



Assessment of Soil Analytical Methods and Black Oats (*Avena strigosa* L.) Morphological and Physiological Responses to Copper Toxicity

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Received: 30 July 2025 / Accepted: 10 January 2026 / Published online: 4 February 2026
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Abstract

Copper (Cu) accumulation is a major concern in agricultural production systems due to potential toxicity to plants and environmental contamination. Soil testing is the best available means to determine nutrient availability, but different analytical methods extract various amounts of Cu. Hence, critical levels of Cu toxicity need to be defined for multiple extractants to ensure adequate interpretation of potential element toxicity to plants. Objective: Our study aimed to (a) evaluate Cu availability in soils using three different chemical extractants (CaCl₂, Mehlich-1, and EDTA) and (b) identify black oats (*Avena strigosa* L.) physiological responses that can be used as indicators of Cu phytotoxicity potential and assist in defining critical Cu toxicity levels. A greenhouse experiment was conducted with three soils previously incubated with different Cu rates. Soil Cu availability was analyzed by CaCl₂ 0.01 mol L⁻¹, Mehlich-1, and EDTA, and the concentrations were correlated to black oats relative dry matter yield and plant photosynthetic parameters (i.e., photosynthetic rate, stomatal conductance, total chlorophylls, and carotenoids) of plant growth in soil without Cu application. Soil testing methods recovered different amounts of Cu in the following order: EDTA > Mehlich-1 > CaCl₂, with CaCl₂ being the method with the lowest sensitivity to assess Cu availability. Soil clay content affected Mehlich-1 Cu extractions, with concentration decreasing for soils with greater clay content. The highest plant Cu concentrations were observed in the roots, with little variation in concentrations in the shoot. The EDTA and Mehlich-1 analytical methods showed different critical Cu toxicity concentrations for black oats dry matter and photosynthetic parameters. The analytical methods showed different Cu extraction capacities, with concentrations increasing in the following order: EDTA > Mehlich-1 > CaCl₂, and concentration differences being influenced by soil clay and organic matter contents. Therefore, critical Cu phytotoxicity levels should consider both soil characteristics and the extraction method used. The inhibitory concentrations IC₂₅ and IC₅₀ values were consistent for biomass and photosynthetic rate, indicating the latter as an effective non-destructive method to monitor plant metal-induced stress. However, interactions with other nutrients and abiotic stresses still require further investigation.

Keywords Cu toxicity · CaCl₂ · EDTA · Mehlich-1 · Soil analysis · *Avena strigosa*

1 Introduction

Copper accumulation in agricultural production systems is a worldwide concern due to increased challenges for sustainable food production and environmental contamination. Frequent applications of Cu-based fungicides in vineyards and orchards can lead to soil Cu accumulation (Silva et al. 2020; Marques et al. 2023a, b; Brunetto et al. 2023; Morsch et al. 2024). Likewise, continuous application of organic amendments (e.g., pig manure) in crop production systems can rapidly build up soil test Cu levels (Brunetto et al. 2016, 2017a). Casali et al. (2008) reported total soil

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Cu concentrations of 503 and 726 mg Cu kg⁻¹ in the soil 0–20 cm layer in two vineyards with approximately 40 years of cultivation. Also, Girotto et al. (2014) reported total soil Cu concentrations of 213, 456, and 520 mg Cu kg⁻¹ in 23-, 30-, and 33-year-old vineyards, respectively. Increased soil Cu content can build up bioavailable Cu fractions (Drescher et al., 2021) that can be toxic to agronomic crops and cover crops, as well as increase the risk of element transfer into the environment (Brunetto et al. 2016; Cesco et al. 2021).

Copper toxicity is a major crop production concern as excess Cu in plant tissues negatively affects cell division and root elongation, resulting in roots with a larger diameter (Ambrosini et al. 2015; Morsch et al. 2021), and reduced volume and surface area (Trentin et al. 2019; De Conti et al. 2020a; Conti et al. 2020b; Silva et al. 2022). Plants with Cu toxicity exhibit reduced root growth and a limited ability to absorb water and essential nutrients, which can lead to chlorosis symptoms and negatively impact plant dry matter production (Girotto et al. 2016; Guimarães et al. 2016; De Conti et al. 2018). Excessive shoot Cu uptake can further damage the plant's photosynthetic apparatus due to impaired electron transport and inhibited enzyme activity during the Calvin-Benson Cycle, resulting in reduced pigment synthesis and overall plant photosynthetic efficiency (Ambrosini et al. 2018; Hammerschmitt et al. 2020; Silva et al. 2022).

In the soil, Cu can bond to mineral or organic functional groups with different energy levels, affecting Cu adsorption and desorption capacity, and consequently, the soil Cu availability. Additionally, intrinsic soil attributes such as pH, the quantity and type of clay minerals, oxides, iron (Fe), aluminum (Al), and manganese (Mn) hydroxides, and soil organic matter content influence soil Cu availability. Sandy soils, with low pH and organic matter content, have a lower Cu adsorption capacity compared to soils with greater buffer capacity, and therefore have an increased risk of Cu transfer potential (Brun et al. 2001; Chaignon & Hinsinger et al., 2003), requiring greater attention to properly assess soil Cu availability and minimize Cu contamination in the environment.

