

# The Dynamics of Macro- and Micronutrients in Native Tree Species Affected by Copper Contamination

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## Abstract

This study was conducted to assess the physicochemical characteristics of urban forest soil contaminated by copper and the dynamics of macro- and micronutrients uptake by *Schinus terebinthifolia* and *Eugenia uniflora* seedlings. The seedlings received 0 (control), 60, 120, 180 or 240 mg Cu kg<sup>-1</sup> soil applied to urban forest soil within São Paulo City, Brazil. Our results showed that K was reduced in Cu-contaminated soil used for *S. terebinthifolia* cultivation and that organic matter was higher in Cu-contaminated soil used for *E. uniflora* cultivation. Other physicochemical properties of soil remained unaltered. *S. terebinthifolia* presented nutritional imbalances in N, K and Mg on leaves, while *E. uniflora* presented nutritional imbalances in K on leaves and in S on roots. It can be concluded that copper contamination can negatively affect chemical and nutritional characteristics of urban forest soil, as well as the nutritional dynamics of *S. terebinthifolia* and *E. uniflora*.

**Keywords:** *Schinus terebinthifolia*, *Eugenia uniflora*, pollution, heavy metal, nutrition.

## 1. INTRODUCTION AND OBJECTIVES

Copper (Cu) is an essential micronutrient for plant growth and development, playing key roles in several physiological processes, such as cell respiration, photosynthesis, ATP generation (Marques et al., 2018), participation in redox reactions and in oxidative stress protection (Rehman et al., 2019). Despite its necessity, copper in high concentrations can be toxic to plants, inducing adverse effects on plant physiology and biochemistry, such as excessive formation of reactive oxygen species (ROS) (Saleem et al., 2020), nutritional imbalance (Zeng et al., 2019), reduced growth and morphological alterations (Hossain et al., 2020).

Many factors influence the uptake of nutrients by terrestrial plants, such as temperature, pH and aeration of soils, organic matter, and soil contaminated with heavy metals (Hu et al., 2018). In general, excess copper in soil can disrupt plant nutritional metabolism by inhibiting the uptake of nutrients from soil and interfering with nutrient translocation from roots to shoots (Kumar et al., 2020). However, the degree of nutritional imbalance caused by Cu toxicity in plants is dependent on environmental factors, including the concentration

and bioavailability of Cu in soil, length of exposure to heavy metal, and environmental growth conditions, as well as the morphophysiological characteristics of each plant species (Adrees et al., 2015).

In recent years, the accumulation of copper in soils has become a worldwide environmental concern. Human activities like mining, excessive use of copper-based pesticides, chemical and organic fertilizers, untreated sewage sludge and combustion of fossil fuels increase copper levels in soils, reaching concentrations that present risks for the biota and human health (Farias et al., 2018; Shabbir et al., 2020). Toxic concentration of Cu can be observed in highly urbanized regions, such as large cities, compared to more forested areas or areas with native vegetation, such as urban forest fragments (Argyrazi et al., 2018; Li et al., 2019). Urban forest fragments are remnants of native and secondary forests inserted in the urban environment. These fragments play a specific role in the existence and long-term maintenance of ecosystem services, conservation of biodiversity and habitat for several plant species (Dislich & Pivelo, 2002). However, the sheer proximity of various pollutants places urban forest fragments at risk of particulate material from the emissions of automotive fleets

and industry. These emissions contain heavy metals and can contaminate both soil and ground water (Nakazato et al., 2021; Ferreira et al., 2019). Air pollution and deposition of particulate matter from human activities can also negatively affect the nutritional status of trees, reducing their development in urban forest remnants (Bulbovas et al., 2020).

Studies with tree species have already reported that excess copper in soil can negatively affect plant growth (Marco et al., 2016) and biomass production (Siqueira et al., 2021). It can also lower CO<sub>2</sub> assimilation by reduced efficiency of the electron transport chain (Li et al., 2019). However, the direct effects of copper contamination on urban forest fragment soils, as well as the nutritional consequences observed in Brazilian native tree species from the Atlantic Forest cultivated in polluted soil, remain under investigated. It is herein hypothesized that the addition of increasing concentrations of copper in the soil of an urban forest fragment will alter its nutritional characteristics, thereby affecting the bioavailability of other elements, such as K or Mg, triggering nutritional imbalance in tree species. Therefore,

this study aimed to validate if copper contamination changes the physicochemical properties of experimental plots of urban forest fragment in a manner that directly affects the nutritional dynamics of two tree species native to the Atlantic Forest.

## 2. MATERIALS AND METHODS

### 2.1. Characterization of plants and soil

Soil used in the experiment (Table 1) was a red-yellow latosol (LVA) (Santos et al., 2018) collected between 0.0 and 40.0 cm in depth in a forest area in the Parque Estadual Fonte dos Ipiranga (PEFI), São Paulo City, São Paulo State, Brazil. The experiment was carried out in a greenhouse at the Institute of Botany (23°30'S and 46°40'W; 770 m altitude) located inside the PEFI. Seedlings of the pioneer tree species *Schinus terebinthifolia* Raddi. (aroeira-vermelha) and the non-pioneer tree species *Eugenia uniflora* L. (pitanga), both native to the Atlantic Forest, were used in the experiment.

