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Effects of copper on physiological and cytological aspects in *Lactuca sativa* L.

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ABSTRACT: (Effects of copper on physiological and cytological aspects in *Lactuca sativa* L.) This study characterized the effect of copper on physiological and cytological aspects in *Lactuca sativa* L. Lettuce seeds were exposed to concentrations of 50, 100, 250 or 500 μM of CuSO_4 and distilled water was used as the negative control, using five replicates of 30 seeds and 3 mL of solution per treatment for phytotoxicity tests, and three replicates for cytotoxicity tests. For proline, enzymatic antioxidant activity, and lipid peroxidation, we used four replicates with 50 seeds and 5 mL of solution. The experimental design was completely randomized. Root elongation and fresh matter were reduced as the concentration increased. This can be attributed to the reduced mitotic index, from 29.02% in the control treatment to 1.5% at the concentration of 500 μM CuSO_4 . Chromosomal abnormalities did not differ significantly. The proline content in the seedlings increased depending on the concentration of the metal. Superoxide dismutase (SOD) was the main enzyme responsible for the elimination of reactive oxygen species (ROS). Ascorbate peroxidase, guaiacol peroxidase and catalase did not change significantly. The action of SOD along with proline attenuated lipid peroxidation in the membranes. Although the concentration of 500 μM CuSO_4 was cytotoxic, it was not clastogenic in the tested conditions. In addition, copper did not influence germination and dry matter. *L. sativa* showed an efficient antioxidant system, which prevented significant damage to cell membranes.

Key words: cytotoxicity, heavy metal, phytotoxicity, enzyme antioxidant activity.

RESUMO: (Efeitos do cobre sobre aspectos fisiológicos e citológicos em *Lactuca sativa* L.) Este estudo caracterizou o efeito do cobre sobre os aspectos fisiológicos e citológicos em *Lactuca sativa* L. Sementes de Alface foram expostas às concentrações 50; 100; 250 e 500 μM de CuSO_4 e água destilada foi utilizada como controle negativo, utilizando-se cinco repetições de 30 sementes e 3 mL de solução por tratamento para os testes de fitotoxicidade e três repetições para testes de citotoxicidade. Para prolina, atividade antioxidante enzimática e peroxidação lipídica foram utilizadas quatro repetições com 50 sementes e 5 mL de solução. O delineamento experimental foi o inteiramente casualizado. O alongamento de raiz e biomassa fresca foram reduzidos conforme o aumento da concentração. Isso pode ser atribuído ao índice mitótico reduzido de 29,02%, no tratamento controle, para 1,5% na concentração 500 μM de CuSO_4 . Anormalidades cromossômicas não mostraram diferença significativa. O conteúdo de prolina nas plântulas aumentou dependendo da concentração do metal. A dismutase do superóxido (SOD) foi a principal enzima responsável pela eliminação das espécies reativas de oxigênio (ERO). Peroxidase do ascorbato, do guaiacol e catalase não apresentaram alteração significativa. A ação da SOD juntamente com a prolina, atenuou a peroxidação lipídica das membranas. Embora a concentração 500 μM de CuSO_4 foi citotóxica não foi clastogênica nas condições testadas. Além disso, o cobre não influenciou a germinação e biomassa seca. *L. sativa* apresentou eficiente sistema antioxidante, o qual preveniu danos significativos às membranas celulares.

Palavras-chave: citotoxicidade, metal pesado, fitotoxicidade, atividade antioxidante enzimática.

INTRODUCTION

In low concentrations, copper is an essential plant micronutrient, as a constituent and activator of many enzymes (Burkhead *et al.* 2009). However, in excess, copper can negatively affect plant growth and metabolism, becoming phytotoxic (Fidalgo *et al.* 2013, Cambrollé *et al.* 2013).