Worldwide, several chemical extraction methods have been employed to assess plant-available Cu concentrations, including adaptations of analytical protocols, such as soil-to-solution ratios, extraction times, and sample shaking intensities. Altogether, these increase the number of analytical methodologies adopted by soil testing labs, and consequently the diversity of results in soil test reports and critical toxicity levels (Kennedy et al. 1997). Furthermore, diverse chemical reagents, including saline solutions (e.g., CaCl₂, MgCl₂, NaNO₃, and NH₄OAc), acidic solutions (e.g., HCl and HNO₃), chelating agents (e.g., DTPA and EDTA), or a mixture of these chemicals, have been used to assess soil

Cu and other metal elements availability (Kennedy et al. 1997; Pastor et al., 2007; Miotto et al. 2017; Brunetto et al. 2017b). Overall, previous research indicates that saline solutions (regardless of concentration) have a lower Cu extraction capacity when compared to chelating solutions. However, regardless of the method of analysis, the Cu concentration values obtained will only be useful and reliable to indicate plant toxicity potential if the method is calibrated with plant physiological or biological responses, such as photosynthesis rate and biomass production.

Several soil test methods and critical Cu concentrations that were initially proposed to guide nutrient rate recommendations have been used in toxicity studies Pham (2024) without proper calibration or validation. Hence, understanding the relationship between soil Cu availability, as measured by diverse analytical methods, and plant Cu toxicity or tissue-Cu concentrations has been challenging to establish. Furthermore, critical soil test Cu concentrations to assess the risk of plant Cu toxicity are still scarce in the literature. With proper calibration, the same analytical method used to recommend Cu fertilizer could be used to interpret excess Cu availability and plant toxicity potential. However, there is still a lack of research to define a “critical Cu toxicity index” for diverse soil testing methods similar to the critical nutrient concentration for optimal plant growth used to guide fertilizer applications (CQFS-RS/SC 2016). One available means to define such a “critical toxicity index” is by using the inhibitory concentration (IC), which has different threshold values depending on the percentage of inhibition to be considered (Visioli et al. 2014; Pokorska-Niewiada et al. 2018). Research by Martins et al. (2019) demonstrated that characterizing the risk of arsenic (As) toxicity based on available extractable concentrations, rather than total As content, allowed the estimation of more realistic As toxicity values through inhibitory concentrations (IC₂₀ and IC₅₀) across different soils in Brazil. These results suggest that the definition of preventive values of metal toxicity should consider plant-available concentrations, which requires element- and analytical method-specific calibrations. To address this knowledge gap, our study aimed to (a) evaluate soil Cu availability by three different analytical methods (CaCl₂, Mehlich-1, and EDTA), and (b) establish method-specific critical soil Cu toxicity concentrations associated with black oats' morphological and physiological responses. More specifically, we aim to define method-specific critical Cu concentrations based on black oats biomass yield and photosynthetic parameters' response to Cu availability in diverse soils. We hypothesize that critical Cu toxicity concentrations will be greater for EDTA, followed by Mehlich-1 and CaCl₂, due to increased extraction power of the analytical method and increased soil Cu solubility.

2 Materials and Methods

2.1 Soil Characteristics and Copper Rate Treatments

The study was carried out using three soils with distinct physical and chemical properties. The soils were collected from different geographic regions from Rio Grande do Sul, southern Brazil, in areas without Cu application in recent years. A Typic Hapludult from Santana do Livramento (SL – 30°47'22" S, 55°22'08" W), a Typic Hapludult from Santa Maria (SM – 29°43'38" S, 53°45'33" W), and a Udipsamment from Bento Gonçalves (BG – 29°09'44" S, 51°31'50" W) were collected from the 0–20 cm layer. The soils were air-dried, crushed, and passed through a 2 mm mesh sieve, and stored for the greenhouse trial. A subsample was analyzed for selected soil physical and chemical characteristics (Table 1). It is worth noticing that the Santa Maria soil has three times greater clay content than the Santana do Livramento soil.

Soil water pH (1:1 v/v soil-to-water mixture) was evaluated, and the soil potential acidity was estimated using the direct titration method with Ca hydroxide (CQFS-RS/SC 2016). After measuring soil potential acidity for each soil, calcium hydroxide [Ca(OH)₂] and magnesium oxide [MgO] were applied to raise the water pH to 6.0 and maintain a 4:1 Ca to Mg ratio. Phosphorus fertilizer (30 mg kg⁻¹ of soil) was applied with the amendments and, subsequently, soils were homogenized and incubated. Throughout the incubation period, distilled water was added to maintain soil moisture at 80% of the maximum water retention capacity (MWRC). The soil incubation was ended after 60 days, when the soil pH stabilized and the target pH of 6.0 was achieved.

After incubation, 3.5 kg of each soil was placed in plastic pots. A nutrient solution containing 20, 30, 80, 5, 2, 2, 1, and 0.5 mg dm⁻³ of N, P, K, S, Zn, Mn, Fe, and B, respectively, was added. The soil Cu levels were determined before treatment application (Table 1). Six Cu rates were applied to each soil, corresponding to 0, 10, 20, 30, 45, and 60 mg kg⁻¹ of Cu for the SL soil, 0, 20, 40, 60, 90, and 120 mg kg⁻¹ of Cu for the SM soil, and 0, 30, 60, 90, 140, and 190 mg kg⁻¹ of Cu for the BG soil. The Cu source used was copper sulfate. The Cu rates values were established based on differences in soil clay and soil organic matter (SOM) contents, which affect Cu availability (Brun et al. 2001; Chaignon & Hinsinger, 2003). The experimental design was completely randomized, with five replications. After the application of nutrients and Cu rates, the soil in each pot was homogenized, moistened again, and incubated for another 40 days with moisture close to 80% of the maximum water retention capacity (MWRC).