**Table 1.** Soil chemical composition at the beginning of the experimentation.

pH	O.M. CaCl <sub>2</sub>	P <sub>resin</sub> mg dm <sup>3</sup>	H+Al	K	Ca	Mg	BS	CEC	V%	B	Cu	Fe	Mn	Zn
	g dm <sup>3</sup>	mg dm <sup>3</sup>	mmol <sub>c</sub> dm <sup>-3</sup>							mmol <sub>c</sub> dm <sup>-3</sup>				
5.1	42.0	5.5	33.6	1.6	50.7	9.6	61.9	95.5	64.8	0.6	1.3	25.7	21.3	9.0

Base summatory (BS) = K+Ca+Mg; Cation exchange capacity (CEC) = Ca+Mg+K+Al+H; Base saturation (V%) = BS/CEC\*100

### 2.2. Experimental conditions and design

The seedlings were transplanted into 2.6 L vases containing natural PEFI soil and remained there for 30 days to allow acclimatization. The experiment consisted of the application of 75 ml Hoagland and Arnon solution n.1. (Hoagland & Arnon, 1950) modified with 0 (control), 60, 120, 180 or 240 mg Cu (CuSO<sub>4</sub>·5H<sub>2</sub>O) kg<sup>-1</sup> soil dry weight (DW) (Table 2). Copper concentrations were based on the Environmental Agency of São Paulo State (CETESB, 2016) prevention value for Cu (60 mg Cu kg<sup>-1</sup> DW soil). Ionic balance of the

solutions was carried out in order to maintain the constancy of the other macro- and micronutrients, and pH was adjusted to 5.8. The plants were irrigated weekly with ¼ of the total concentration, repeating the procedure 4 times to obtain the total values of copper in the soil for each treatment. During the experimental period, distilled water was used to irrigate the plants whenever necessary. The experiment lasted 120 days after soil contamination with copper between the spring and summer seasons in Brazil (September to December 2019) with a mean temperature of 26 °C and relative humidity of 56% inside the greenhouse.

**Table 2.** Ion balance of Hoagland and Arnon nutritional solution modified with 0, 60, 120, 180 or 240 mg Cu kg<sup>-1</sup>.

Ion source	Treatments (mg Cu kg <sup>-1</sup> soil DW)				
	0	60	120	180	240
	Released Ions (mmol L <sup>-1</sup> )				
NH <sub>4</sub> <sup>+</sup> - (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	18	13.65669	9.313379	4.970069	0.626759
NH <sub>4</sub> <sup>+</sup> - NH <sub>4</sub> NO <sub>3</sub>	1	3.171655	5.34331	7.514966	9.686621
NO <sub>3</sub> <sup>-</sup> - NH <sub>4</sub> NO <sub>3</sub>	1	3.171655	5.34331	7.514966	9.686621
[NH <sub>4</sub> <sup>+</sup> ] + [NO <sub>3</sub> <sup>-</sup> ]	20	20	20	20	20
SO <sub>4</sub> <sup>2-</sup> - (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	9	6.828345	4.65669	2.485034	0.313379
SO <sub>4</sub> <sup>2-</sup> - CuSO <sub>4</sub> ·5H <sub>2</sub> O	0	2.171655	4.34331	6.514966	8.686621
[SO <sub>4</sub> <sup>2-</sup> ]	9	9	9	9	9
Cu <sup>2+</sup> - CuSO <sub>4</sub> ·5H <sub>2</sub> O	0	2.171655	4.34331	6.514966	8.686621
[Cu <sup>2+</sup> ]	0	2.171655	4.34331	6.514966	8.686621

### 2.3. Analyses of soil and macro- and micronutrient content in plant tissues

After the experimental period, soil samples of each treatment were homogenized, fractioned and oven-dried at 60 °C until constant weight. The pH, total organic matter (O.M.), potential acidity (H+Al) and content of total macro- and micronutrients were determined following the method adopted and described by Raij et al. (2001). Soil pH was potentiometrically determined in 0.01 mol L<sup>-1</sup> CaCl<sub>2</sub>. O.M. was determined by the colorimetric method after soil digestion with sulfochromic solution. H+Al was determined using 1 mol L<sup>-1</sup> calcium acetate at pH 7. Calcium (Ca) and magnesium (Mg) were determined by atomic absorption spectrophotometry (AAS); potassium (K) by flame spectrophotometry; and phosphorus (P) by Ultraviolet-visible (UV/Vis) spectrophotometry after extraction with ion exchange resin. Boron (B) was determined by the colorimetric method (azomethine-H) after extraction with barium chloride. Iron (Fe), manganese (Mn) and zinc (Zn) were determined by atomic absorption spectrometry (AAS) after extraction in DTPA solution at pH 7.3.

