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Root morphology and leaf gas exchange in *Peltophorum dubium* (Spreng.) Taub. (Caesalpinioideae) exposed to copper-induced toxicity

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ABSTRACT

Excess copper (Cu) in the soil causes physiological and morphological changes in plants. The objective of this research was to evaluate root morphology and photosynthetic responses in *Peltophorum dubium* plants exposed to excess Cu. Growth, leaf gas exchange, root morphology and bioaccumulated Cu in the different plant tissues were evaluated. Excess Cu reduced plant height and diameter, and altered root system morphology in *P. dubium*. Length, surface area, mean diameter and root volume also decreased with excess Cu treatment. The effect of excess Cu on roots was observed by the inhibited development of very fine roots. In general, accumulation of Cu occurred mainly in the root system, and this heavy metal in excess inhibited the growth of *P. dubium*. As a consequence of Cu toxicity, there was a reduction in root and shoot dry weight, as well as significant affects on photosynthesis (A and A_{max}) and transpiration (E) that were dependent on Cu concentration.

1. Introduction

Copper (Cu) is a micronutrient required for plant growth and metabolism, as well as functioning as a structural component for some enzymes (Yruela, 2013; Küpper and Andresen, 2016). Cu can influence all phases of the plant life cycle, both through deficiency or excess in soils. In addition, Cu excess can cause physiological, biochemical and morphoanatomical changes in plants, leading to reduced development (Küpper and Andresen, 2016; Gautam et al., 2016). Disorders in photosynthesis (Cambrollé et al., 2013), oxidative stress and the production of reactive oxygen species (ROS) (Adrees et al., 2015), lipid peroxidation (Thounaojam et al., 2012), changes in N metabolism (Yruela, 2013), remodeling of the root system (Batool et al., 2015; Ivanov et al., 2016; Marques et al., 2018a), reduction in carbon fixation (Küpper and Andresen, 2016) and reduction or inhibition of plant development are consequences of Cu toxicity (Cambrollé et al., 2015; Marco et al., 2016). However, it is noteworthy that metal sensitivity and toxicity depend on the plant developmental stage (Zortéa et al., 2016).

Heavy metal pollution is a serious environmental problem because they are non-biodegradable and can accumulate in the environment, which leads to contamination of the food chain. In addition, severely metal-polluted soils frequently lack vegetation and consequently result in soil erosion and off-site pollution (Ali et al., 2013). The use of different tree species can be an alternative method for soil recovery, immobilizing the heavy metal in plant tissues. Plants that grow in contaminated soils adapt tolerance mechanisms ranging from metal exclusion (non-absorption) to metal sequestering in specific tissues (Viehweger, 2014). In addition, understanding morphophysiological responses of plants exposed to heavy metals is important for the selection and identification of potential species that could be used in the recovery of contaminated areas.

Peltophorum dubium (Caesalpinioideae) is a native Brazilian tree with a wide geographic distribution. These plants are rustic and have good resistance to various environmental conditions (Alves et al., 2011). Due to their adaptive and tolerant characteristics in a wide range of environments, *P. dubium* can be used in recovery or revegetation strategies for degraded areas and in phytoremediation programs in both tropical and temperate environments. Therefore, the objective of this research project was to evaluate root morphology and photosynthetic responses of *P. dubium* exposed to excess Cu.

2. Material and methods

2.1. Experimental design

The experiment was carried out at the Institute of Agricultural Sciences of the Universidade José do Rosário Vellano (UNIFENAS), in

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Alfenas, MG, Brazil, which is located at the geographic coordinates: 21° 25′ 45″ S, 45° 56′ 50″ W, and average altitude of 881 m.

Peltophorum dubium plants ("Canafistula") were used in experiment assembly. A randomized block design (RBD) was used and the treatments consisted of four copper (Cu) concentrations, 0 (control), 100, 200 and 400 mg kg^{-1}, and were conducted in four replicates. Copper sulphate ($CuSO_4.5H_2O$) was used as a source at 25% Cu.

Seeds were sown in 180-g tubes filled with Plantmax® commercial substrate and 3 kg m^{-3} of Yorim Master® basic fertlizer (16% P_2O_5, 18% Ca, 7% Mg). Three side dressing fertilizations were provided at 45, 60 and 90 d after sowing with 20 g N and 15 g K_2O, using urea and potassium chloride as sources. These compounds were dissolved in 10 l water and applied at 10 ml solution per plant.

