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Morpho-physiological and biochemical mechanisms of copper tolerance in *Handroanthus heptaphyllus*

Mecanismos morfofisiológicos e bioquímicos de tolerância ao cobre em plantas de *Handroanthus heptaphyllus*

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ABSTRACT

Copper (Cu)-contaminated soils are becoming more common, and phytoremediation is an effective strategy for reducing the negative effects of Cu on soils. Tree species are commonly used for this technique because they act as a barrier to this type of contamination. It is necessary to evaluate Cu tolerance and toxicity thresholds together with the harm this metal causes to plants. The objective of the current study was to investigate the tolerance of *Handroanthus heptaphyllus* plants to Cu through morpho-physiological, biochemical, and tissue Cu concentration analyses. *H. heptaphyllus* seedlings were subjected to nutrient solutions with varying concentrations of copper: 0, 5, 32, 64, 96, and 128 µM Cu in a hydroponic system. We conducted a morpho-physiological trait analysis on shoot height, root length, dry weight, morphological variables of the root system, leaf area, and photosynthetic variables. In addition, we also looked into photosynthetic pigments, antioxidant enzymes, lipid peroxidation, hydrogen peroxide concentration, and Cu accumulation in tissues. The values obtained for variables such as dry weight and pigments did not show significant differences, regardless of Cu concentration. Moreover, photosynthetic and transpiration rates were negatively affected only at the highest Cu concentration (128 µM). Overall, excess Cu had no effect on *Handroanthus heptaphyllus* below 128 µM. Cu accumulated mainly in the roots without a decrease in biomass, which could indicate the tolerance of the species to this metal as well as its great potential in the phytostabilization of Cu in contaminated soils.

Index terms: Contaminated areas; oxidative stress; phytoremediation.

RESUMO

Solos contaminados com cobre (Cu) são um problema crescente, e o uso da fitorremediação tem evidenciado resultados positivos na redução dos efeitos nocivos desse elemento nos solos. Espécies arbóreas têm sido amplamente utilizadas para esta técnica, pois funcionam como barreira a esse tipo de contaminação. Portanto, é necessário avaliar os limites de tolerância/toxicidade ao Cu, bem como os danos causados por este metal nas plantas. Assim, o presente estudo teve como objetivo investigar a tolerância de plantas de *Handroanthus heptaphyllus* ao Cu através de análises morfofisiológicas e bioquímicas, bem como a concentração de Cu nos tecidos. Mudas de *H. heptaphyllus* foram submetidas a diferentes concentrações de Cu adicionadas na solução nutritiva: 0,5, 32, 64, 96 e 128 µM Cu em sistema hidropônico. Foram analisados atributos morfofisiológicos, como altura da parte aérea e comprimento da raiz, massa seca, variáveis morfológicas do sistema radicular, área foliar e variáveis fotossintéticas. Além disso, foram investigados pigmentos fotossintéticos, enzimas antioxidantes, peroxidação lipídica, concentração de peróxido de hidrogênio e Cu acumulado nos tecidos. Os valores registrados para variáveis como massa seca e pigmentos não apresentaram diferença significativa, independentemente das concentrações de Cu. Além disso, as taxas fotossintéticas e transpiratórias foram afetadas negativamente apenas na maior concentração de Cu (128 µM). No geral, a espécie *Handroanthus heptaphyllus* foi afetada pelo excesso de Cu apenas em 128 µM. O Cu se acumulou principalmente nas raízes, sem registrar diminuição da biomassa, o que pode indicar a tolerância da espécie a esse metal, bem como seu grande potencial para ser utilizado na fitoestabilização de Cu em solos contaminados com este metal.

Palavras-chave: Áreas contaminadas; estresse oxidativo; fitorremediação.

INTRODUCTION

Copper (Cu) is a heavy metal naturally found in the soil. Cu concentrations are affected by the original geological materials and soil formation processes (Schmitt et al., 2021). However, human activities such as mining, industrialization, and agriculture can significantly raise the concentrations of Cu in the soil (Briffa; Sinagra; Blundell,

2020). Applying pesticides, bactericides, and fungicides can also help increase soil Cu levels (Yang et al., 2017).

Copper, a component of the electron transport chain of photosynthesis (in plastocyanin) and respiration (in cytochrome c oxidase), is an essential micronutrient for plants at low concentrations (Marchi et al., 2020). Cu is also required for high-affinity binding between ethylene and its receptor (Navarro et al., 2021) and is a component of proteins such as ascorbate oxidases and amine oxidases (Smirnoff, 2018). However, the excessive increase in Cu concentration in agricultural soils is of concern due to its potential toxicity when taken up in large amounts by plants and animals (Okereafor et al., 2020). High Cu concentrations in crops, such as cereals, fruits, and vegetables, pose a risk to food security (Schmitt et al., 2021).

Crops grown in soils with high levels of available Cu can accumulate this metal in their tissues and develop toxicity symptoms (Tiecher et al., 2017). The adverse effects of such disorders on physiological and biochemical variables vary depending on plant species, organ, element concentration, and tissue tolerance to high Cu levels (Marastoni et al., 2019). Therefore, understanding the morpho-physiological and biochemical responses of plants exposed to heavy metals is crucial for the remediation of contaminated areas (Gautam; Anjani; Srivastava, 2016).

An interesting strategy for remediating areas contaminated with metals is to use tree species because of their long life cycles, ability to produce large amounts of biomass, and dense root systems. In addition, tree species can concentrate large amounts of metals in their trunks and roots, immobilizing metals in plant tissues and delaying their return to the soil (Yan et al., 2020). Due to their deeper soil penetration and larger root systems, trees contribute more effectively to the phytoremediation of contamination sources like mining activities that reach these soil layers. As a result, these species are crucial to phytoremediation efforts in areas with heavy metal contamination (Silva et al., 2019). The ability of various tree species to grow in these Cu-excess conditions and the physiological and biochemical effects of toxic metal presence on plants can help determine the nature of plant adaptation under metal overload and the best species for phytoremediation. However, research on the tolerance of tropical tree species in Cu-contaminated soils is still limited.