2.2 Greenhouse Experiment Setup

Fifteen pre-germinated black oat seeds (cultivar BRS Madrugada) were sown in each pot. Each pot had three 1 cm diameter holes at the base for irrigation purposes. A 60 µm lock screen was used at the base of each pot to prevent soil loss. Each pot was placed on a plastic plate, and the pots were weighed every two days to check the soil moisture. When necessary, distilled water was added to the plastic plates to maintain soil moisture at 80% of the MWRC. At 7, 14, and 21 days after plant emergence (DAE), 15 mg N per pot was applied in the form of urea, and at 33, 40, and 47 DAE, 10 ml of modified Hoagland's solution was applied per pot to ensure adequate nutrient availability for plant growth (Marques et al. 2023a, b).

Table 1 Soil classification and selected chemical and physical properties at the 0–20 cm depth of three soils from Southern Brazil before experiment setup and copper rate application

Chemical and physical parameters	Soil Santana do Livramento (Typic Hapludult)	Santa Maria (Typic Hapludult)	Bento Gonçalves (Udipsamment)
pH in water (1:1)	5.8	5.7	5.8
H+Al (cmol _c kg ⁻¹)	1.4	4.0	3.2
Al (cmol _c kg ⁻¹)	0.0	0.1	0.1
Ca (cmol _c kg ⁻¹)	0.5	3.4	4.4
Mg (cmol _c kg ⁻¹)	0.6	4.8	0.7
K (mg kg ⁻¹)	32	143	76
P (mg kg ⁻¹)	16.4	8.4	71.5
Cu extracted with CaCl ₂ (mg kg ⁻¹)	0.00	0.00	0.00
Cu extracted with Mehlich-1 (mg kg ⁻¹)	0.15	0.04	0.02
Cu extracted with EDTA (mg kg ⁻¹)	0.77	1.16	7.03
Soil organic matter (g kg ⁻¹)	10	32	27
CTC (cmol _c kg ⁻¹)	2.7	12.6	8.5
Clay (g kg ⁻¹)	67	205	210
Silt (g kg ⁻¹)	39	417	570
Sand (g kg ⁻¹)	894	377	283

2.3 Soil and Plant Sampling

The experiment was carried out until 65 DAE, which corresponded to the beginning of flowering. At 65 DAE, plant physiological assessments were carried out in the middle third of the last fully expanded leaf, using a portable Infra-Red Gas Analyzer (LI-COR, model LI-6400 XT, Japan), with photosynthetic radiation of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and CO_2 concentration of $400 \mu\text{mol mol}^{-1}$. With the obtained data, the CO_2 liquid assimilation rate ($A - \mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) and the stomatal conductance ($G_s - \text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$) were calculated. Thereafter, leaves were collected, immediately frozen in liquid N, and stored in an ultra-freezer at -80°C for subsequent determination of the photosynthetic chlorophylls (mg g^{-1} FM [fresh matter]) and carotenoids (mg g^{-1} FM) pigments. The photosynthetic pigment concentrations were analyzed based on the methodology proposed by Hiscox and Israelstam (1979), and therefore 0.005 g of leaf tissue was incubated with dimethylsulfoxide (DMSO) at 65°C , until full pigment removal. The supernatant extract was subjected to absorbance readings in a spectrophotometer at wavelengths of 663, 645, and 470 nm. Total chlorophyll (chlorophyll a + chlorophyll b) and carotenoid concentrations were estimated based on the equation outlined by Lichtenthaler (1987) and the results were expressed as mg g^{-1} FM.

Following the gas exchange readings and leaf sampling, the plants' aboveground biomass was cut close to the soil surface, and roots were manually separated from the soil. Shoots and roots were oven-dried at 65°C until constant mass and ground in a mill to pass through a sieve with 1-mm openings. Thereafter, plant above and belowground samples were acid-digested, and the Cu concentrations (mg kg^{-1}) were determined using an atomic absorption spectrometer (EAA; Varian SpectraAA-600, Australia).

At the end of the experiment, a soil sample was collected from each pot. The soil was air-dried, ground, and passed through a 2-mm mesh sieve. Thereafter, soil Cu was extracted using CaCl_2 (0.01 mol L^{-1}), Mehlich-1 ($0.05 \text{ mol L}^{-1} \text{HCl}$; $0.0125 \text{ mol L}^{-1} \text{H}_2\text{SO}_4$), and $0.01 \text{ mol L}^{-1} \text{EDTA} + 1.0 \text{ mol L}^{-1} \text{NH}_4\text{CH}_3\text{COO}$, with pH adjusted to 7.0. Copper concentration (mg kg^{-1}) in the extracts was analyzed in an atomic absorption spectrometer (EAA; Varian SpectraAA-600, Australia).

2.4 Statistical Analyses

Data from each soil were evaluated separately regarding the assumption of normality of errors (Shapiro-Wilk); transformations were applied when necessary. ANOVA was performed to evaluate the effect of Cu concentrations for each soil separately, and when significant ($p < 0.05$), regression

analysis was performed to evaluate the effect of soil Cu concentration on the results. Pearson linear correlation was performed between CaCl_2 , Mehlich-1, and EDTA extractable Cu concentrations and plant photosynthetic parameters and relative crop biomass production (considering the no-Cu control as 100%). Copper toxicity limit values for black oats were evaluated by IC_{25} and IC_{50} (the 25% and 50% inhibitory concentrations (IC_{25} and IC_{50}) of Cu) in relation to the no-Cu control treatment, using normalized nonlinear regression models with Mehlich-1 and EDTA extractable Cu concentrations. The GraphPad prism program was used to fit the logarithmic models to determine the IC_{25} and IC_{50} . The use of IC_{25} and IC_{50} parameters is essential to quantify the toxic effects of Cu on black oats, allowing an accurate assessment of the exposure levels that cause reductions of 25% and 50% in plant growth or physiological parameters compared to the untreated control.