The soil of the subsurface layer was used for chemical characterization (Table 1). In a 360 dm^3 soil sample, 3.6 kg earthworm humus, 160 g limestone and 230 g single superphosphate were added. After 120 d, *P. dubium* plants were transferred to pots (18 kg) and kept in a greenhouse with a 150-μm plastic cover and a black photoconverter on the sides to provide 50% shading. During the experiment, soil moisture was controlled every two days by weighing the pots and replacing the water so that the soil moisture was maintained at 70% of the retention capacity. Thirty days after transplanting (150-day-old plants), copper treatment solutions were applied to the soil surface as $CuSO_4{\bullet}5H_2O$. Sixty days after the application of copper (210 days old plants), nitrogen fertilization was applied at a dose of 10 mg kg^{-1} N in the form of urea.

2.2. Biometric measurements

Plant height, stem diameter, and root and shoot dry weight were evaluated at 120 days after Cu application (270 days old plants). Measurements for plant height and stem diameter were made using a digital caliper and a tape measure. Three roots were measured per treatment at the end of the experiment (120 days) to evaluate changes in root morphology. After the washing process, plants were separated into root systems and shoots, at collar height. The image analysis system WinRhizo Pro 2007a (Regent Instruments, Sainte-Foy, QC, Canada) was coupled to a professional scanner (Epson, Expression 10000 XL, Epson America, Inc., USA) equipped with an additional light unit (TPU) to evaluate root morphology. To obtain images, we followed the method previously described by Souza et al. (2012), which were then used to measure the following characteristics: root length (cm), root surface area (cm^2), root mean diameter (mm) and root volume (cm^3). Three root categories were defined according to Bohm (1979): very thin roots (VTR) with a diameter less than 0.5 mm, thin roots (TR) with a diameter between 0.5 and 2 mm and thick roots (THR) with more than a 2-mm diameter. Root length, surface area and volume were measured within each category. The roots were then stored in paper bags and transported to a forced air oven set at 72 °C until a constant mass was obtained.

2.3. Gas exchange

Gas exchange parameters were measured using a portable photosynthesis system (IRGA, LI-6400XT, Li-Color, Lincoln, Nebraska, USA), with an artificial light chamber (LI-6400-02B RedBlue, Li-Cor) at 15 (initial) and 120 (final) days of cultivation (165- and 270-day-old plants, respectively) after copper application to the soil. Measurements were performed in the morning from 09.00 to 11.00 on fully expanded leaves at the fourth node counting from the apical region. The parameters for leaf photosynthesis rate (A) and transpiration (E) were measured. Measurements were performed on a 6-cm^2 leaf area and the airflow in the chamber was at a CO_2 concentration of 380 mmol mol^{-1}. The air was collected from outside the greenhouse and mixed with one gallon of buffer and then pumped into the chamber. A photon flux density (PPFD) of 1100 μmol m^{-2} s^{-1} with a blue-red LED light source was used and the chamber temperature was maintained at 28 °C.

The curves of the net photosynthetic rate (A) in response to the flux of photosynthetically active photons (PAR) were also determined by the same portable photosynthesis system. All measurements were performed on the same leaf used for the spot measurements using three plants per treatment. The PARs of 1600, 1200, 800, 200, 100, 50, 25 and 0 μmol m^{-2} s^{-1} were implemented for 5 min at 28 °C, with ambient CO_2 conditions of approximately 380 μmol mol^{-1}. The curves were performed at 15 (initial) and 120 (final) days of cultivation. Data on the curves were adjusted to the rectangular hyperbolic function: $A = a + [(A_{max} \times PAR)/(b + PAR)]$, where A_{max} is the maximum net photosynthetic rate and 'a' and 'b' are adjustment coefficients of the equation. Using the curve, the following parameters for maximum net photosynthetic rate (A_{max}) and dark respiration rate (R_d) were measured in leaves adapted to the dark, light compensation point (LCP) and light saturation point (LSP).

2.4. Copper contend in soil and plant tissues

Soil analysis was performed during the initial planting and the harvesting of the plants. The initial analysis (Table 1) was performed in a composite sample for soil chemical characterization (pH, P, K, Ca Mg, Al, Organic Matter, Fe Mn, Zn, Cu) according to Raij et al. (2011). To analyze the plants at harvesting, exchangeable Cu ions were extracted with DTPA (Raij et al., 2011) from soil samples of each pot. Nitroperchloric digestion was conducted according to Carmo et al. (2000) by analysis of Cu in plant leaf, stem and root tissues. Cu content in soil extracts and plants samples were measured with atomic absorption spectroscopy (AAS) (Carmo et al., 2000).