Handroanthus heptaphyllus (Vell.) Mattos, which belongs to the Bignoniaceae family and is popularly known as ipê-roxo, is one such tree species with phytoremediation potential. The species has significant ecological and economic value, making it useful for afforestation and urban landscaping projects (Berghetti et al., 2021). They are also commonly used in reforestation programs and to restore degraded areas (Silva et al., 2021). The timber from this species is used in construction due to its high density, strength, and malleability (Parcianello et al., 2021). Due to important physiological properties resulting from the genus to which it belongs, which allow it to adapt to different environmental conditions and ecosystems, this species is often used to rehabilitate damaged areas (Berghetti et al., 2021).

The current study aimed to gather critical information for developing soil remediation strategies by examining the tolerance of *H. heptaphyllus* plants to Cu using morpho-physiological, biochemical, and tissue Cu concentration analyses. We hypothesize that *H. heptaphyllus* trees can tolerate high Cu concentrations in their tissues by reducing the bioavailability of this metal while maintaining their biomass production.

MATERIAL AND METHODS

Experimental setup

We conducted the study in a greenhouse at the Federal University of Santa Maria (UFSM) - Santa Maria *Campus* - RS, Brazil, with a controlled temperature of about 25 °C and an average humidity of 60%.

Handroanthus heptaphyllus seedlings (120 days old) were distributed in a completely randomized experimental design with four replicates per treatment and 16 plants per replicate. We exposed these seedlings to various Cu concentrations, with the Hoagland and Arnon nutrient solution at a standard Cu concentration of 0.5 µM as the control treatment (Hoagland; Arnon, 1950). The nutrient solution was fortified with 32, 64, 96, and 128 µM Cu for the other treatments. Copper sulfate ($CuSO_4.5H_2O$) was the reagent used as the Cu source.

For the production of seedlings of *H. heptaphyllus*, we used seeds from the Forest Nursery of UFSM - Santa Maria Campus. The seeds were sown in plastic trays (38 cm x 56 cm) with the commercial substrate Carolina Soil®, composed of *Sphagnum* sp. and vermiculite.

From the 15th day after sowing (DAS), the seedlings received weekly fertirrigation using a complete nutrient solution with a pH of 5.5 ±0.1. The nutrient solution consisted of (in µM): 6090.5 nitrogen; 974.3 magnesium; 4986.76 chlorine; 2679.2 potassium; 2436.2 calcium; 359.9 sulfur; 243.592 phosphorus; 0.47 copper; 2.00 manganese; 1.99 zinc; 0.17 nickel; 24.97 boron; 0.52

molybdenum, and 47.99 iron ($FeSO_4$/EDTA) (Hoagland; Arnon, 1950).

About three months after sowing in the trays and when the seedlings (at 120 days of age) had reached a uniform height of about 10 cm, they were carefully removed from the substrate and transferred to the hydroponic system. Each seedling was placed in a 16 L tray containing a complete nutrient solution (Hoagland; Arnon, 1950). A Styrofoam sheet with sixteen holes in the center covered the surface of each tray to let the plants through. It allowed us to fix the plants and reduce the evaporation of the solution contained in each tray.

The seedlings acclimatized for seven days in the original nutrient solution of Hoagland and Arnon (1950). PVC microtubes were inserted into the solution through the Styrofoam sheet and connected to an air compressor to aerate the solution. In its original form, the nutrient solution contained the following concentrations in mg L^{-1}: NO_3^- = 196; NH_4 = 14; P = 31; K = 234; Ca = 160; Mg = 48.6; S = 70; Fe-EDTA = 5; Cu = 0.02; Zn = 0.15; Mn = 0.5; B = 0.5; and Mo = 0.01.

Following acclimatization, the treatments were applied, and the seedlings were subjected to various Cu availability conditions for 14 days. Samples were collected when symptoms of Cu toxicity were observed, especially at the highest Cu concentrations, totaling 21 days in the hydroponic system. We replaced the nutrient solution in each tray once a week and adjusted its pH daily to 5.5 ±0.1, with 1.0 mol L^{-1} HCl or 1.0 mol L^{-1} NaOH.

Photosynthetic variables

Photosynthetic variables were evaluated on the third fully expanded leaf using an Infrared Radiation Gas Analyzer (IRGA) model Li-COR® 6400 XT at photosynthetic radiation of 1500 µmol $^{-2}$ s^{-1} and a CO_2 concentration of 400 µmol mol^{-1}. Readings were taken between 8:00 and 10:00 am before the collection of plant samples for growth analysis, focusing on the following variables: net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), Rubisco instantaneous carboxylation efficiency (A/Ci - obtained by the ratio between net CO_2 assimilation rate and intercellular CO_2 concentration), and water use efficiency (WUE – based on the ratio between assimilation and transpiration rates).

Determination of growth variables

Four plants were taken from each experimental unit for growth evaluation. After collection, the plants were dissected into shoot and root parts. The separated samples were then taken to the UFSM Plant Physiology and Plant Nutrition laboratory to determine growth variables. The shoot height and root length were measured using a millimeter ruler. Measurements taken before and after the application of the treatments were considered growth increments during the period.

To determine the shoot dry weight (SDW; g $plant^{-1}$) and root dry weight (RDW; g $plant^{-1}$), plants were harvested and separated into shoot and root systems, washed under running water, and dried in an oven with forced air circulation at approximately 65 °C until weight became constant.

Roots were morphologically characterized using digitized images taken with WinRhizo Pro 2013 software and an EPSON Expression 11000 scanner with auxiliary light (TPU) and 600 DPI resolution. We measured total root length (cm^3 $plant^{-1}$), root volume (cm^3 $plant^{-1}$), root surface area (cm^2 $plant^{-1}$), and mean root diameter (mm). Leaf area was measured using the WinRhizo 2013 system, based on the methodology proposed by Tennant (1975). Samples were digitized using a professional scanner (EPSON Expression 11000), and images were analyzed in TIFF format.

