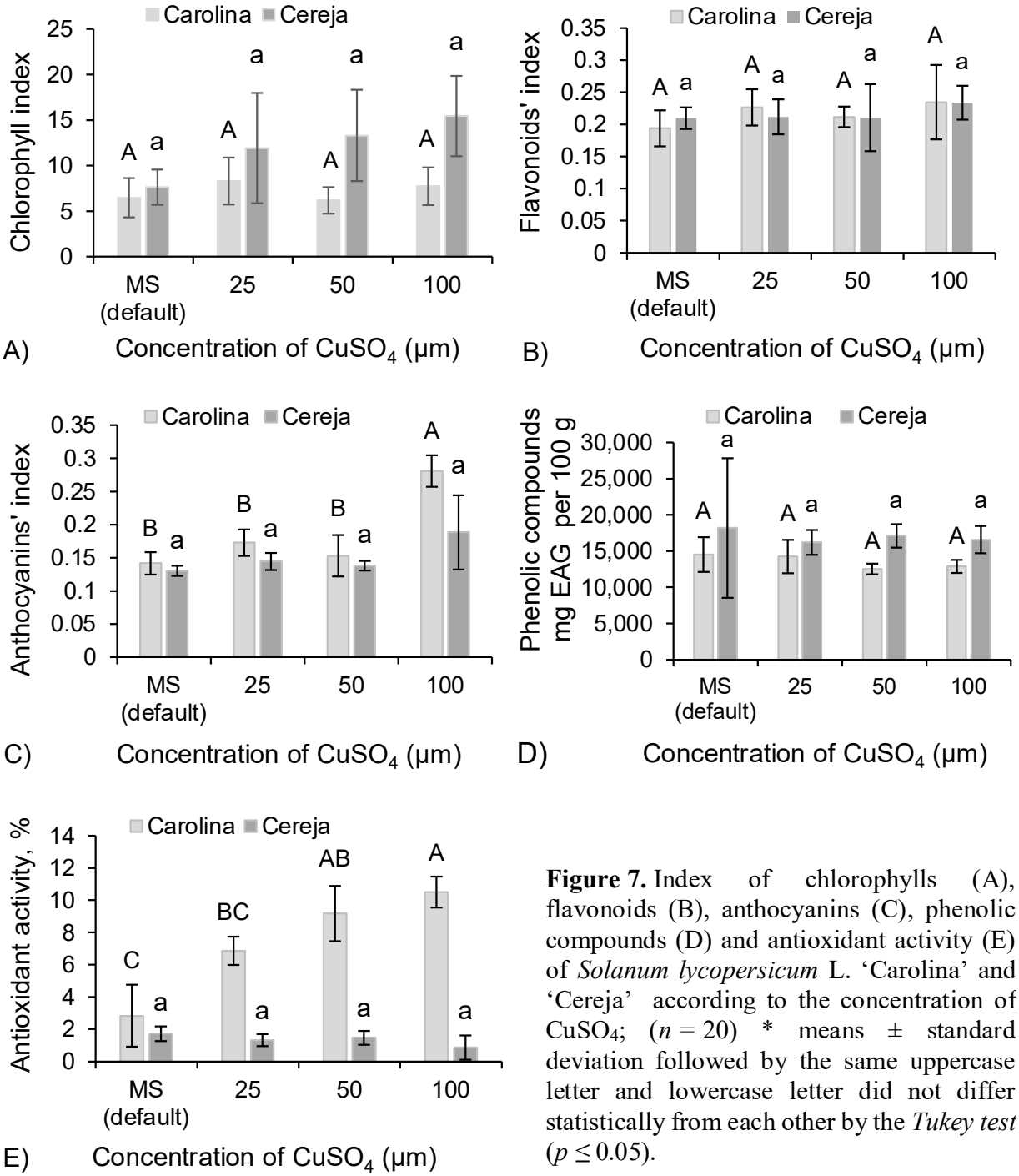

Figure 7. Index of chlorophylls (A), flavonoids (B), anthocyanins (C), phenolic compounds (D) and antioxidant activity (E) of *Solanum lycopersicum* L. 'Carolina' and 'Cereja' according to the concentration of $CuSO_4$; ($n = 20$) * means ± standard deviation followed by the same uppercase letter and lowercase letter did not differ statistically from each other by the *Tukey test* ($p \leq 0.05$).