Bioassays are highly efficient for monitoring environmental pollutants, and they are important for evaluating the level of environmental damage caused by contamination with heavy metals (Pandard *et al.* 2006, Rodrigues 2013). Higher plants are widely used in bioassays and are commonly used in studies of heavy metal phytotoxicity (Pandard *et al.* 2006, Czerniawska & Kusza 2011). *Lactuca sativa* L. (Asteraceae) is widely used

as a test organism because of its sensitivity to chemical agents, high germination rate, rapid and linear growth over a wide range of pH, and low sensitivity to osmotic potentials (Rice 1984, Araújo & Monteiro 2005, Ribeiro *et al.* 2012).

Studies on the effects of bioaccumulation of heavy metals such as copper show that they are phytotoxic, causing oxidative damage and can negatively affect structures and cellular processes, such as photosynthesis, mitochondrial respiration, carbon metabolism, nitrogen assimilation, permeability of the cell membrane, and others metabolic mechanisms, inhibiting the plant development (Ke 2007, Burkhead *et al.* 2009, Yruela 2009, Cambrollé *et al.* 2013). This occurs due to the chemical properties that make the transition metals important components of

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the cellular oxidation-reduction system, and can also be responsible for undesirable reactions and cell damage (Kim & Kwak 2010, Ravet & Pilon 2013).

Copper, when in excess, is capable of catalyzing the production of Reactive Oxygen Species (ROS), causing damage to biological molecules (DNA, RNA and proteins) and also lipid peroxidation in cell membranes (Ke 2007). To combat oxidative stress, plants possess a complex antioxidant metabolism that regulates the detoxification and/or elimination of ROS, and consequently reduces oxidative damage (Choudhury *et al.* 2013).

In phytotoxicity tests, we commonly evaluate the inhibition of the germination process and root elongation that occurs in the presence of low concentrations of the toxic compound (Palácio *et al.* 2012). In addition, inferences regarding cytotoxicity can be made through analyzing changes in the cellular cycle (Fernandes *et al.* 2007). Here, we characterized the effect of different concentrations of copper on germination, initial growth, change in the cellular cycle, and antioxidant enzyme activity in *L. sativa*.

MATERIAL AND METHODS

Plant material and treatments

For the evaluation of germination (G%), root elongation (RE), fresh matter (FM) and dry matter (DM), mitotic index (MI), and chromosomal abnormalities (CA), 30 seeds of *L. sativa* L. cv. Grand Rapids were randomly distributed in Petri dishes, lined with a double layer of Whatman No. 2 filter paper, moistened with 3 mL of CuSO₄ solution in concentrations of 50, 100, 250 or 500 µM, using distilled water as the negative control. For the quantification of proline, enzyme antioxidant activity, and lipid peroxidation, we used 50 seeds and 5 mL of solution per treatment. The dishes were maintained in a Biochemical Oxygen Demand (B.O.D.) chamber at 20 °C with a photoperiod of 12 h (Simões *et al.* 2013). The pH and osmotic potential of the solutions were evaluated using, respectively, a digital pH meter (Tecnopon mPA 210) and automatic osmometer (Model 3320 Micro-Osmometer, Advanced Instruments).

Phytotoxicity and cytotoxicity

The G% was evaluated 24 and 48 h after the experiment began. The RE was obtained by measuring ten randomly selected roots at the end of the seven days of cultivation. Subsequently, these plants were weighed on a precision analytical balance (Model AY 220, Marte) to determine the FM, and placed in a circulating-air oven (Nova Era 400 ND) at 45 °C until reaching constant weight, for the determination of dry matter.

To determine the MI and CA, *L. sativa* root tips were obtained 24 h after exposure to each treatment. The roots were fixed in Carnoy solution, hydrolyzed in 5 M HCl at 25 °C and stained with Schiff reactive. The cytogenic preparations were performed according to Pereira *et al.*

(2013). We evaluated 6000 cells/treatment for the MI and CA evaluation.

The statistical experimental design was completely randomized, with five treatments and five replicates for phytotoxicity and three replicates for cytological parameters.