2.5. Data analysis

For all analyzed parameters, the means and standard error (SE) were calculated. For statistical analysis of significant results, linear regression, analysis of variance (ANOVA) and the Skott-Knott test at 5% significance ($p \leq .05$) was consucted using the Sisvar program version 4.3 (Universidade Federal de Lavras, Lavras, Brazil).

3. Results

3.1. Biometric measurements

The growth of *P. dubium* was affected by excess Cu. Increased Cu concentrations in the soil resulted in a linear decrease in height (H), stem diameter (SD), shoot and root dry weight ($p \leq .05$) (Fig. 1). *P. dubium* grown in soil with 400 mg kg^{-1} Cu had a reduced growth of 1.4× for H, 1.3× for SD, and 2.4× and 2.7× for shoot and root dry weight, respectively, when compared to control plants (Fig. 1).

Table 1
Chemical characterization of the soil used in the experiment.

pH (H_2O)	P (Mehlich)	K	Ca^{+2}	Mg^{+2}	Al^{+3}	SB*	V*	M.O.*	Zn	Fe	Mn	Cu
	mg kg^{-1}	$cmol_c$ kg^{-1}					%	dag kg^{-1}	mg kg^{-1}			
5.7	0.4	0.2	0.5	0.3	0.1	1.0	29	0.9	0.2	3.0	2.5	0.2

* Sum of bases (SB); base saturation index (V); organic matter (M.O.).

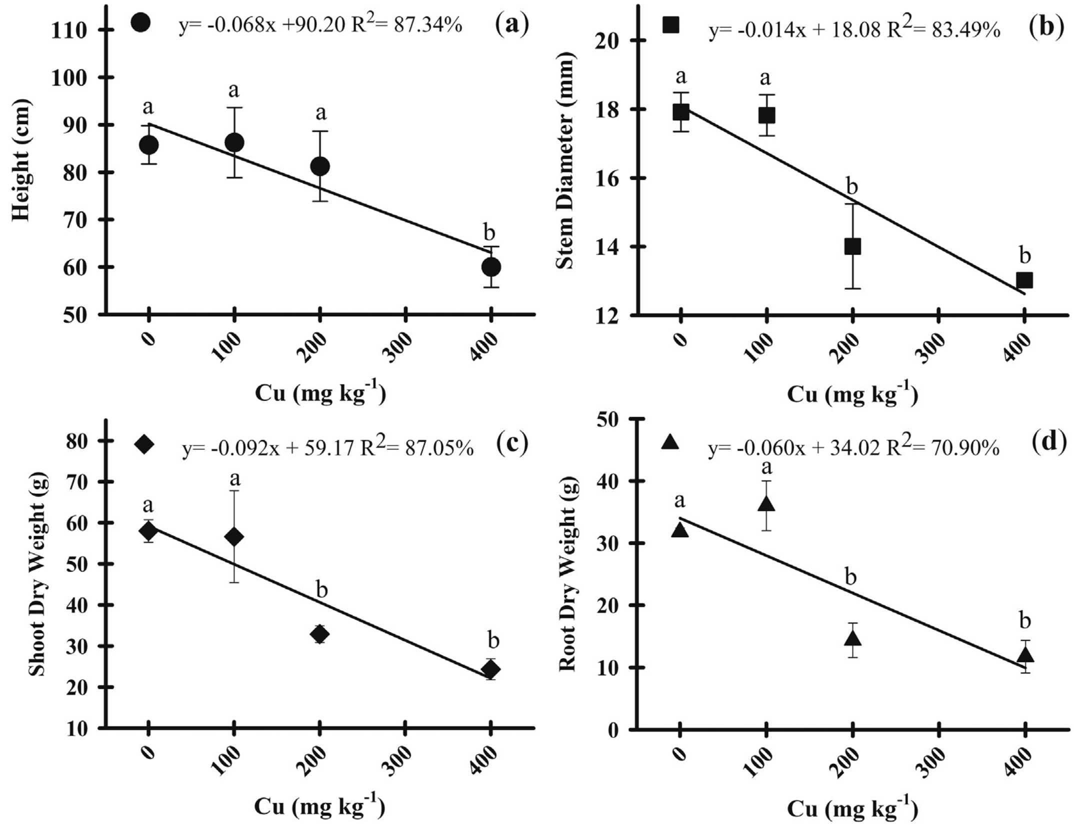

Fig. 1. (a) Height (H), (b) stem diameter (SD), (c) shoot dry weight and (d) dry weight of *P. dubium* roots that were exposed to different concentrations of Cu that was applied to the soil ($p \le .05$). Means between treatments followed by the same letter are not statistically different by the Skott-Knott test at 5% probability ($p \le .05$). Each value indicates mean ± SE.