3 Results

3.1 Copper Recovery Capacity by Different Analytical Methods and Black Oats' Cu Uptake in Cu-contaminated Soils

Copper recovery differed among the analytical methods for all three soils (Fig. 1). The EDTA method extracted the greatest amount of Cu compared to Mehlich-1 and CaCl_2 . The Cu concentration extracted by EDTA increased linearly with the increased Cu rates, showing a consistent trend across all soils tested. The EDTA extractable Cu concentrations were around 77, 65, and 63% of Cu added to SL, SM, and BG soils, respectively. Mehlich-1 recovered a smaller amount of Cu than EDTA, but also showed a linear relationship for the three soils. However, Mehlich-1 Cu extraction capacity was affected by soil type, with 35, 6.2, and 4.1% of Cu recovery for SL, SM, and BG, respectively. The CaCl_2 method showed low extraction power (less than 1% of added Cu) with no Cu recovery in treatments receiving $< 30 \text{ mg kg}^{-1}$ of Cu in SL and BG soils and $< 40 \text{ mg kg}^{-1}$ of Cu in SM soil. For SL and SM, CaCl_2 extractable Cu showed a quadratic relation with the Cu rate applied prior to incubation.

Black oats' Cu uptake was significantly affected by Cu rate in all soils, with shoots showing a relatively small Cu uptake increase with increased Cu availability in comparison to the roots (Fig. 2). The Cu concentration in plant shoots ranged from 9 to 16, 11–14, and 8–14 mg kg^{-1} of Cu for plants grown in SL, SM, and BG soil, respectively. Copper uptake in plant roots increased linearly with increased Cu rate for all soils and ranged from 18 to 739, 55–452, and 33–644 mg kg^{-1} of Cu for plants grown in SL, SM, and BG soil, respectively.

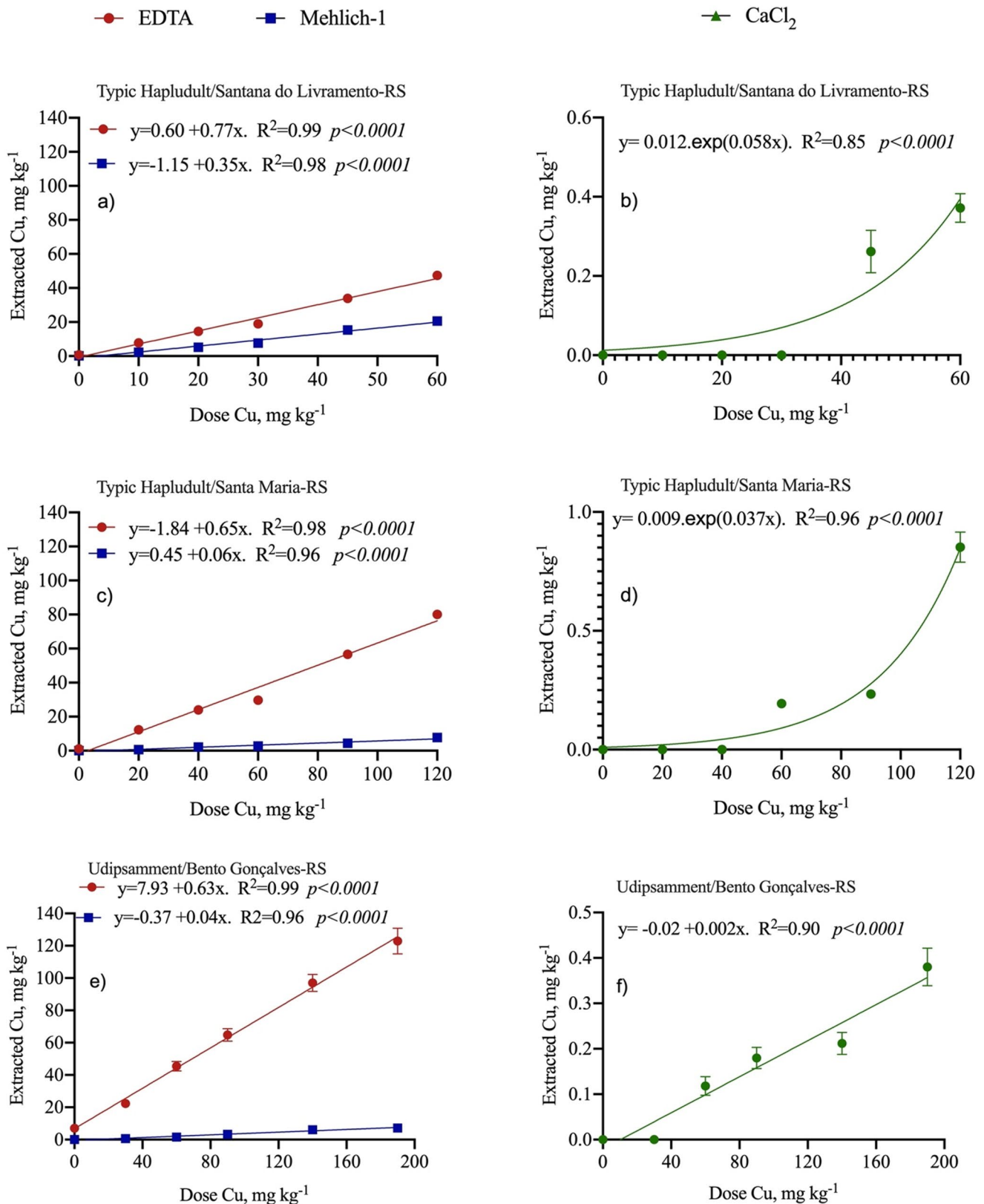


Fig. 1 Relationship of soil Cu availability measured by Mehlich-1 and EDTA (a, c, and e), and CaCl_2 (b, d, and f) as a function of Cu rates applied to the three different soils from Southern Brazil without a his-

tory of Cu application. The equations shown correspond to the fitted models for each analytical method