Enzymatic antioxidant activity and proline content

For the extraction of the antioxidant enzymes, 0.2 g of whole plant fresh matter was macerated in liquid nitrogen and homogenized in 1.5 mL of extraction buffer. The homogenized solutions were centrifuged at 15,000 rpm for 10 min at 4 °C, collecting the supernatant for the enzyme analyses of the superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and guaiacol peroxidase (GPX) (Biemelt *et al.* 1998).

Superoxide dismutase activity was determined by the enzyme's capacity to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), proposed by Giannopolitis & Ries (1977). The tubes containing the buffer along with the sample, as well as the control (same incubation medium without the sample) were illuminated with 20 W fluorescent light for seven min and the readings were performed at 560 nm. One unit of SOD was defined as the capacity of the enzyme to reduce 50% of NBT.

Ascorbate peroxidase activity was determined by the decrease of ascorbate absorbance ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) at 290 nm every 15 s, during three min, according to Nakano & Asada (1981). For the analyses, an aliquot of 100 µL of the enzyme extract was added to 2000 µL of the incubation buffer. A unit of APX was defined by the amount of enzyme that oxidizes 1 µmol min⁻¹ of ascorbic acid.

Catalase activity was determined by the decrease of absorbance at 240 nm every 15 s, during three min, monitored by the consumption of hydrogen peroxide, as described by Havir & Michale (1987). The reaction was initiated by the addition of H₂O₂ ($\epsilon = 36 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of CAT was defined as the amount of enzyme necessary to decompose 1 µmol min⁻¹ of H₂O₂.

Guaiacol peroxidase activity was determined by observing the change in absorbance at 470 nm every ten s, during 0.5 min, caused by the reduction of guaiacol ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$), as described by Urbanek *et al.* (1991). One unit of GPX was defined by the capacity of the enzyme to oxidize 1 µmol min⁻¹ of guaiacol.

The proline content was determined after seven days of cultivation according to Torello & Rice (1986). For this, 0.1 g samples of whole plant fresh matter were homogenized with 10 mL of 3% sulfosalicylic acid, shaken vigorously for one hour, filtered, and centrifuged at 15,000 rpm for ten min. Next, 2 mL of the supernatant was collected and added to 2 mL of acid ninhydrin and 2 mL of acetic acid. This mixture was heated at 100 °C for one hour and cooled in an ice bath. The intensity of the color was measured at 520 nm in a spectrophotometer (Halo SB-10, Dynamica Scientific) and the absorbance was compared with the standard curve of proline. The results were expressed in µmol of proline per gram of fresh matter.

Lipid peroxidation

Lipid peroxidation was determined by quantifying the species which are reactive to thiobarbituric acid (TBA), according to Buege & Aust (1978). For this, 0.1 g samples of whole plants were macerated in liquid nitrogen, added to 20% polyvinylpyrrolidone (m/v), and homogenized in 1.5 mL of 20% trichloroacetic acid (TCA) (m/v). The homogenized solution was centrifuged at 15,000 rpm for ten min. Aliquots (0.75 mL) of the supernatant were added to 0.75 mL of TBA, and the tubes were heated at 90 °C for 20 min. After heating, the tubes were rapidly cooled in an ice bath and read in a spectrophotometer (Halo SB-10) at 540 nm. The concentration of the malondialdehyde (MDA)/thiobarbituric acid (TBA) complex was calculated from the equation: $[MDA] = (A_{535} - A_{600}) / (\xi \cdot b)$, in which: ξ (extinction coefficient = $1.56 \times 10^{-5} \text{ cm}^{-1}$); b (optic length = 1).

The statistical experimental design used for the physiological parameters was completely randomized, with five treatments and four replicates.

The data were submitted to analysis of variance and the means were compared by the Scott-Knott test with 5% significance. The variables that did not meet the assumptions of residue normality and/or homogeneity were square root transformed.