Determination of biochemical variables

For biochemical analyses, we collected 12 plants from each treatment, for a total of 240 plants. The plants were sectioned into shoots and roots, washed with distilled water, packed in aluminum foil envelopes, and immediately frozen with liquid nitrogen to avoid sample degradation, and taken to the UFSM Plant Physiology and Plant Nutrition laboratory. Samples were stored in an ultra-low temperature freezer at –80 °C until analyzed. Samples were manually macerated with liquid nitrogen to obtain a fine powder. The sample amount for each variable was specific and preweighed on a precision digital scale: 0.05 g of fresh sample for the determination of leaf pigments, 0.5 g for antioxidant enzymes, 0.3 g for hydrogen peroxide, and 0.5 g for lipid peroxidation.

Concentration of pigments

Total chlorophylls and carotenoids were extracted using the method of Hiscox and Israelstan (1979) and estimated using the equation of Lichtenthaler (1987). Five-milliliter Dimethyl sulfoxide (DMSO) was added to each 0.05 g sample. The tubes were incubated at 65 °C for approximately one and a half hours until the complete release of pigments, resulting in a dark green solution.

This solution was then divided into two 2 mL replicates. Absorbance of the solution was measured in a UV-visible spectrophotometer (1105, Bel Photonics) at wavelengths 663, 645, and 470 nm for chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively.

Determination of antioxidant enzyme activity

Of the sample, 0.5 g was mixed with 3 mL of 0.05 M homogenate extraction buffer (pH 7.8) containing 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP) to determine antioxidant enzymes. The homogenate was centrifuged at 13,000 x g for 20 min at 4 °C in a high-speed refrigerated centrifuge (CR22 N). The supernatant was used to determine enzyme activity and protein concentration (Zhu et al., 2004).

The activity of the guaiacol peroxidase enzyme (POD) was determined using guaiacol as a substrate (Zeraik; Souza; Fatibello-Filho, 2008). The reaction mixture included 1.0 mL potassium phosphate buffer (100 mM, pH 6.5), 1.0 mL guaiacol (15 mM), and 1.0 mL H_2O_2 (3 mM). 50 µL of the plant extract was added to the above-homogenized solution. Oxidizing guaiacol to tetra guaiacol and measuring the increase in absorbance at 470 nm at 15-second intervals measured the enzyme activity. The results were expressed as enzyme units per milligram of protein (U mg^{-1} protein). The molar extinction coefficient of 26.6 mM^{-1} cm^{-1} was used for the calculation.

The activity of superoxide dismutase (SOD) was determined according to the spectrophotometric method described by Giannopolitis and Ries (1977). The reaction mixture (MIX) was kept in the dark and contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 0.1 mM EDTA, 75 µM nitrobluetetrazolium (NBT), and 2 µM riboflavin. The increase in absorbance at 560 nm mirrored the photochemical production of blue formazan from NBT. The reaction was carried out in test tubes (13 × 100 mm) at 25 °C, each containing 2.8 mL of the reaction mixture (MIX) and 200 µL of the enzyme extract of the respective samples. After pipetting, the tubes were placed in a reaction chamber illuminated by a 15 W fluorescent lamp. The reaction was light-sensitive and allowed for two minutes, after which the samples were read in the UV-visible spectrophotometer (1105, Bel Photonics).

One unit of SOD is the amount of enzyme that inhibits the photoreduction of NBT by 50% (Beauchamp; Fridovich, 1971). In the assay, methionine reduces photochemically excited riboflavin to semiquinone, which donates an electron to oxygen, forming the superoxide radical, which converts NBT to blue formazan. Superoxide dismutase catalyzes this reaction.

Hydrogen peroxide concentration

The amount of hydrogen peroxide was measured by the method of Loreto and Velikova (2001). Root and leaf samples (0.3 g) were homogenized in 3.0 mL of 0.1% trichloroacetic acid (TCA) and centrifuged. After adding the supernatant to 0.5 mL potassium phosphate buffer (10 mM) and 1 mL KI (1M), the samples' absorbance was measured at 390 nm using a spectrophotometer. The H_2O_2 concentration of the supernatant was determined by comparing the measured values with a calibrated standard curve. The H_2O_2 concentration was expressed as fresh weight µmol g^{-1}.

Membrane lipid peroxidation

Lipid peroxidation was determined by malondialdehyde (MDA) concentration (El-Moshaty et al., 1993). Leaf and root samples (0.5 g) were homogenized in 4.0 mL of sodium citrate buffer (pH 6.5) and centrifuged. One milliliter of supernatant was mixed with one milliliter of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated to 95 °C for 40 min, cooled in an ice bath for 15 min, and centrifuged at 10,000 x g for 15 min. The absorbance of the supernatant was measured at 532 and 600 nm (to correct for non-specific turbidity). Lipid peroxidation was expressed as MDA mg^{-1} protein nmol.

Analysis of Cu in plant tissues

Dry shoot and root samples were ground in a Wiley mill after being dried and sieved through a 2 mm sieve. Plant tissue was nitric-perchloric acid digested (3.0 mL HNO 65% PA and 1 mL HClO 70% PA) (Empresa Brasileira de Pesquisa Agropecuária - Embrapa, 2009). The total Cu concentration was determined using an atomic absorption spectrophotometer (AAS, Perkin Elmer Analyst 200, USA). The Cu concentration in tissues was calculated (Tedesco; Gianello; Biassani, 1995) and expressed as mg kg^{-1}.

Statistical Analysis

The data obtained were analyzed for the normality of errors by the Shapiro-Wilk test and homogeneity of variances by Bartlett's test (Storck et al., 2016). Using the statistical program Sisvar, the data were subjected to analysis of variance, and the means were compared using the Tukey test with a 5% probability of error (Ferreira, 2019).