Fig. 2 Copper concentration in black oats roots and shoots biomass as affected by different Cu rates in three soils from Southern Brazil without a history of Cu application: **(a)** Santana do Livramento, **(b)** Santa Maria, and **(c)** Bento Gonçalves. The equations shown correspond to the fitted models for each analytical method

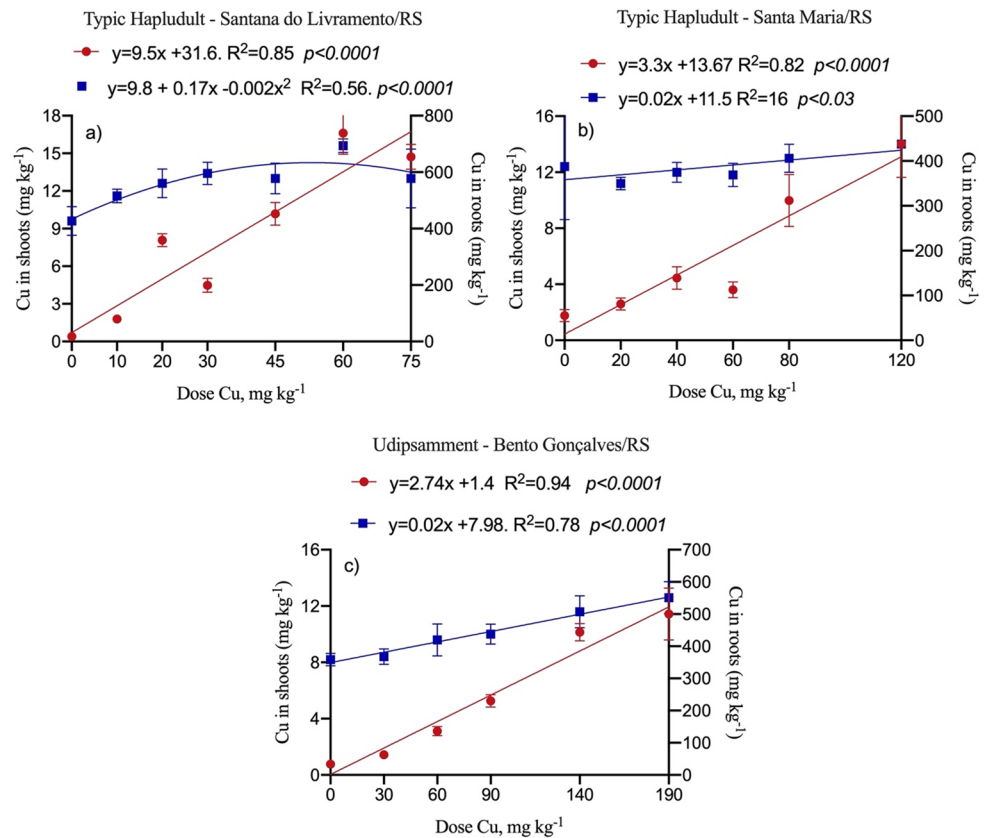
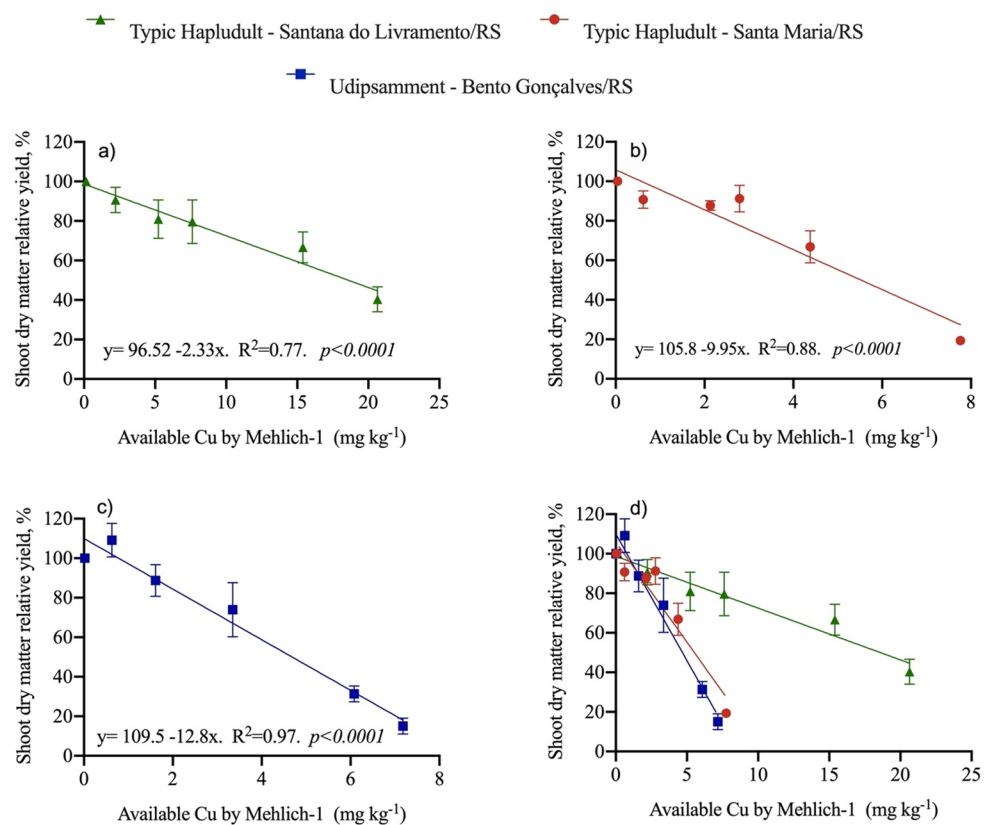