Plants were removed from the pots, and the leaves, stems and roots were sectioned. The roots were washed under distilled running water. After oven-drying at 60 °C until constant weight, the plant material was weighed and milled in a knife mill to obtain a homogeneous powder. The total contents of macro- and micronutrients in vegetable material were determined according to the methods described by Malavolta (1997). Cu, Ca, Mg, Zn, Fe, and Mn were determined by atomic absorption spectrophotometry (AAS); P was determined by the ammonium metavanadate colorimetric method; K was determined by flame spectrophotometry; and sulfur (S) was determined by the turbidimetric method (BaCl<sub>2</sub>·2H<sub>2</sub>O) after nitric-perchloric acid digestion. Nitrogen (N) was evaluated by the Kjeldahl method after sulfuric digestion, and B was assessed by the colorimetric method (azomethine-H) after incineration.

### 2.4. Absolute copper content in tissues

The absolute copper content (mg Cu kg<sup>-1</sup> DW) in leaves and roots of *S. terebinthifolia* and *E. uniflora* was determined by multiplying the total copper content in each tissue by the tissue's dry weight (DW) as  $Cu_{\text{tissue}} \cdot DW_{\text{tissue}}$ .

### 2.5. Statistical analyses

The experimental design adopted completely randomized blocks, consisting of 5 blocks with 5 treatments and 20

plants per plot, totaling 100 plants of each species. Data were submitted to Shapiro-Wilk normality test and Brown-Forsythe homoscedasticity test (Tables S1, S2, S3, S4, S5 and S6) using the statistical software GraphPad, v 9.0. If the data were determined to meet assumptions of normality and homogeneity of variance, then they were submitted to analysis of variance (One-Way ANOVA), and means were compared by Tukey's test at 5% probability ( $p \leq 0.05$ ). Otherwise, data were submitted to the Kruskal-Wallis non-parametric test, followed by Dunn's multiple comparison test at 5% probability ( $p \leq 0.05$ ).

## 3. RESULTS

### 3.1. Soil

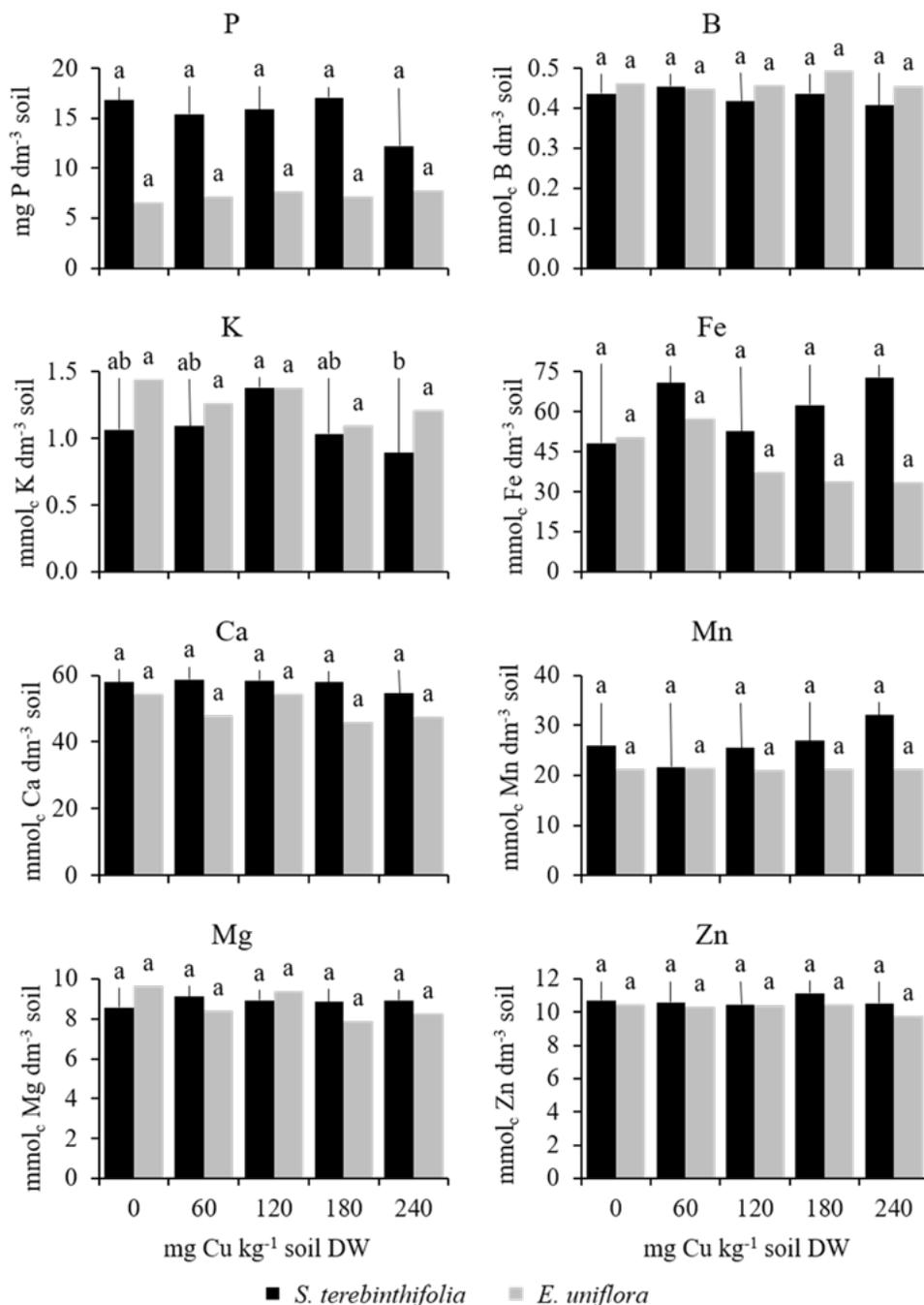
O.M. levels in the soil cultivated with *E. uniflora* showed an increase after treatment with 240 mg Cu kg<sup>-1</sup> when compared to 180 mg Cu kg<sup>-1</sup>, while no change in O.M. levels was observed in the soil cultivated with *S. terebinthifolia* (Table 3). No difference was observed for pH and H+Al among treatments in soil cultivated with *S. terebinthifolia* and *E. uniflora*.