RESULTS AND DISCUSSION

The pH levels in the treatments ranged from 5.14 to 5.45, while the osmotic potential ranged from -0.001 to -0.002 MPa. According to Borella & Pastorini (2010), *L. sativa* tolerates pH levels between 4 and 10. Gatti *et al.* (2004, 2014) observed that solutions with an osmotic potential of up to -0.2 MPa have no effect on lettuce growth.

Lettuce showed G% of 90% and 95% 24 and 48 h after sowing, respectively. The analysis of variance indicated no significant difference between the tested concentrations of copper ($p > 0.05$). Salvatore *et al.* (2008) also found no influence of copper on germination of *L. sativa*, *Solanum lycopersicum*, *Raphanus sativus* and *Brassica oleracea*. This is probably because germination does not necessarily imply growth by cellular division, which is known to be inhibited by heavy metals (Liu *et al.* 2003, Pereira *et al.* 2013); germination can also be attributed to cellular elongation or stretching of the embryonic axis after soaking (Haber & Luippold 1960, Bewley & Black 1984, Chon *et al.* 2004).

We observed significant differences among the treatments for RE ($p < 0.001$) (Fig. 1A). The control differed from the remaining treatments, showing the largest root (18.7 mm). The plants exposed to the concentrations of 50 and 100 μM , differed between each other and from the remaining treatments, with lengthening of 11.9 and 1.3 mm, respectively. In addition, we observed apical necrosis of the roots in these concentrations, which may have influenced the absorption of water by the plants. This reveals that the initial growth of the plants is more sensitive than is germination. Root elongation is a parameter widely

used in metal toxicity tests (Voigt *et al.* 2006, Kopittke *et al.* 2011, Pereira *et al.* 2013), and in general, the roots are more affected than other plant structures by substances present in the environment, since roots are responsible for absorbing these substances (Turk & Tawaha 2002).