Fig. 3 Relative black oats shoot biomass yield as a function of Mehlich-1 Cu availability in three soils from Southern Brazil artificially contaminated with increasing Cu concentrations: **(a)** Santana do Livramento, **(b)** Santa Maria, **(c)** Bento Gonçalves, and **(d)** all three soils grouped together. The equations shown correspond to the fitted models for each soil



3.2 Black Oats' Biomass production, Photosynthetic response, and Toxicity Values in Cu-contaminated Soils

Black oats' aboveground biomass production was significantly affected by increased Cu rates, with biomass production reducing linearly with increasing Mehlich-1 (Fig. 3) and EDTA (Fig. 4) extractable-Cu concentrations. Unlike Mehlich-1 and EDTA methods, there was no significant relation between relative biomass production and CaCl_2 extractable-Cu concentration, indicating that this method is not sensitive to predict Cu toxicity (Data not presented).

The IC_{25} and IC_{50} values were calculated for EDTA and Mehlich-1 methods, but not for CaCl_2 , since the soil Cu concentrations at the low Cu rates were equal to zero. The IC_{25} and IC_{50} values for EDTA extractable Cu concentrations were in the following order: BG>SM>SL (Table 2). On the other hand, for Mehlich-1, the order was SL>SM>BG. When using EDTA, the IC_{25} determination for shoot dry matter was 2.04 times greater in BG soil compared to SL. When comparing the IC_{25} for Mehlich-1 in SL and BG soils, the IC_{25} value was 4.09 times greater in SL than in BG soil. The IC_{25} inhibitory concentrations for black oats shoot biomass production ranged from 13.8 to 28.1 mg kg^{-1} of Cu for EDTA and from 1.5 to 6.1 mg kg^{-1} of Cu for Mehlich-1. Likewise, the IC_{50} inhibitory concentrations were also

greater for EDTA, with the BG soil showing concentrations of 84.4 and 4.5 mg kg^{-1} of Cu for EDTA and Mehlich-1, respectively.

Likewise observed for biomass production, plant photosynthetic rate and stomatal conductance linearly decreased with increasing Cu availability for both Mehlich-1 and EDTA analytical methods (Fig. 5). The IC_{25} values for plant photosynthetic rate in SL soil were 3.15 times greater than for BG soil, when calculated based on Mehlich-1 extractable Cu (Table 2). However, when calculating the IC_{50} using EDTA extractable Cu concentration, we observed that the BG soil had 2.48 times greater IC_{50} values than the SL soil. For stomatal conductance, IC_{25} and IC_{50} presented the following order for the soils evaluated: BG>SM>SL, when determined considering EDTA extractable Cu concentrations; and SL>SM>BG, for Mehlich-1 extractable Cu concentrations.

Black oats total chlorophyll and carotenoid concentrations decreased with increasing Cu availability, regardless of the analytical method (Fig. 6). However, when using EDTA, the IC_{25} values for total chlorophyll varied from 16.0 to 61.1 mg kg^{-1} of Cu; and values for carotenoids ranged from 13.3 to 83.1 mg kg^{-1} of Cu among soils (Table 2). Likewise for other plant variables, the IC_{25} based on Mehlich-1 extractable Cu concentrations were lower than for EDTA, and ranged from 3.9 to 10.7 and 3.4–5.8 mg kg^{-1} of Cu for total chlorophyll and carotenoids, respectively (Table 2).

Fig. 4 Relative black oats shoot biomass yield as a function of the EDTA Cu availability in three soils from Southern Brazil artificially contaminated with increasing Cu concentrations: (a) Santana do Livramento, (b) Santa Maria, (c) Bento Gonçalves, and (d) all three soils grouped together. The equations shown correspond to the fitted models for each soil