RESULTS AND DISCUSSION

Excessive Cu application can severely affect plant growth and productivity by causing changes in root system architecture and nutrient imbalances (Marastoni et al., 2019). Heavy metals can inhibit shoot and root growth and consequently reduce the biomass production of plants of different species (Trentin et al., 2019).

There was a significant effect ($p \leq 0.05$) for the evaluated factor (different concentrations of Cu) for the morphological growth variables. For the variables increment in shoot (IS) (Figure 1a), shoot dry weight (SDW) (Figure 1e) and root dry weight (RDW) (Figure 1f), there was no significant difference independent of the tested Cu concentrations. For the variables leaf number increment (NLI) (Figure 1c) and leaf area (Figure 1d), Cu promoted a significant reduction in these variables only with 8 mg L^{-1} Cu. For root increment (RI) we can notice that Cu promoted a reduction in this variable at concentrations of 4, 6 and 8 mg L^{-1} Cu (Figure 1b).Thus, in this study, we found that growth in shoots (IS) (Figure 1a) was less affected by the addition of Cu to the nutrient solution compared to growth in roots (Figure 1b).

It may have occurred because reduced root growth in plants is the most evident physiological response to heavy metal stress, as roots are in direct contact with contaminants (Huang et al., 2017). Since Cu has potentially toxic effects on cell division in root apical meristems, the high Cu concentration reduced root elongation. The impact on the apical meristem may also have prevented lateral root growth (Llugany et al., 2003).

In addition, high Cu levels also constrained the length (Figure 2a) and surface area of the root system (Figure 2b). De Conti et al. (2020) observed similar results in a study using iron fertilization to increase tolerance mechanisms to Cu toxicity in *Lolium multiflorum* (Ryegrass) plants. These morphological alterations in roots may be due to hormonal imbalances induced by high Cu levels, which modify or inhibit cell division, particularly in meristematic regions (De Conti et al., 2020).

The root volume (Figure 2c) and root diameter (Figure 2d), however, did not differ between the control and the highest Cu concentration (128 µM). The increase in root diameter observed at 32 and 96 µM concentrations could be related to the inhibition of root length associated with cell division disorders. In addition, the free ionic form of the metal (Cu^{2+}) can bind to the carboxyl groups ($-COO^{-}$) on the cell wall and affect cortical cell division and organization. It might increase the cortex area and its diameter (Ambrosini et al., 2015), which would then cause a decrease in root length.

These observed changes in root volume and diameter (Figure 2c and Figure 2d) may account for the lack of variation in root dry weight (Figure 1f) despite the decrease in root increment (Figure 1b). Furthermore, the results imply that even though the Cu-exposed roots were shorter, the increased production of lateral roots partially offset the lower root dry biomass production in the Cu-exposed, maintaining biomass production. Regardless of the Cu concentrations used, there were no differences in shoot dry weight (SDW, Figure 1e), but the variable increment in leaf number (NLI) was less in the presence of 128 µM Cu compared to the control (Figure 1c). It might be because most of the Cu accumulated in the plants is located in the root region (Figure 3f), reducing the fraction supplied to the shoot (Figure 3e) (De Conti et al., 2020). The high Cu concentrations in the root system of *H. heptaphyllus* indicate that this species is suitable for Cu phytostabilization in soils contaminated with this metal. This phenomenon is described in several plant species as a tolerance mechanism triggered to prevent or limit the occurrence of toxic symptoms at the stem level (Baldi et al., 2018). Thus, we can conclude that the *H. heptaphyllus* seedlings adopt the exclusion strategy by accumulating most of the Cu in the roots to avoid the effects of Cu toxicity.

Although the Cu concentration in shoot tissues (Figure 3e) was lower than the Cu values in root tissues (Figure 3f), a detrimental effect of Cu on photosynthetic variables was observed (Figure 4), especially at the highest Cu concentration (128 µM).

The net photosynthetic rate reduced in the presence of 128 µM Cu in the nutrient solution (Figure 4a), and the actual Cu concentration in the shoot tissues increased to 69 mg kg^{-1} (Figure 3e). Previous studies have shown that Cu concentrations greater than 20 mg kg^{-1} in stem tissues inhibit photosynthetic rates (Trentin et al., 2019). High Cu concentrations in leaf tissues can cause degradation of internal chloroplast structure, oxidative pigment decomposition, damage to Chl-protein complexes, poor nutrient uptake, and a reduction in cell energy balance (Rouphael et al., 2008; Rocchetta; Küpper, 2009; Giannakoula; Therios; Chatzissavvidis, 2021). It can lead to decreased synthesis and pigment concentration in leaves. In addition, Cu-induced oxidative stress can damage lipids, proteins, and chloroplast pigments, causing the thylakoid membrane to rupture (Giannakoula; Therios; Chatzissavvidis, 2021) and impairing photosynthesis.