**Table 3.** Chemical properties of soils cultivated with *S. terebinthifolia* and *E. uniflora*. Lowercase letters compare treatments within each species.

Species	Treatments mg Cu kg <sup>-1</sup> soil DW	pH CaCl <sub>2</sub>	O.M. g dm <sup>-3</sup>	H+Al mmol <sub>c</sub> dm <sup>-3</sup>
<i>S. terebinthifolia</i>	0	4.8 a	40.0 a	38.6 a
	60	4.9 a	42.4 a	40.4 a
	120	4.9 a	38.9 a	39.4 a
	180	5.0 a	41.0 a	40.1 a
	240	5.0 a	40.0 a	35.8 a
<i>E. uniflora</i>	0	5.0 a	40.7 ab	36.9 a
	60	4.9 a	40.7 ab	34.2 a
	120	5.0 a	43.4 ab	38.5 a
	180	4.9 a	38.9 b	37.7 a
	240	4.9 a	46.4 a	37.6 a

Means followed by same letters in each column do not differ by Tukey's test at 5% probability.

The K contents in soil cultivated with *S. terebinthifolia* decreased after treatment with 240 mg Cu kg<sup>-1</sup> compared to 120 mg Cu kg<sup>-1</sup>, but copper-contaminated soil did not affect K content in the soil cultivated with *E. uniflora* (Figure 1). No difference was observed for Ca and Mg or the micronutrients B, Fe, Mn, and Zn among treatments in soil cultivated with *S. terebinthifolia* and *E. uniflora*.

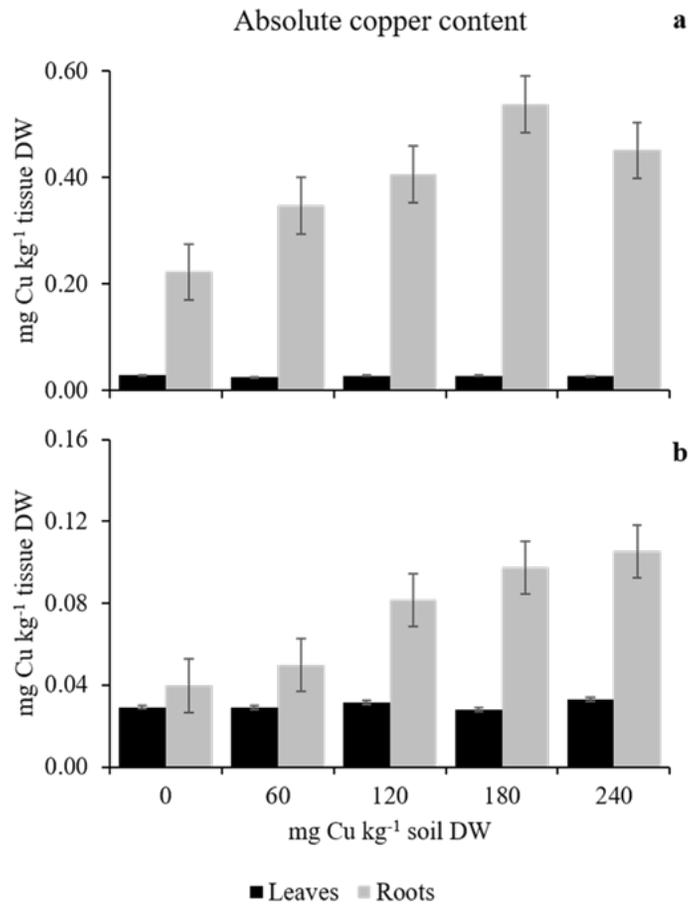


**Figure 1.** Nutritional composition of soils cultivated with *S. terebinthifolia* and *E. uniflora*. Lowercase letters compare treatments within each species. Means followed by same letters in each column do not differ by parametric Tukey's or non-parametric Dunn's test at 5% probability.