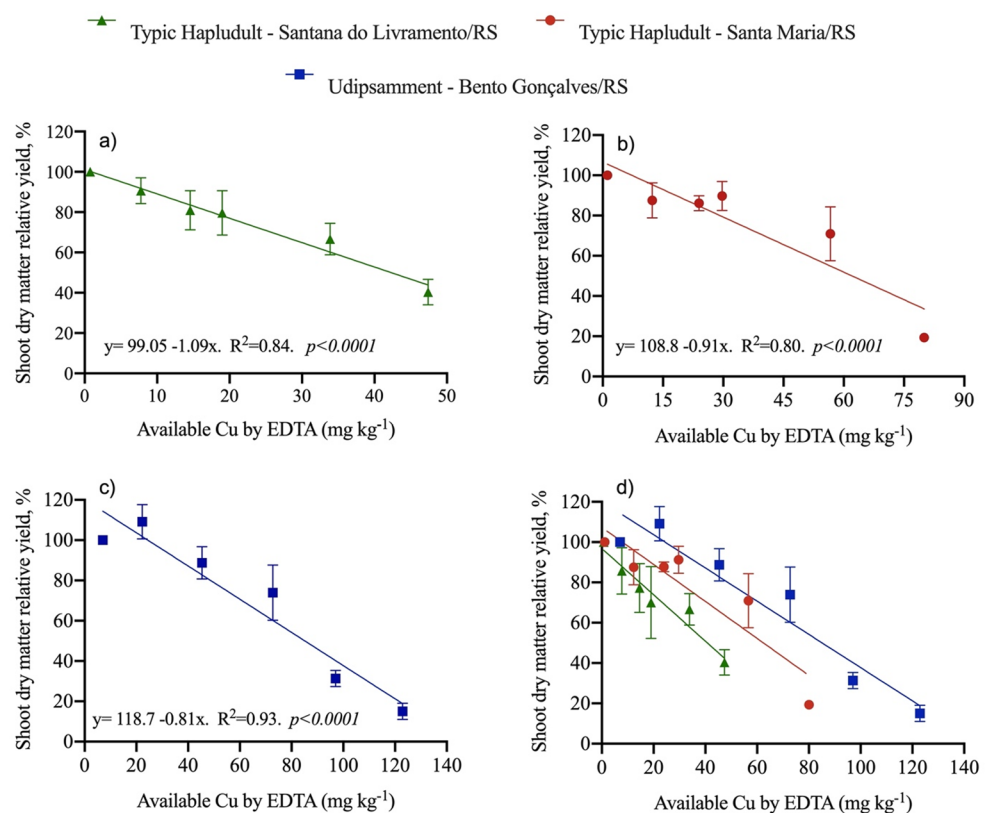


Table 2 Mehlich-1 and EDTA IC_{25} and IC_{50} Cu concentrations for black Oats shoot dry matter production, net photosynthesis, stomatal conductance, and total chlorophyll and carotenoids grown in three soils from Southern Brazil, artificially contaminated with increasing Cu concentrations. Values are expressed in $Mg\ kg^{-1}$

Soil	Mehlich-1	EDTA		
	*IC ₂₅	*IC ₅₀	IC ₂₅	IC ₅₀
	----- Shoot dry matter -----			
Santana do Livramento	6.13±1.31	18.39±3.24	13.77±3.71	41.32±3.14
Santa Maria	1.79±0.41	5.36±1.05	20.71±4.55	62.12±7.65
Bento Gonçalves	1.50±0.14	4.51±1.32	28.13±2.11	84.40±10.23
	----- Net photosynthesis -----			
Santana do Livramento	5.08±1.47	15.23±4.05	11.65±2.04	34.94±5.11
Santa Maria	2.74±0.21	8.22±1.88	5.79±1.32	17.38±3.07
Bento Gonçalves	1.61±0.09	4.82±1.05	28.84±4.87	86.51±1.55
	----- Stomatal conductance -----			
Santana do Livramento	2.92±0.69	8.75±1.58	7.39±2.04	22.16±3.18
Santa Maria	2.37±0.31	7.10±2.12	20.95±3.22	62.86±7.95
Bento Gonçalves	1.44±0.11	4.33±0.87	25.74±4.87	77.21±9.23
	----- Total chlorophyll -----			
Santana do Livramento	10.67±1.94	32.00±2.56	15.96±2.54	47.89±3.65
Santa Maria	2.59±0.54	7.76±0.94	26.77±3.87	80.30±5.87
Bento Gonçalves	3.92±0.69	11.78±1.69	61.17±0.5.79	183.50±11.78
	----- Carotenoids -----			
Santana do Livramento	5.79±0.87	17.38±2.87	13.26±2.87	39.79±5.69
Santa Maria	3.39±0.56	10.17±1.39	33.40±3.35	100.20±9.71
Bento Gonçalves	4.59±0.81	13.77±3.84	83.13±9.50	249.40±19.73

* (the 25% and 50% inhibitory concentrations (IC_{25} and IC_{50}) of Cu)

4 Discussion

The greatest soil extractable Cu concentration values were observed for EDTA, followed by Mehlich-1 and $CaCl_2$, mainly in SM and BG soils (Table 2; Fig. 1). This occurred because EDTA forms complexes with the metal that is adsorbed to soil colloids. Thus, the portion of Cu removed from the mineral or organic colloid surface is maintained soluble in the extract until Cu is quantified (Melo et al. 2006; Schenkeveld et al. 2007). This behavior also helps explain why the Cu recovery is very similar to the applied Cu rate, regardless of soil physical or chemical characteristics (Fig. 1; Table 1). On the other hand, the extraction mechanism of Mehlich-1 is based on the partial dissolution of the colloid surface by acidic action (i.e., presence of HCl and H_2SO_4 in the extractant solution). Therefore, part of the Mehlich-1 extractable Cu can be reabsorbed if concentrations are not quantified immediately following the extraction procedure, with the magnitude of reabsorption being proportional to the soil's metal adsorption capacity (Mumbach et al. 2018). Therefore, the amount of Mehlich-1 Cu recovery is lower than EDTA and varies between soils, depending on the soil's intrinsic colloidal characteristics and buffer capacity (Pham 2024). The lower Cu extraction power by Mehlich-1, compared to EDTA, discriminates