The antioxidant activity was increased in 'Carolina' shoots exposed to 100 µm $CuSO_4$. When the concentration of 100 µm $CuSO_4$ was compared, 72.97% more activity was observed in the control treatment, and 34.63% and 12.75% concerning the

concentrations of 25 and 50 µm $CuSO_4$, respectively (Fig. 7, E). Still, in 'Cereja', the percentage of antioxidant activity did not change in the conditions studied here, maintaining an average of 1.47 (Fig. 7, E).

The activity of the superoxide dismutase enzyme (SOD), when evaluated in the aerial part of the shoot, did not change due to exposure to different concentrations of $CuSO_4$ in either the cultivars (Fig. 8, A), whose mean value was 2,163.982 U SOD mg^{-1} FM min^{-1} in 'Carolina' and 2,252.63 U SOD mg^{-1} FM min^{-1} in 'Cereja' (Fig. 8, A).

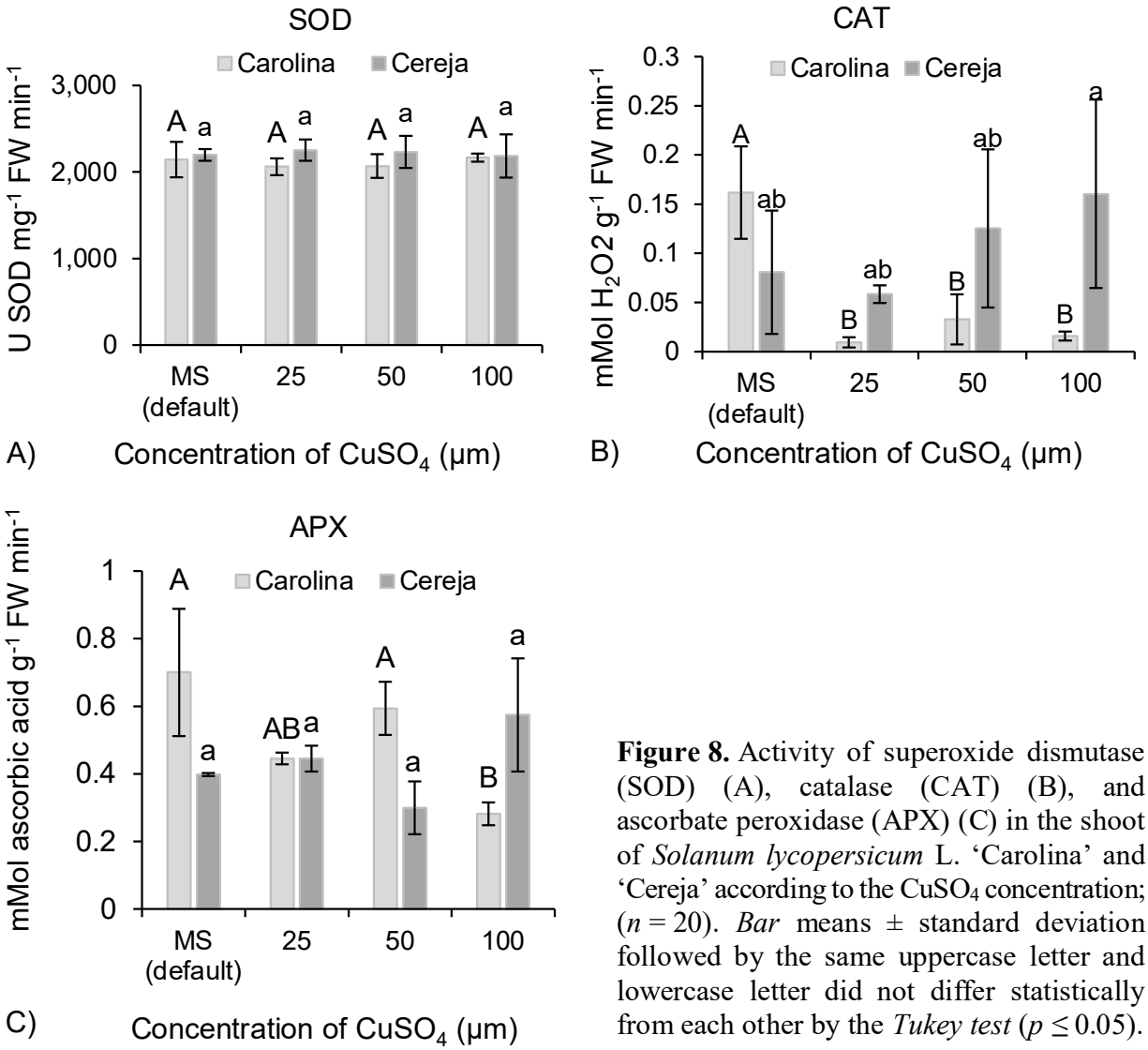

Figure 8. Activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) in the shoot of *Solanum lycopersicum* L. 'Carolina' and 'Cereja' according to the $CuSO_4$ concentration; ($n = 20$). *Bar* means ± standard deviation followed by the same uppercase letter and lowercase letter did not differ statistically from each other by the *Tukey test* ($p \leq 0.05$).

There was a reduction in the catalase activity (CAT) in the shoot of the 'Carolina' cultivar when exposed to Cu. The addition of copper to medium decreased 94.18%, 79.77%, and 90.10% the activity of this enzyme in treatments with 25, 50, and 100 µm of $CuSO_4$, respectively, compared to the control treatment (Fig. 8, B). Drazkiewicz et al. (2004) observed in studies with Arabidopsis thaliana that Cu