### 3.2. Absolute concentration of copper content in tissues

The absolute concentration of copper in both species increased as the concentration of copper applied to the soil increased (Figure 2). The absolute concentration of copper

in the leaves of *S. terebinthifolia* varied from 0.025 to 0.028 mg Cu kg<sup>-1</sup> leaf DW, while in roots, it increased from 0.222 to 0.537 mg Cu kg<sup>-1</sup> root DW (Figure 2a). *E. uniflora* showed variation in absolute concentration of copper in the leaves from 0.028 to 0.033 mg Cu kg<sup>-1</sup> leaf DW and in the roots from 0.040 to 0.105 mg Cu kg<sup>-1</sup> root DW (Figure 2b).



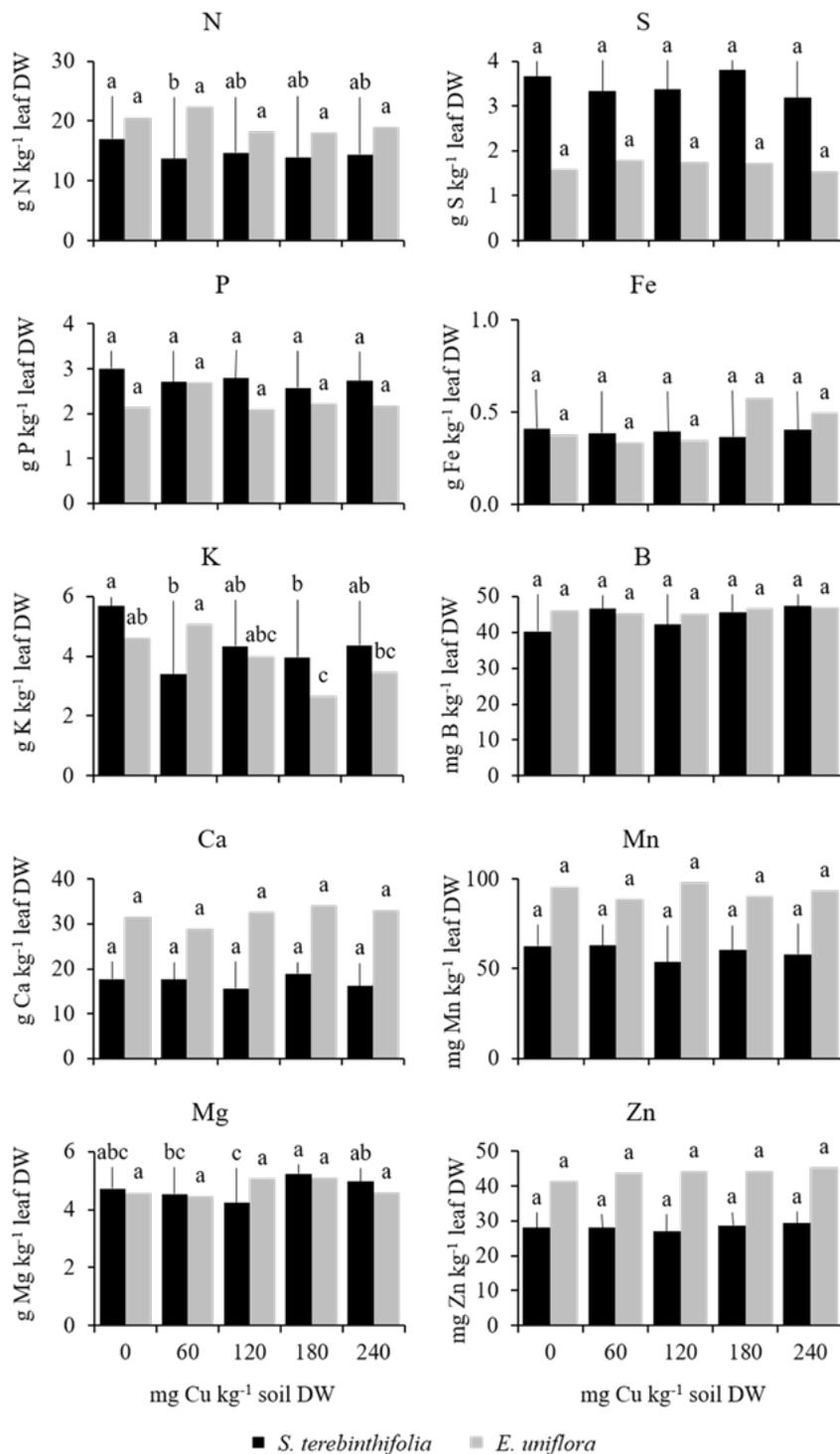
**Figure 2.** Absolute copper contents in leaves and roots of *S. terebinthifolia* (a) and *E. uniflora* (b). Bars represents standard error.