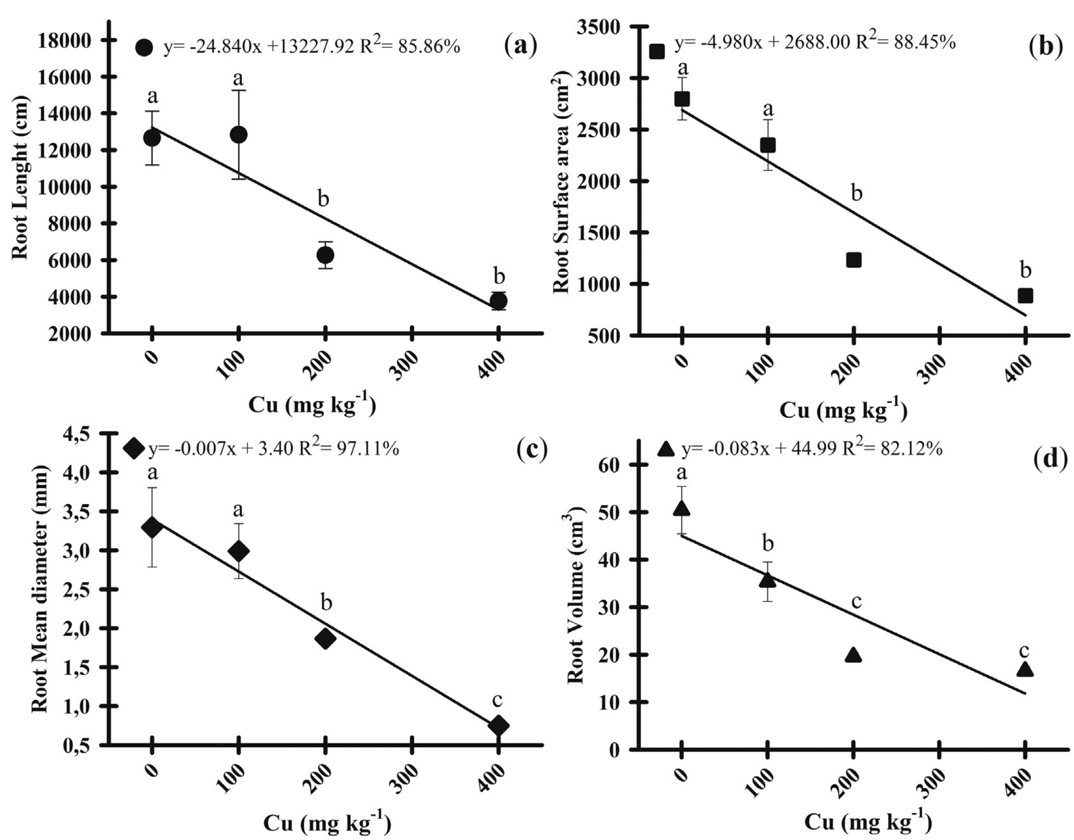

Fig. 2. Root and dry weight characteristics of *P. dubium* roots including the (a) length, (b) surface area, (c) mean diameter and (d) volume after plants were exposed to different copper concentrations ($p \le .05$). Means between treatments followed by the same letter are not statistically different by the Skott-Knott test at 5% probability ($p \le .05$). Each value indicates mean ± SE.