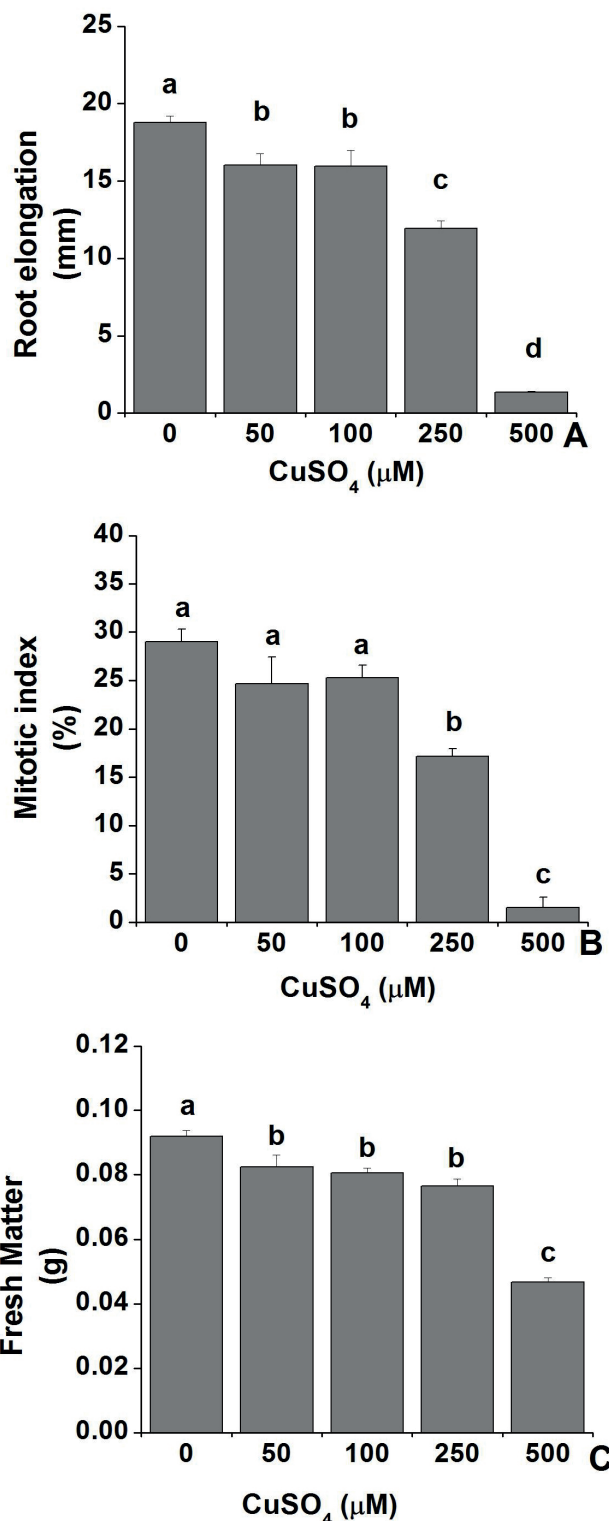


Figure 1. (A) Root Elongation (mm), (B) Mitotic Index (%) and (C) Fresh matter (g) of *Lactuca sativa* exposed to different concentrations of CuSO_4 . Bar: standard error. Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level.

Pasternak *et al.* (2005) and Potters *et al.* (2009) reported the inhibition of root elongation in plants exposed to copper, as a morphological response that is sensitive to the environmental stress caused by metals. Marschner (1995) reported that an immediate response to exposure to elevated concentrations of copper consists of inhibition of root growth, as well as damage to cell membranes due to the increase of potassium efflux. In addition, the toxicity level can be based on the increase or decrease of the MI, and can therefore be used as a cytotoxicity parameter (Fernandes *et al.* 2007). Another consequence of copper toxicity, in addition to inhibiting cellular division, is the inducement of chromosomal abnormalities (Souguir *et al.* 2008).

The root meristem of *L. sativa* exposed to distilled water showed an MI of 29.01% (Fig. 1B). Treatment with copper significantly reduced ($p < 0.05$) the MI at the concentrations of 250 (17.1%) and 500 μM (1.5%), respectively, differing from each other and from the remaining treatments. Therefore, the increase in the level of copper had a concentration-dependent cytotoxic effect on the meristematic cells of the test-organism root.

The reduction of MI indicates a toxic effect of the copper, which can interfere with the normal development of mitosis, reducing the number of cells that enter prophase and blocking mitosis during interphase (Rijstenbil & Poortvliet 1992). Studies conducted by Yildiz *et al.* (2009) with *Allium cepa* L. showed that exposure to copper retards root growth in a concentration-dependent manner, with the effective concentration (EC_{50}) being approximately 1.5 mg/L, and a reduction of the MI of around 64% in relation to the control. In addition, we observed the presence of chromosomal abnormalities such as, stickiness, fragments, chromosomal bridges, c-metaphases, and multipolarity in anaphase-telophase cells after they were exposed for 4 d to the chemical solutions.

Pereira *et al.* (2013), working with *L. sativa* exposed to lead, identified changes in the cellular cycle, as well as structural abnormalities in the chromosomal complement, both aneugenic and clastogenic, after exposure to the chemical solutions for 24 h, that same evaluation period used in this study. This concurs with the results observed for *Vicia faba* and *Pisum sativum*, for which copper had clastogenic and aneugenic effects on meristematic root cells, inducing the formation of micronuclei, suggesting that this metal acts as a genotoxic agent (Souguir *et al.* 2008). However, the present study found no significant difference in the occurrence of CA, suggesting that, for the tested concentrations and conditions, copper does not have a genotoxic effect. The observed CA may correspond to a background.