### 3.3. Macro- and micronutrient content in plant tissues

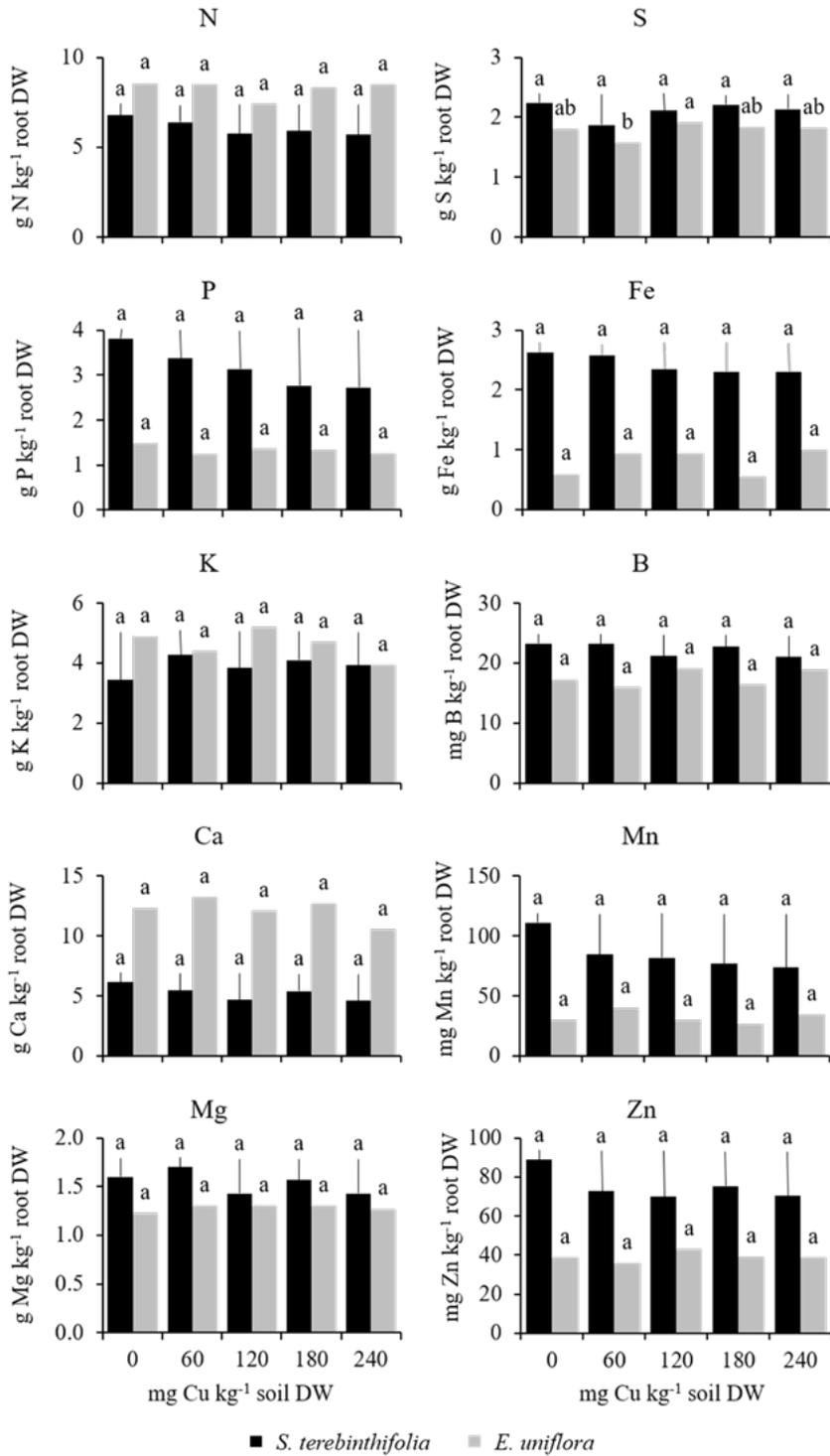
*S. terebinthifolia* showed a reduction in N levels in the leaves after treatment with 60 mg Cu kg<sup>-1</sup> when compared to the control, while *E. uniflora* showed no statistical differences for N (Figure 3). *S. terebinthifolia* leaves showed a reduction in K levels after treatment with 60 and 180 mg Cu kg<sup>-1</sup> when compared to the control. *E. uniflora* leaves also showed a reduction in K levels after treatment with 180 mg Cu kg<sup>-1</sup>

compared to control and 60 mg Cu kg<sup>-1</sup>. *S. terebinthifolia* leaves showed an increase in Mg levels after treatments with 180 and 240 mg Cu kg<sup>-1</sup> compared to 120 mg Cu kg<sup>-1</sup>, but no difference compared to control. No difference was observed for P, Ca, S, Fe, B, Mn, Zn in *S. terebinthifolia* and *E. uniflora* leaves among treatments (Figure 3).