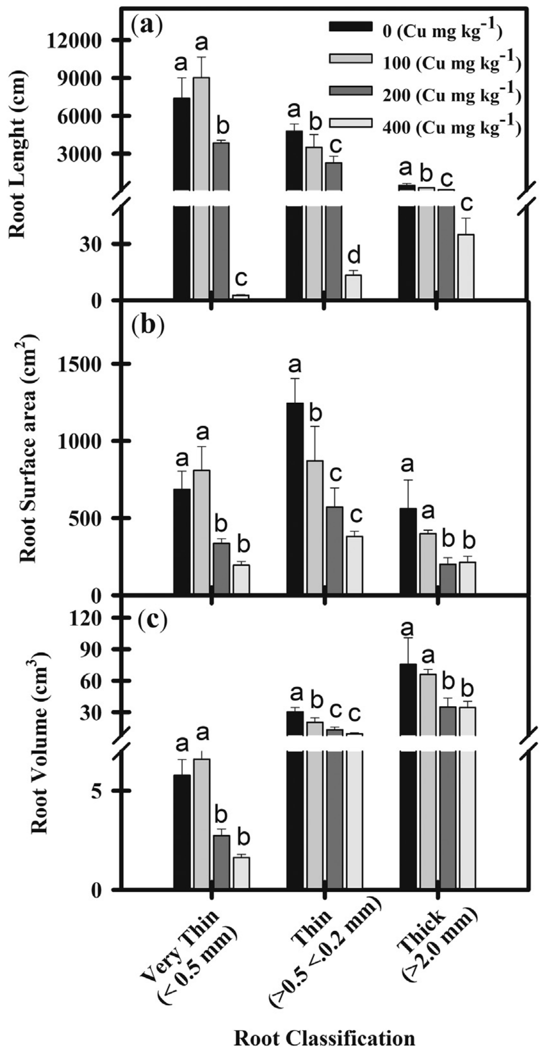

Fig. 3. (a) Root length, (b) root surface area and (c) root volume organized by diameter class in *P. dubium* exposed to different Cu concentrations. Means between treatments followed by the same letter do not statistically differ from one another by the Skott-Knott test at 5% probability ($p \le .05$). Each value indicates mean ± SE.

3.2. Root morphology

As soil Cu concentrations increased, there was a linear decrease in length, surface area, mean diameter and root volume in *P. dubium* ($p \le .05$) (Fig. 2). Plants grown in soil that contained 200 and 400 mg kg^{-1} Cu had reduced root length (2.5×), root surface area (2.4×) and root mean diameter (2.4×) compared to plants treated with 100 mg kg^{-1} Cu or the control treatments (Fig. 2a–c). In addition, plants exposed to 400 mg kg^{-1} Cu presented a significant reduction in their root volume, which was 3× less than that measured in the the control plants (Fig. 2d).

In the distribution of length, surface area and volume by diameter classes of *P. dubium* roots, a behavior similar to the previous parameters was observed (Figs. 2 and 3), with Cu increasing in the soil, causing a decrease in the evaluated parameters. Approximately 90% of the *P. dubium* root system consists mainly of very thin roots (<0.5 mm). These thin roots were significantly reduced in length, surface area and volume (Fig. 3a–c) when grown in soil with 200 and 400 mg kg^{-1} Cu compared to control plants ($p \le .05$). Low Cu treatment (100 mg kg^{-1}) affects negatively TR and THR, but improved VTR, although this later effect was not significant. (Fig.3). In addition, the number fine (TR) and thick roots (THR) were also reduced with the application of 400 mg kg^{-1}Cu compared to control conditions (Fig. 3a–c).

3.3. Gas exchange

Excess Cu in the soil after 15 d (initial) of cultivation negatively affected all gas exchange parameters (A, E, A_{max}, R_d, LPC, LPS) in *P. dubium*, and these affects were the greatest at a concentration of 400 mg kg^{-1} (Fig. 4; $p \le .05$). However, after 120 d of cultivation, no significant difference observed in any of the evaluated parameters between the different Cu doses tested (Fig. 4; $p \le .05$).

3.4. Copper content in soil and plant tissues

After plants were harvested, Cu content available in the soil in the collected samples increased, ranging from 3.56 to 85.48 mg kg^{-1} (Fig. 5). Higher levels of Cu were observed in the root systems of *P. dubium* compared to that in the other tissues such as the stem and leaf (Fig. 6). In general, as the Cu concentration increased in the soil, Cu content increased in the plant tissues as well.