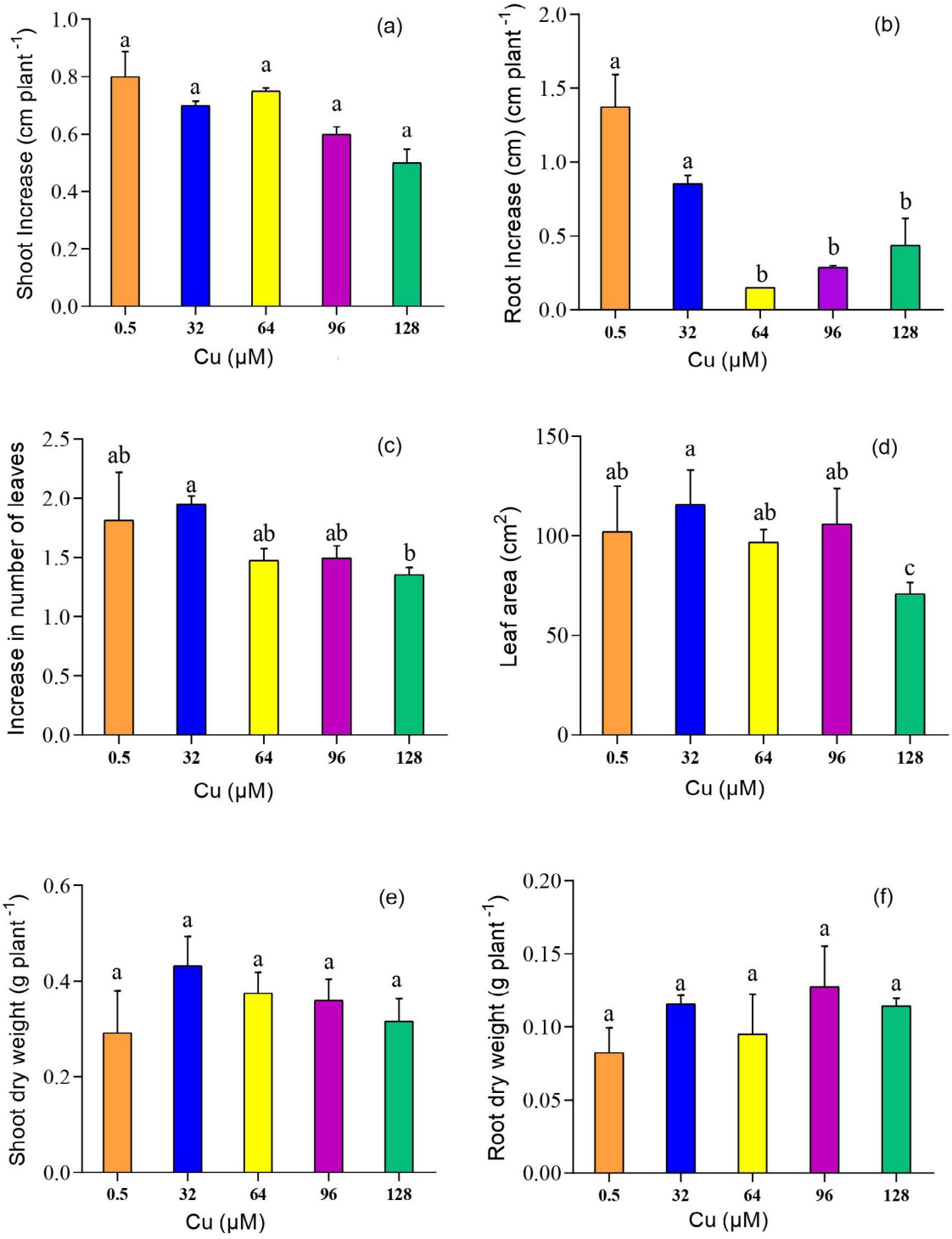

Figure 1: Mean values for the increase in the shoot (SI) (a), root (RI) (b), number of leaves (NLI) (c), leaf area (d), shoot dry weight (SDW) (e), and root dry weight (RDW) (f) in *Handroanthus heptaphyllus* seedlings in response to various Cu concentrations. Different letters represent statistical differences between treatments as determined by Tukey's test with a 5% margin of error. The bars represent the mean ±standard deviation.

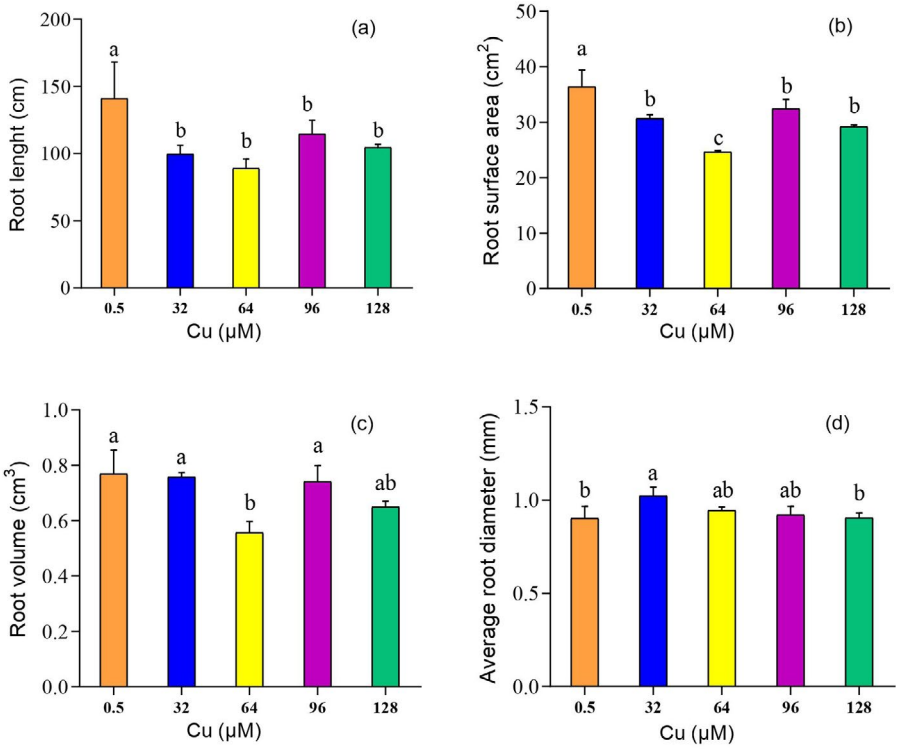

Figure 2: Mean values recorded for root length (a), root surface area (b), root volume (c), and average root diameter (d) in *Handroanthus heptaphyllus* seedlings in response to various Cu concentrations. Different letters represent statistical differences between treatments as determined by Tukey's test with a 5% margin of error. The bars represent the mean ± standard deviation.

The reduced transpiration rate (Figure 4d) brought on by different Cu concentrations may be related to the roots' reduced ability to absorb nutrients due to the toxic effects of Cu on root development (Ambrosini et al., 2018). However, net photosynthetic rate (Figure 4a) and stomatal conductance (Figure 4c) were not correlated because excess Cu did not lead to a drop in intercellular CO_2 concentration (Figure 4b).