For FM, the treatments differed significantly ($p < 0.05$) (Fig. 1C). However, for DM, there was no significant difference ($p < 0.05$). The non-significant loss of dry matter can be attributed to the expansin accumulation involved in cell wall plasticity influenced by the increase of copper concentration, resulting in larger caliber roots. This explains why plants exposed to higher concentrations have a dry mass equivalent to the control (Pena *et al.* 2015). The

FM of plants exposed to concentrations of 50, 100 and 250 μM CuSO_4 were equivalent to each other and lower than the control. The concentration of 500 μM resulted in the lowest FM. Kováčik *et al.* (2010) attributed the reduction of total biomass to the decrease in root growth of different plants exposed to copper, which corroborates with that exposed by Cambrollé *et al.* (2013), who reports that copper, in high concentrations, similarly to any trace metal, can be translocated to the aerial part of the plant and affect many physiological processes. Ahsan *et al.* (2007) observed a significant reduction in biomass of rice seedlings between the control and plants exposed to 500 μM copper, four days after germination. Studies conducted by Ali *et al.* (2000) and Iannacone *et al.* (2005) with *Allium cepa*, *Beta vulgaris*, *Oriza sativa* and *Raphanus sativus* found that exposure to copper caused reductions in fresh and dry matter, that were inversely proportionate to the copper concentration.

With respect to the proline content, in *L. sativa* plants exposed to copper, the control showed 4.8 $\mu\text{mol g}^{-1}$ of fresh matter and did not differ statistically from the concentrations of 50 and 100 μM CuSO_4 , which resulted in contents of 7.8 and 6.9 $\mu\text{mol g}^{-1}$ fresh matter, respectively (Fig. 2). The concentration of 250 μM CuSO_4 differed significantly from the remaining treatments, resulting in 22.1 $\mu\text{mol g}^{-1}$ of fresh matter. The highest proline content was observed for the highest concentration of CuSO_4 (500 μM), presenting value of 33.86 $\mu\text{mol g}^{-1}$ of fresh matter. This reveals an increase of 250% in the proline content for the concentration of 250 μM CuSO_4 and 590% for the highest concentration tested (500 μM) when the plant was exposed to this metal.

The highest production of proline is directly correlated to oxidative stress caused by the accumulation of metal in plants (Kováčik *et al.* 2009, Radič *et al.* 2010). Therefore, due to its importance for osmotic adjustment, proline is the compound most studied in plants under abiotic stress (Kavi Kishor *et al.* 2005). Stress induces proline

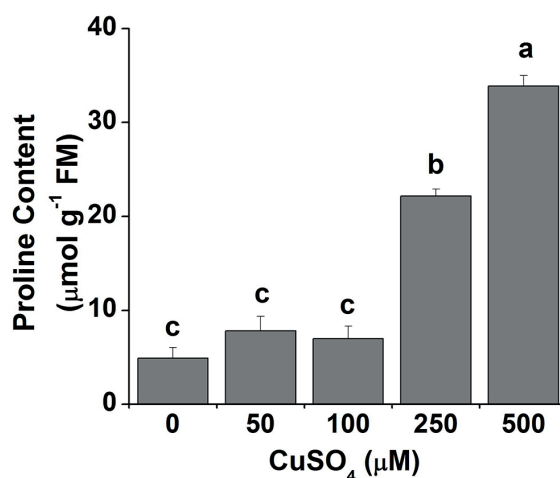


Figure 2. Proline content of *Lactuca sativa* exposed to different concentrations of CuSO_4 . Bar: standard error. Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level.

synthesis and inhibits its degradation; its concentration varies among plant species and depends on the level of stress, and plants may accumulate 100 times more proline than under normal conditions (Verbruggen & Hermans 2008). This amino acid is also capable of removing ROS, inhibiting lipid peroxidation of cellular membranes (Rajeb *et al.* 2014).

The increase in the proline content can be related to a response of the plant under stress to prevent the loss of water, since this amino acid is important in osmotic adjustment in plant cells (Delauney & Verma 1993). The osmotic adjustment allows water absorption, plant growth and the expansion of foliar turgidity during stress, associated with the partial opening of the stomata, allowing CO₂ assimilation (Alves & Setter 2004). However, in the present study, the high levels of proline in the higher metal concentrations were not able to prevent mass loss, especially at the concentration of 500 µM CuSO₄. The increase in the proline content can also activate many cellular functions such as carbon and nitrogen supply used during growth for reestablishment after stress (Kavi Kishor *et al.* 2005). This explains the behavior of *L. sativa* DM, since there was no significant difference among treatments, even in the highest copper concentrations.