4. Discussion

Copper toxicity was detrimental to the development of *P. dubium*, indicated by limited plant growth in plants grown in soil supplemented with exogenous copper. The increased Cu content in soil negatively affected all parameters analyzed in *P. dubium* (Fig. 1). High concentrations of Cu can affect plant morphology, including shoot and root growth in several plant species (Adrees et al., 2015. The deleterious effects of excess Cu caused a reduction in the formation of primary and lateral roots, which reflected a decrease in root length, surface area, mean diameter and volume (Fig. 2). Batool et al. (2015) reported that the decrease in the growth of the root system could be due to a reduction in cell division, which leads to an increase in root cell wall thickness when plants are exposed to heavy metals (Viehweger, 2014). In addition, Sheldon and Menzies (2005) described Cu toxicity as detrimental to plant roots, based on their observations of symptoms that ranged from root cuticle tearing and root trichome reduction, to severe deformation of root morphology, which could be due to the root being the first organ that comes into contact with the heavy metal contaminant. Excess Cu can result in a metabolic imbalance that leads to an increase in reactive oxygen species (ROS) and lipid peroxidation in cell membranes (Yruela, 2013) that cause root structure deformation. The VTR are important for the absorption of water and nutrients (Souza et al., 2012). In low Cu treatments, plants invest more in the formation of VTR compared to the other drivers of root diameter. However, we report that high Cu concentrations (200 and 400 mg kg^{-1}) affected root length development, surface area and volume of VTR (Fig. 3). These results are consistent with the high sensitivity of VTR to nutrient and soil conditions in the presence of high Cu (Feigl et al., 2013).

Low root volume indicates that the plant has limited soil exploration and reduced absorption of water and nutrients, which consequently limit shoot development and is consistent with the reduced dry weight of the plants grown in increased Cu conditions compared to the control (Figs. 2 and 3). Ivanov et al. (2016) reported that the toxic levels of Cu significantly inhibited growth and development in *Pinus sylvestris* L., which led to the loss of the main taproot as well as the inhibition of lateral root development, indicating that Cu toxicity has a negative effect on root system architecture.

The limited shoot and root development in *P. dubium* grown in high Cu concentrations (Figs. 1–3) can also be explained by the deleterious effects CY toxicity has on photosynthetic pigment biosynthesis, cell membrane permeability, homeostasis and nutrient balance (Adrees et al., 2015; Gautam et al., 2016). In addition, lower photosynthetic (A and A_{max}) and transpiration (E) rates may be related to excess Cu ions disturbing the electron transport chain during the photochemical phase of photosynthesis, which may lead to oxidative stress and an increase in reactive oxygen species (ROS) that inhibit cellular metabolic processes (Sytar et al., 2013). Similarly, high levels of Cu ions can