Cu can damage the plant photosynthetic apparatus (Tiecher et al., 2018) and cause oxidative stress by disrupting the balance between antioxidant defenses and an increase in reactive oxygen species (ROS) production (Hammerschmitt et al., 2020). In cells, particularly in chloroplasts and mitochondria, ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet-}$), and singlet oxygen ($^1O_2$) are naturally produced (Ferreira et al., 2015) as a result of photosynthetic electron transport and cellular respiration. However, under stress conditions, such as high Cu, the production of ROS is dramatically increased.

Plants exposed to Cu excess commonly activate their enzymatic antioxidant system after ROS formation (Brunetto et al., 2019). The activity of superoxide dismutase (SOD), which is part of the primary defense system of plants to eliminate superoxide anions ($O_2^{\bullet-}$), is frequently reported in this context (Tiecher et al., 2017).

We observed increased SOD activity in roots at the highest Cu concentrations (96 and 128 μM) (Figure 5d). This could be due to an increase in the production of $O_2^{\bullet-}$ radicals in this organ, which could activate the existing enzyme stock (Hassan et al., 2020). Since SOD is involved in the dismutation of the superoxide free radical into H_2O_2, which successfully prevents cell damage, increased SOD activity in roots results in the neutralization of stress-generated free radicals and high H_2O_2 accumulation (Schwalbert et al., 2019). According to Madejón et al. (2009), maize root tips with higher SOD activity had higher Cu tolerance. The activation of the enzyme guaiacol peroxidase (POD) in roots at the same Cu concentrations suggests that POD dismutases the H_2O_2 produced by SOD activity. The root tissues showed a similar response pattern for SOD and POD (Figure 5d and Figure 5f), H_2O_2 concentration (Figure 3b), and Cu concentration in root tissue (Figure 3f).

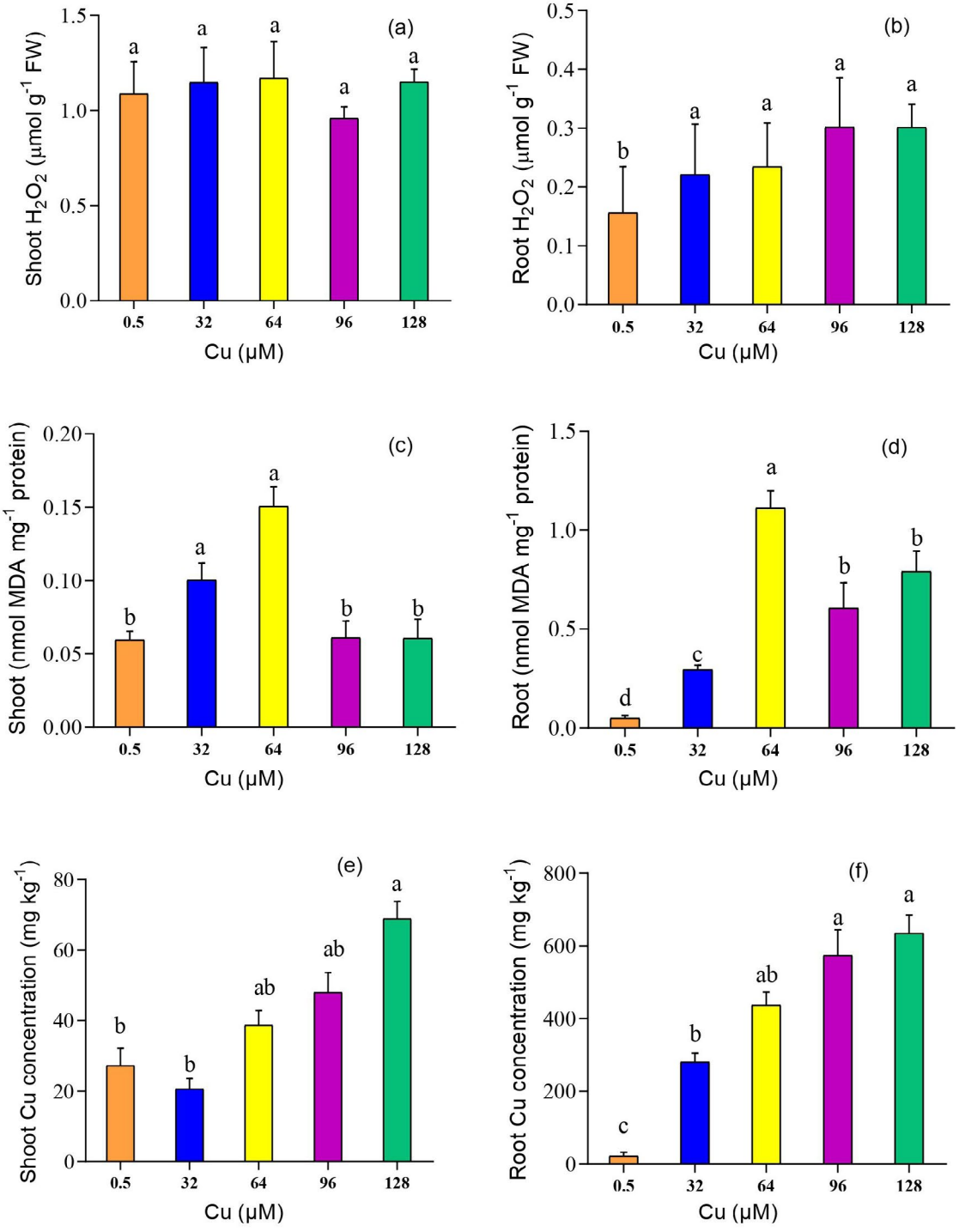

Figure 3: Mean values hydrogen peroxide (H_2O_2) concentration in the shoot (a) and root (b), membrane lipid peroxidation in shoots (c) and roots (d), Cu concentration in the shoot (e) and root (f) tissues of *Handroanthus heptaphyllus* seedlings in response to various Cu concentrations. Different letters represent statistical differences between treatments as determined by Tukey's test with a 5% margin of error. The bars represent the mean ±standard deviation.