*E. uniflora* roots showed an increase in S levels after treatment with 120 mg Cu kg<sup>-1</sup> compared to 60 mg Cu kg<sup>-1</sup>, while no alteration of macro- and micronutrient levels in the roots of *S. terebinthifolia* among treatments was observed (Figure 4).



**Figure 3.** Macro- and micronutrient content in leaves of *S. terebinthifolia* and *E. uniflora*. Lowercase letters compare treatments within each species. Means followed by same letters in each column do not differ by Tukey's test at 5% probability.



**Figure 4.** Macro- and micronutrient content in roots of *S. terebinthifolia* and *E. uniflora*. Lowercase letters compare treatments within each species. Means followed by same letters in each column do not differ by Tukey's test at 5% probability.

#### 4. DISCUSSION

Copper contamination can induce different effects on the physicochemical properties of soils (Seguel et al., 2019). Changes in soil characteristics caused by excess of copper result from the effects of copper on the chemical bonds between the soil and the aggregated particles that adhere strongly to O.M., preventing its decomplexation (Karkush & Ali, 2019). The difference in the contents of O.M. between species is likely related to the heterogeneity of soils from urban forest fragments, which have different levels of O.M. from soil microbiota activities and particulate matter deposition (Ferreira et al., 2019). More specifically, the release of exudates of each species and the chelation of metals in the soil surrounded by plant roots (rhizosphere) can also change nutrient contents and physicochemical characteristics of soils (Campillo-Cora et al., 2019).

Decrease in total K content in soil caused by excess of copper can be related to competition for adsorption sites in soil particulates (Wyszkowski, 2019), as well as the negative influence of excess copper on the natural cycling of nutrients (Bulbovas et al., 2020). However, the availability of nutrients in copper-contaminated soils is dependent on the plant species present and their uptake capacity (Vendruscolo et al., 2018), as well the release of exudates from each species, which may modify the availability of nutrients in soil (Campillo-Cora et al., 2019). Therefore, the distinct responses in the nutritional status of soil with *S. terebinthifolia* or *E. uniflora* is a result of the difference between species uptake capacity and nutrient availability.

The copper we added to the soil was effectively uptake by *S. terebinthifolia* and *E. uniflora* roots and incorporated into plant tissues, mostly accumulated in the root system. Copper uptake by roots is carried out through several specific and non-specific carriers of heavy metals located in the plasma membrane of root cells (Printz et al., 2016). Copper naturally has a strong affinity for groups of enzymes and proteins present in the apoplast and cell wall of root cells, promoting its retention in plant roots (Giroto et al., 2016). In addition, under situations of toxicity and excess of copper, several species of plants have physiological mechanisms of tolerance to heavy metals, such as release of exudates by the roots, as well as chelation, sequestration and compartmentalization of metal ions in the vacuoles of root cells (Kumar et al., 2020). The increase in copper concentration in tissues, with preferential accumulation in the roots of *S. terebinthifolia* and *E. uniflora*, is a defense response for these tree species, restricting heavy metal to the roots and preventing translocation to leaves and causing a negative impact on the photosynthetic efficiency of plants (Marques et al., 2018).

Heavy metal stress can cause disturbances in the regulation of N uptake by roots and its translocation to shoots, leading to a decrease in the total N levels in leaves (Hippler et al., 2018). Toxicity produced by excess of Cu in plants reduces N uptake and translocation by decreasing the expression level of genes encoding  $\text{NO}_3^-$  transporters (Huo et al., 2020). The changes in K levels could be triggered by an impairment in uptake and translocation of K from roots to leaves as a result of copper toxicity. Cu toxicity induces the reduction of macronutrient content in shoot (i.e., K) as a result of interferences from ion uptake and translocation from root to shoot (Marastoni et al., 2019), producing an imbalance in homeostasis and distribution of nutrients throughout the plant (Souza et al., 2014; Zaouali et al., 2020). Furthermore, since cationic transporters can act in the transport of different ions in a nonspecific way, a decrease in potassium concentration ( $\text{K}^+$ ) in the aerial part of plants may be related to K in competition with copper ions ( $\text{Cu}^+$  and  $\text{Cu}^{2+}$ ) in the translocation processes (Cao et al., 2017; Kobayashi et al., 2019). Excess of copper can induce an increase of Mg translocation from roots to leaves, thus maintaining the homeostasis of mineral composition in shoots (Zeng et al., 2019). With enough concentration of Mg in plant tissues, the effects of excess Cu caused by the ionic competition between elements can be mitigated, preventing excess Cu from binding to vital action sites and causing toxicity (Juang et al., 2014).