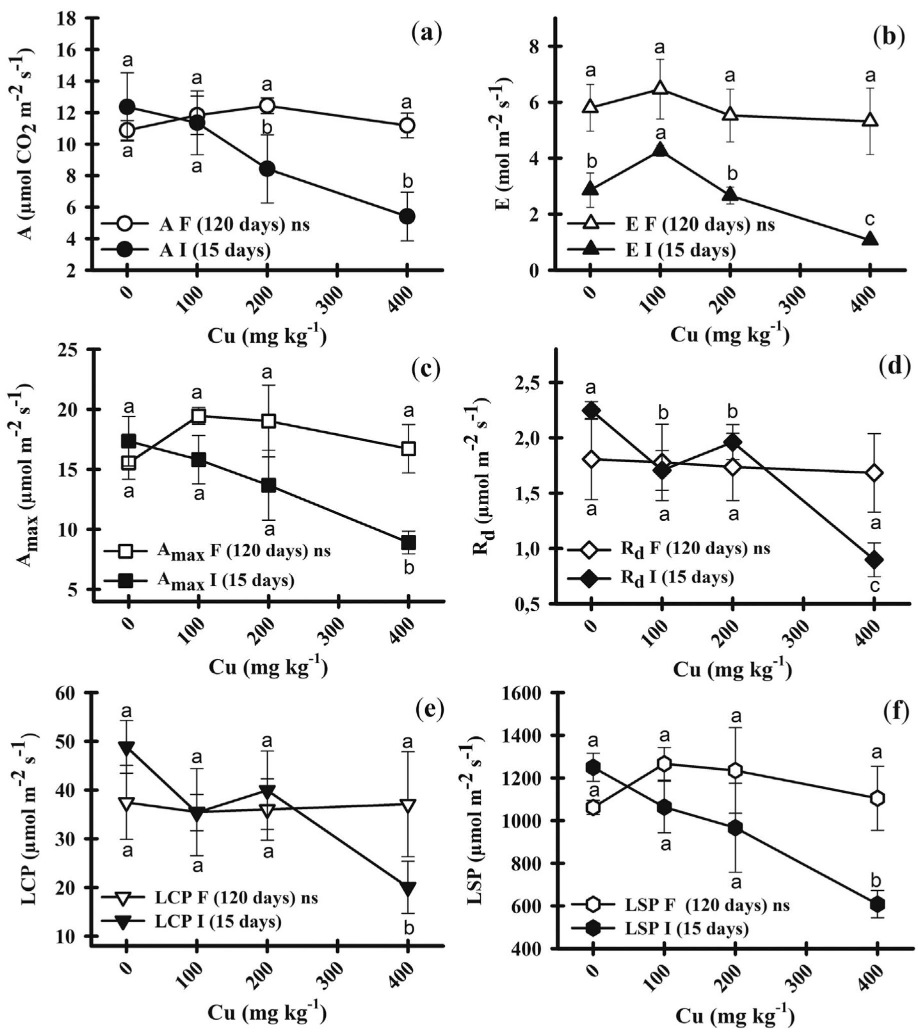

Fig. 4. Gas exchange in *P. dubium* exposed to different Cu concentrations. (a) photosynthesis A, (b) transpiration E, (c) maximum net photosynthetic rate A_{max}, (d) dark respiration rate R_d, (e) light compensation point LCP, (f) light saturation point LSP, initial (I) and final (F) measurements. Means between treatments for each initial and final analysis followed by the same letter are not statistically different by the Skott-Knott test at 5% probability (p ≤ .05). Each value indicates mean ± SE.

decrease carbon fixation and energy production, which have directly reduce or inhibit plant development (Küpper and Andresen, 2016).

The reduction of A and A_{max} in the initial growth period (Fig. 4a, c) can also be attributed to protein denaturation of the antenna complex or the direct inhibition of the FSII reaction center by Cu insertion into pheophytin (Yruela, 2009), which leads to the impairment of PSII electron donation (Bazihizina et al., 2015). Another possible explanation for effected/reduced A and A_{max} is the increase in fluorescence (Zhang et al., 2013), which may lead to decreased photosynthesis at higher Cu concentrations. In addition, DalCorso et al. (2014) reported that Cu toxicity increases plant susceptibility to photoinhibition, and the decrease in the transpiration (E) we observed in our plants (Fig. 4b) may be related to stomatal closure due to Cu-dependent increase in stomatal resistance to CO_2 absorption in the mesophyll (Bazihizina et al., 2015). All these results may also be related to the high levels of Cu that alter the photosynthetic metabolism in the leaves, which are responsible for the plant's energy supply (ATP and NADPH). Changes in energy supply could alter the balance of plant development.

After 15 d of treatment with increased soil Cu concentrations, we observed a reduced light compensation point (LCP), light saturation point (LSP) and dark respiration rate (Rd) in plants compared to the control treatment (Fig. 4e, f,). The decrease in both LCP, which reflects the equilibrium between CO_2 uptake and release, and LSP, which reflects the ability of the plant to use the highest light levels (i.e. photons), is possibly related to the toxic levels of Cu in the soil that result in a large amount of intracellular ions and damage the photosynthetic system responsible for photochemistry. These results indicate that excess Cu is an effective photosynthesis inhibitor (Fig. 4a and c), similar to results found by Han et al. (2006) in their study on heavy metal effects on *Betula schmidtii*.

Cu-treated plants had increased A and A_{max} compared to the control plants at 120 d (Fig. 4a and c), but this affect did not result in increased

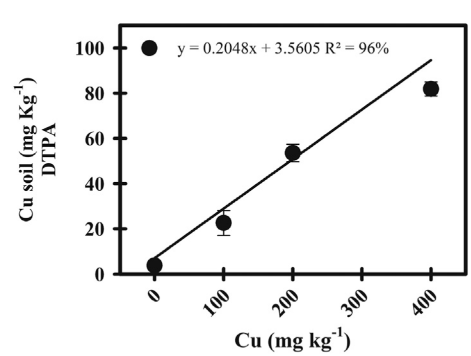

Fig. 5. Cu content in the soil after *P. dubium* was harvested, as a function of the Cu concentrations. Means between treatments followed by the same letter do not differ statistically by the Skott-Knott test at 5% probability (p ≤ .05). Each value indicates mean ± SE.