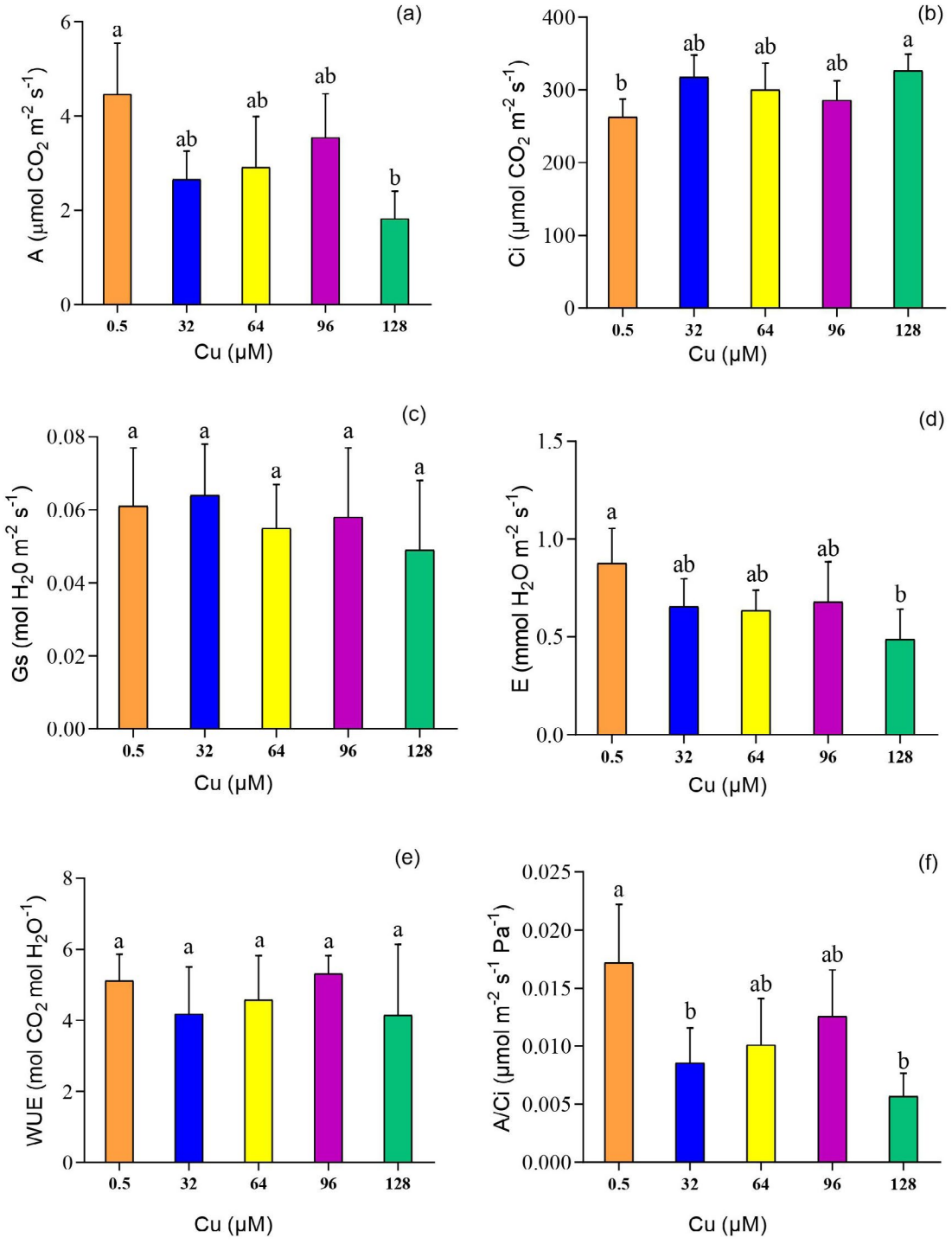

Figure 4: Mean values of CO_2 net assimilation rate (A) (a), CO_2 intercellular concentration (Ci) (b), stomatal conductance (Gs) (c), transpiration rate (E) (d), water use efficiency (WUE) (e), and instantaneous carboxylation efficiency (by Rubisco) (A/Ci) (f) in *Handroanthus heptaphyllus* seedlings in response to various Cu concentrations. Different letters represent statistical differences between treatments as determined by Tukey's test with a 5% margin of error. The bars represent the mean ±standard deviation.