inhibited CAT activity, while other antioxidant enzymes intensified its activity. This author reported that his results corroborate the hypothesis that high concentrations of O_2^- have a direct effect on CAT inactivation (Salin, 1988). On the other hand, in the 'Cereja' cultivar, there was no significant difference in CAT activity (Fig. 8, B).

Ascorbate peroxidase (APX) in the shoot of the 'Carolina' shoots in the control treatment showed 60% more activity concerning 100 µm $CuSO_4$, not differing statistically from the treatment with 50 µm $CuSO_4$ (Fig. 8, C). In the 'Cereja' shoots, APX activity did not change due to the different Cu concentrations (Fig. 8, C).

When evaluating SOD activity in the root system, it was observed that shoots exposed to 100 µm $CuSO_4$ increased 39.20% in activity compared to 50 µm $CuSO_4$ in the 'Carolina' cultivar. In contrast, in the other treatments, the activity was constant, with an average of 2242.34 U SOD mg^{-1} FM min^{-1} (Fig. 9, A). In the 'Cereja' cultivar, the activity of SOD in the root system of shoots exposed to 100 µm $CuSO_4$ showed an increase of 38.79% compared to that obtained in the control treatment (Fig. 9, A). As previously reported, roots tend to accumulate Cu to protect the shoot (Drazkiewicz et al., 2004; Küpper & Andresen, 2016). The function of SOD is to dismute the anionic superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) (Bhattacharjee, 2010). Thus, we believe that, in both cultivars, SOD intensified its activity in the root system, dismuting the O_2^- to H_2O_2 to prevent these molecules from damaging the shoot.