The results show that, in order to combat a possible oxidative stress caused by the elevation in copper content, there was a significant increase in SOD activity, so that the highest copper concentrations (250 and 500 µM) resulted in the highest SOD activities (Fig. 3). This response can be attributed to the accumulation of superoxide radicals (O₂⁻) induced by copper, allowing, according to Meng *et al.* (2007), the codification of genes for the synthesis of the enzyme. For catalyzing the dismutation of the superoxide anion, which is the first ROS formed, the SOD participates in the first line of defense against oxidative stress (Gill & Tuteja 2010). Also, the removal of the superoxide radical may prevent the formation of hydroxyl radicals before their interaction with target

molecules (Xu *et al.* 2013). Although the activity of the enzymes APX, GPX and CAT changed, the differences were not significant.

Olteanu *et al.* (2013) found high SOD activity and a decrease in CAT in *Triticum aestivum* submitted to different sources of copper (citrate and acetate) for seven days, suggesting that the SOD has a primordial function in the prevention of oxidative stress. Therefore, although CAT shows high specificity for H₂O₂, its affinity is lower than that of APX (Meng *et al.* 2007, Sharma *et al.* 2012), and it is less efficient in removing H₂O₂, which possibly explains its lower activity in relation to APX. The decline in CAT, although not significant, might be attributed to the modifications induced by copper in the assembly of the enzyme subunits (Verma & Dubey 2003). In addition, CAT is sensitive to stress caused by copper, which in low concentrations can increase enzyme activity, but at high concentrations causes inhibition (Srivastava *et al.* 2006).

The accumulation of malondialdehyde is frequently used as an indicator of damage to cell walls (Bouazizi *et al.* 2010, Ke 2007, Sharma *et al.* 2012), however, we observed no significant difference in the level of lipid peroxidation among treatments. This can be attributed to the accumulation of proline and the activity of SOD (Figs. 2 and 3), acting concomitantly in the removal of ROS, and thus contributing to maintaining the integrity of the cellular membrane and of the remaining cellular components. These results diverge from those of Devi & Prasad (2005), who demonstrated that the accumulation of copper resulted in an increase of lipid peroxidation and changes in the activity of antioxidant enzymes in *Brassica juncea*.

CONCLUSIONS

The present results show that copper concentrations above 250 µM caused oxidative damage and resulted in the reduction of the mitotic index in the root tips, which were responsible for root growth and fresh matter reduction. However, a clastogenic effect was not observed. Therefore, copper can be considered phytotoxic and cytotoxic, but not genotoxic in the test conditions.

Lactuca sativa showed an efficient antioxidant system, initiated by the activity of SOD, that, along with proline, effectively removed the ROS and prevented significant damage to the lipid membranes.

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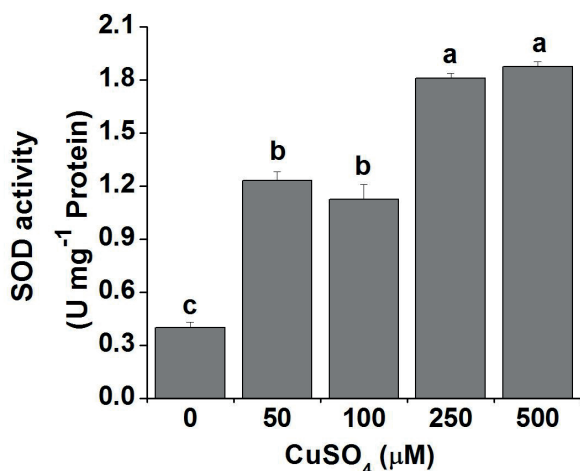


Figure 3. Activity of superoxide dismutase (SOD) in *Lactuca sativa* exposed to different concentrations of CuSO₄. Bar: standard error. Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level.

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