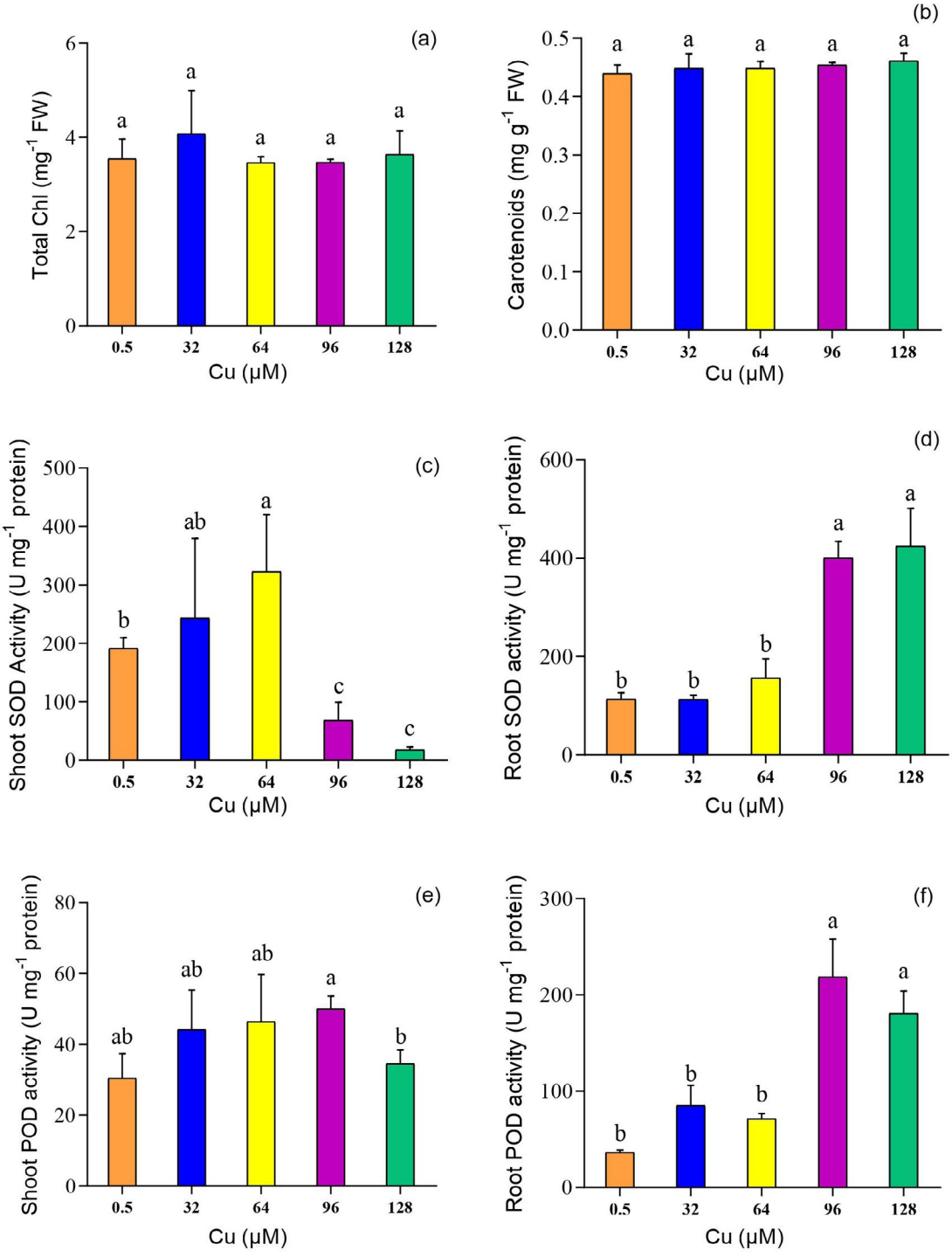

Figure 5: Mean values total chlorophyll (Total chl) (a), carotenoids (b), superoxide dismutase (SOD) activity in shoots (c) and roots (d), and guaiacol peroxidase (POD) activity in shoot tissues (e) and root tissues (f) of *Handroanthus heptaphyllus* seedlings in response to various Cu concentrations. Different letters represent statistical differences between treatments as determined by Tukey's test with a 5% margin of error. The bars represent the mean ± standard deviation.

However, the shoot tissue of *H. heptaphyllus* seedlings did not show an increase in H_2O_2 concentration (Figure 3b). This finding suggests that the presence of antioxidants in the roots is more vital than in the shoot because SOD and POD activity is lower in the shoot, which leads to less H_2O_2 buildup. It could be because Cu causes less stress in the shoot than in the roots, which are the primary target in plants and are more vulnerable to Cu damage, necessitating a stronger response from the antioxidant system. Large-scale H_2O_2 buildup is highly detrimental to cellular metabolism. POD converts H_2O_2 into water and oxygen by dismutating H_2O_2, thus, playing an essential role in providing tolerance to unfavorable conditions in plants (Bernardy et al., 2020).

POD activity in the shoot did not differ between the control treatment and the other Cu concentrations (Figure 5e). Studies have shown that both increases and decreases in enzyme activities are frequently seen in response to heavy metal exposure (Zhao et al., 2021). The increase in POD activity in roots (Figure 5f) may be due to Cu stress stimulation and increased substrate concentration, as SOD was activated in roots and released more H_2O_2. However, the increased POD activity in the roots (Figure 5f) was insufficient to prevent higher H_2O_2 production in these organs by adding Cu (Figure 3b), as the increase was quite steep. The observed increase in H_2O_2 under Cu stress is likely responsible for lipid peroxidation, as evidenced by the excessive accumulation of MDA.

The concentration of membrane lipid peroxidation (MDA) in roots increased significantly with increasing Cu in the nutrient solution (Figure 5d). The rise in MDA may be a direct effect of Cu toxicity, indicating oxidative stress in *H. heptaphyllus* seedlings and possibly resulting in irreparable harm to long-term plant tissue development and function.

Excessive Cu availability can significantly alter membrane permeability besides impairing the functionality of transmembrane and ion channels (Marastoni et al., 2019), leading to a nutrient imbalance in heavy metal-loaded environments (De Conti et al., 2019). We conclude that different Cu concentrations can alter the balance of plasma membrane permeability, increase the production of ROS, and induce the expression of antioxidant proteins as an adaptive response to neutralize excess ROS and minimize damage.

Cu had no detrimental effects on shoot and root dry weight, increase in leaf number, or photosynthetic pigments. Moreover, the net assimilation and transpiration rates in *H. heptaphyllus* dropped only after 128 µM Cu. This result supports our original theory and shows that this plant is tolerant to high Cu concentrations.

CONCLUSIONS

The species *Handroanthus heptaphyllus*, in general, was affected by excess Cu only at concentration of 128 µM. Copper was primarily accumulated in the roots without a concomitant decrease in root and shoot biomass, which may indicate that species tolerance to this metal has significant potential for the phytostabilization of Cu-contaminated soils.

AUTHOR CONTRIBUTION

Conceptual Idea: Kuinchtner, C. C.: Tabaldi, L. A.; Methodology design: Kuinchtner, C. C.; Aguilar, M. V. M.; Data collection: Senhor, D. F.; Birck, T. P.; Data analysis and interpretation: Senhor, D. F.; Birck, T. P.; and Writing and editing: Kuinchtner, C. C.: Aguilar, M. V. M.: Brunetto, G.: Tabaldi, L. A.

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