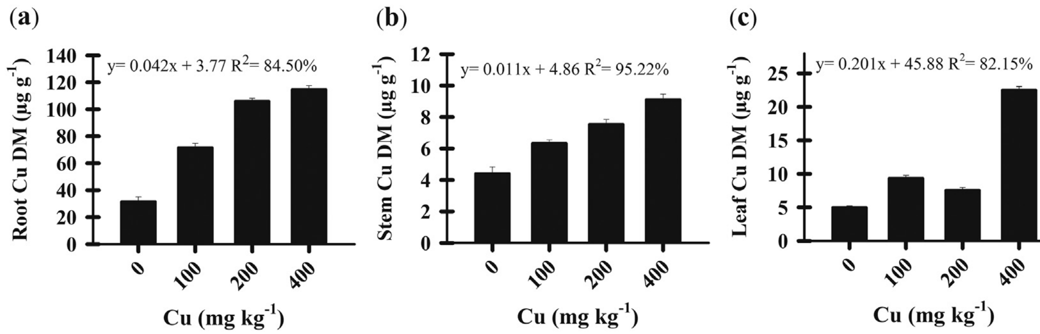

Fig. 6. Bioaccumulated Cu in the roots, stems, and leaves of *P. dubium* exposed to different exogenous Cu treatments. Cu bioaccumulation was measured from the total mass of *P. dubium* plants ($p \leq .05$). The equation illustrates the correlation of the Cu treatments with the amount that accumulated in the plants.

plant growth (Fig. 1a). This finding may be explained by plant defense mechanisms against toxicity that use photosynthetic energy for the production of enzymes and antioxidant compounds than for plant growth. According to Gill and Tuteja (2010), ROS influence the expression of several genes that regulate growth, cell cycle, programmed cell death, abiotic stress response, and plant development, which is consistent with the results of this research.

Clay soils that contain above 100 mg kg $^{-1}$ Cu are considered toxic for most plants (Silva et al., 2011) and can cause a reduction in plant development. In the present work, we detected values of 4, 24, 45 and 86 mg kg $^{-1}$ when 0, 100, 200 and 400 mg kg^{-1} Cu was added to the soil, respectively, indicating that on average, 21% of the Cu detected in the soil was in an available form at the end of the experiment. The Cu that was retained in the soil was converted into unavailable forms through specific adsorption to produce oxides and complexation reactions by the organic matter of the soil (Joris et al., 2012).

The Cu that could bioaccumulate in *P. dubium* plant tissues did so mainly in the roots (Fig. 6). Marques et al. (2018a, 2018b) reported that tolerant plants limit Cu transport from the roots to the shoots. This is a strategy adopted by plants as a defense mechanism against toxic elements; they detoxify by excluding free ionic forms of the metal from the cytoplasm. This accumulation of the metal in the root system is preventative response that helps preserve the photosynthetic apparatus present in the leaves (Marco et al., 2016). The strategy commonly used by plants to limit heavy metal toxicity is to hinder the entry of excess metal ions into the root cells by trapping them in the apoplast, where they are bound to exudate organic acids or to anionic groups in cell walls (Rascio and Navari-Izzo, 2011). Heavy metals that get absorbed by root cells can also be detoxified through complexation with amino acids, organic acids or peptides, and/or sequestration in the vacuoles (Adrees et al., 2015; Wang et al., 2016). Apoplastic barriers in the endodermis and exodermis can also limit metal translocation to the shoot (Gomes et al., 2011; Freitas et al., 2015). Cell wall thickening of the root system occurs mainly in the exodermis and endodermis to provide a larger retention area of heavy metals, thus reducing their translocation to the shoot (Adrees et al., 2015). In the present work, it is worth mentioning that *P. dubium* exposure to Cu was sufficient to cause a reduction in plant growth, especially in the highest soil concentrations (200 and 400 mg kg $^{-1}$).

Excess Cu in the soil negatively affected the growth of *P. dubium*; however, the plants were able to survive even when exposed to high concentrations of this heavy metal in the soil. The results from our analysis suggest that *P. dubium* is a good candidate for revegetation strategies in areas contaminated by copper.

5. Conclusion

High concentrations of copper in the soil caused reduced growth and foliar gas exchange in *Peltophorum dubium* plants. The increased Cu inhibited the development of the root system, especially that of the very fine roots, which altered root morphology of this species. The accumulation of Cu occurred mainly in the root system of these plants.

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