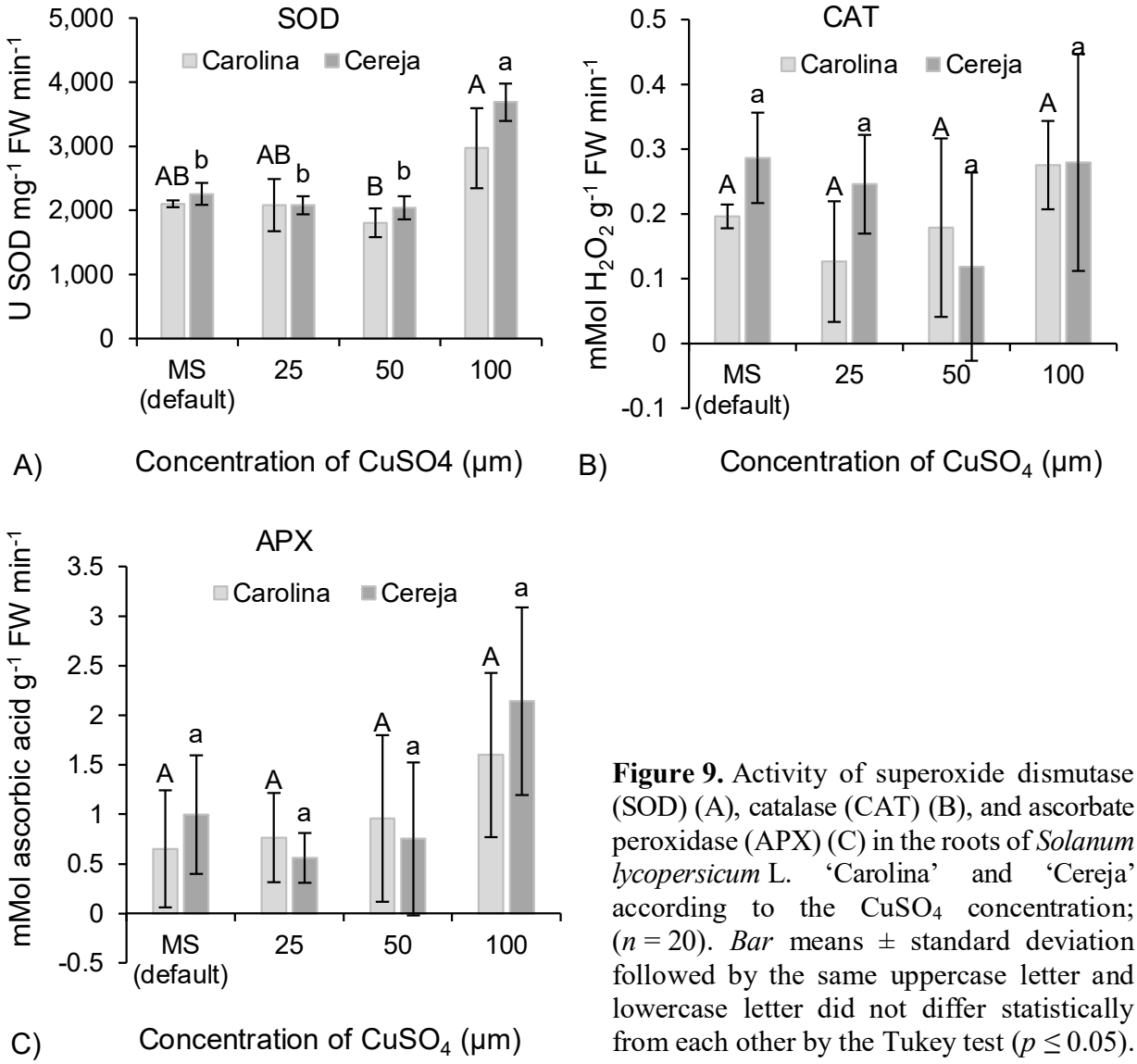

Figure 9. Activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) in the roots of *Solanum lycopersicum* L. 'Carolina' and 'Cereja' according to the $CuSO_4$ concentration; ($n = 20$). *Bar* means ± standard deviation followed by the same uppercase letter and lowercase letter did not differ statistically from each other by the Tukey test ($p \leq 0.05$).

The evaluation of CAT (Fig. 9, B) and APX (Fig. 9, C) activity in the root system demonstrated that the exposure of 'Carolina' and 'Cereja' shoots to different Cu concentrations did not cause statistical differences in those enzymes activity.

The present study provided evidence that the excess of Cu requires the 'Carolina' tomato to redirect its metabolites to combat ROS, which hinders the growth of both shoot and roots. The 'Cereja' tomato, on the other hand, favored SOD activity, especially in the root system, thus controlling the effects of ROS on the shoot. The absence of Cu, in turn, causes an increase in the predisposition of shoots to physiological disorders. Studies still need to be carried out to clarify and control the occurrence of hyperhydricity in 'Carolina' and 'Cereja' tomatoes grown *in vitro* under the $CuSO_4$ concentrations described here.

CONCLUSION

It was concluded that the 'Carolina' and 'Cereja' tomato plants have their growth impaired when exposed to 100 µm de $CuSO_4$. Concentrations higher than 50 µm of $CuSO_4$ cause an increase in the antioxidant activity in the shoot of tomato plants from the 'Carolina' cultivar. Concentrations higher than 50 µm $CuSO_4$ increase SOD activity in the root system of tomato plants from the 'Cereja' cultivar.

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