Cu toxicity can also cause impairment in nutrient uptake by roots by the competition among ions for uptake and nutritional imbalance as a consequence of saturation of nonspecific ionic transporters (Freitas et al., 2015). However, these effects are highly dependent on copper concentrations and method of cultivation. For example, plants cultivated in nutritive solutions have higher copper toxicity by the elevated availability of elements and fast uptake by the roots (Mezzavilla & Neto, 2017), while plants grown in soils present lower copper uptake by the strong adhesion of copper to soil particles and a host of soil-system biotic and abiotic relationships that can lead to less adverse effects on nutritional balance (Kelepertzis et al., 2015; Chua et al., 2019). Since our study was carried out with soil, it is possible that copper remained partially aggregated to the soil, suggesting that the concentrations uptake by both tree species were insufficient to cause nutritional imbalance in all measured elements. It is also possible that higher doses or longer exposure to Cu could have increased the amount uptake by roots, causing nutritional imbalances and negatively interfering with the homeostasis of more elements. In addition to factors related to experimental condition, copper effects on the nutritional imbalance in plants are associated with synergism and/or competition between copper and mineral nutrients for specific absorption sites and transporters (Printz et al., 2016), and some nutrients have weak competition against

copper as a result of different ionic characteristics and lack of affinity with the same carriers (Andrés-Bordería et al., 2017). Thus, the concentration and translocation of the unaffected elements may be a consequence of their weak competition with copper for specific absorption sites and carriers (Kobayashi et al., 2019). The irregular responses in nutrient concentration of *S. terebinthifolia* and *E. uniflora* to copper stress can also be explained by the fact that different species grown in copper-contaminated soils present different dynamics of accumulation, transport, and nutritional homeostasis in relation to their uptake capacity and the rate of development for each tissue (Zeng et al., 2019; Zabotto et al., 2020). Furthermore, a variety of tolerance mechanisms and strategies are adopted by plants which can result in distinct nutritional imbalances for each species when cultivated in copper-contaminated soils (Yruela, 2009).

In our study, we observed that excess copper can modify the chemical properties of urban forest fragment soil. However, the magnitude of these changes can be affected by the species inhabiting that soil. Moreover, when cultivated in soils with high concentrations of copper, we found that the level of nutrients of *S. terebinthifolia* and *E. uniflora* can be affected in different ways. For instance, while the pioneer species *S. terebinthifolia* presents nutritional imbalance only in the leaves, the non-pioneer species *E. uniflora* presents nutritional imbalance in both leaves and roots. During the experiment, a small number of characteristics of soil and plant nutrition were altered. Therefore, it can be assumed that the applied dose or time was insufficient to cause major nutrient imbalance, but that larger doses or longer cultivation time could possibly result in more significant nutritional imbalance. We conclude that the excess of copper can change the chemical and nutritional characteristics of soil, as well as the nutritional dynamics of *Schinus terebinthifolia* and *Eugenia uniflora* seedlings.

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Shoey Kanashiro: Investigation (Supporting); Methodology (Equal); Validation (Supporting); Writing-review & Editing (Equal).

Marisa Domingos Investigation (Equal); Funding acquisition (Equal); Project Administration (Equal); Methodology (Equal); Validation (Equal); Writing-review & Editing (Equal).

Mirian Cilene Spasiani Rinaldi: Investigation (Supporting); Methodology (Equal); Validation (Equal); Writing-review & Editing (Equal).

Armando Reis Tavares: Investigation (Equal); Funding acquisition (Supporting); Project Administration (Equal); Methodology (Equal); Supervision (Equal); Validation (Equal); Writing-review & Editing (Equal).

## SUPPLEMENTARY MATERIAL

The following online material is available for this article:

Table S1 - Shapiro-Wilk Normality test, Brown-Forsythe Heteroscedasticity test and ANOVA table of chemical and nutritional data of soil cultivated with *Schinus terebinthifolia*.

Table S2 - Shapiro-Wilk Normality test, Brown-Forsythe Heteroscedasticity test and ANOVA table of chemical and nutritional data of soil cultivated with *Schinus terebinthifolia*.

Table S3 - Shapiro-Wilk Normality test, Brown-Forsythe Heteroscedasticity test and ANOVA table of *Schinus terebinthifolia* leaf nutritional data.

Table S4 - Shapiro-Wilk Normality test, Brown-Forsythe Heteroscedasticity test and ANOVA table of *Eugenia uniflora* leaf nutritional data.

Table S5 - Shapiro-Wilk Normality test, Brown-Forsythe Heteroscedasticity test and ANOVA table of *Schinus terebinthifolia* root nutritional content.

Table S6 - Shapiro-Wilk Normality test, Brown-Forsythe Heteroscedasticity test and ANOVA table of *Eugenia uniflora* leaf nutritional content.

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