less the differences in soil Cu availability. The different Mehlich-1 Cu recovery capacities across the three soils evaluated suggest that certain soil properties, such as clay and SOM contents, which influence metal absorption capacity, need to be considered to improve the interpretation of the Mehlich-1 Cu analytical results and plant toxicity potential. The use of additional soil properties that are related to the element adsorption capacity, such as clay content (CQFS-RS/SC 2016) or P-remaining content (Ribeiro et al. 1999), is already used for the interpretation of Mehlich-1 extractable P and guide P recommendations in southern Brazil production systems. Hence, critical Mehlich-1 Cu levels for plant toxicity must be established according to the method's relationship with other soil variable(s), allowing the establishment of different critical values based on soil clay and/or SOM contents. In such a framework, the critical Mehlich-1 levels will decrease as Cu adsorption capacity in soil increases, according to the results presented in Fig. 3.

Unlike EDTA and Mehlich-1, the $CaCl_2$ method relies on the principle of ion exchange reactions (outer sphere complexes) for nutrient extractions. Thus, $CaCl_2$ has a lower capacity to recover Cu that is adsorbed in soil by more stable mechanisms (internal sphere complexes; Casali et al. 2008) than the other two methods. As most of soil Cu is strongly adsorbed (Brunetto et al. 2017b), $CaCl_2$ extraction capacity

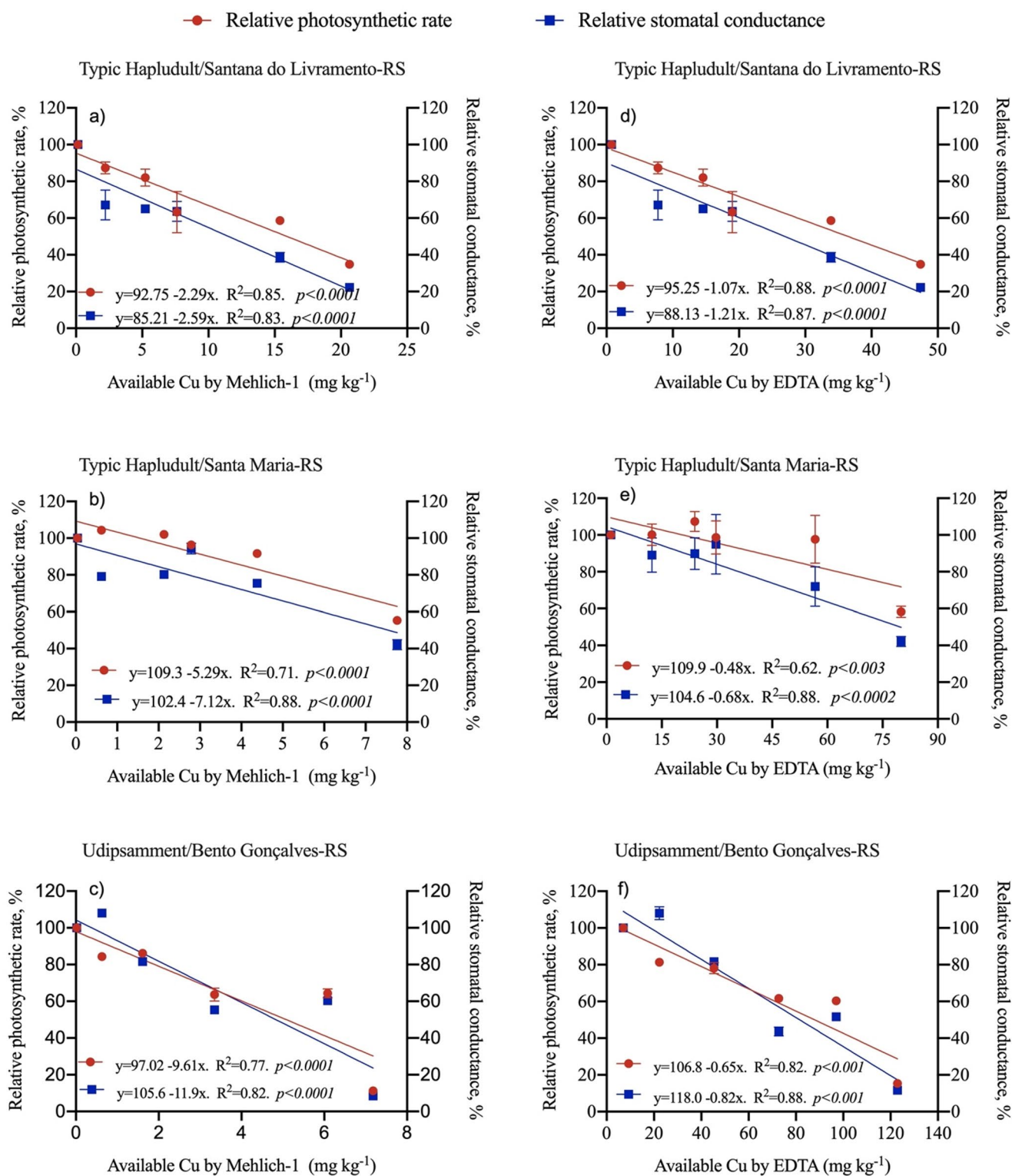


Fig. 5 Relative black oats shoots photosynthetic rate and stomatal conductance as a function of the availability of Mehlich-1 (a, b, and c) and EDTA (d, e, and f) Cu availability in three soils from Southern Brazil

artificially contaminated with increasing Cu concentrations. The equations shown correspond to the fitted models for each photosynthetic parameter

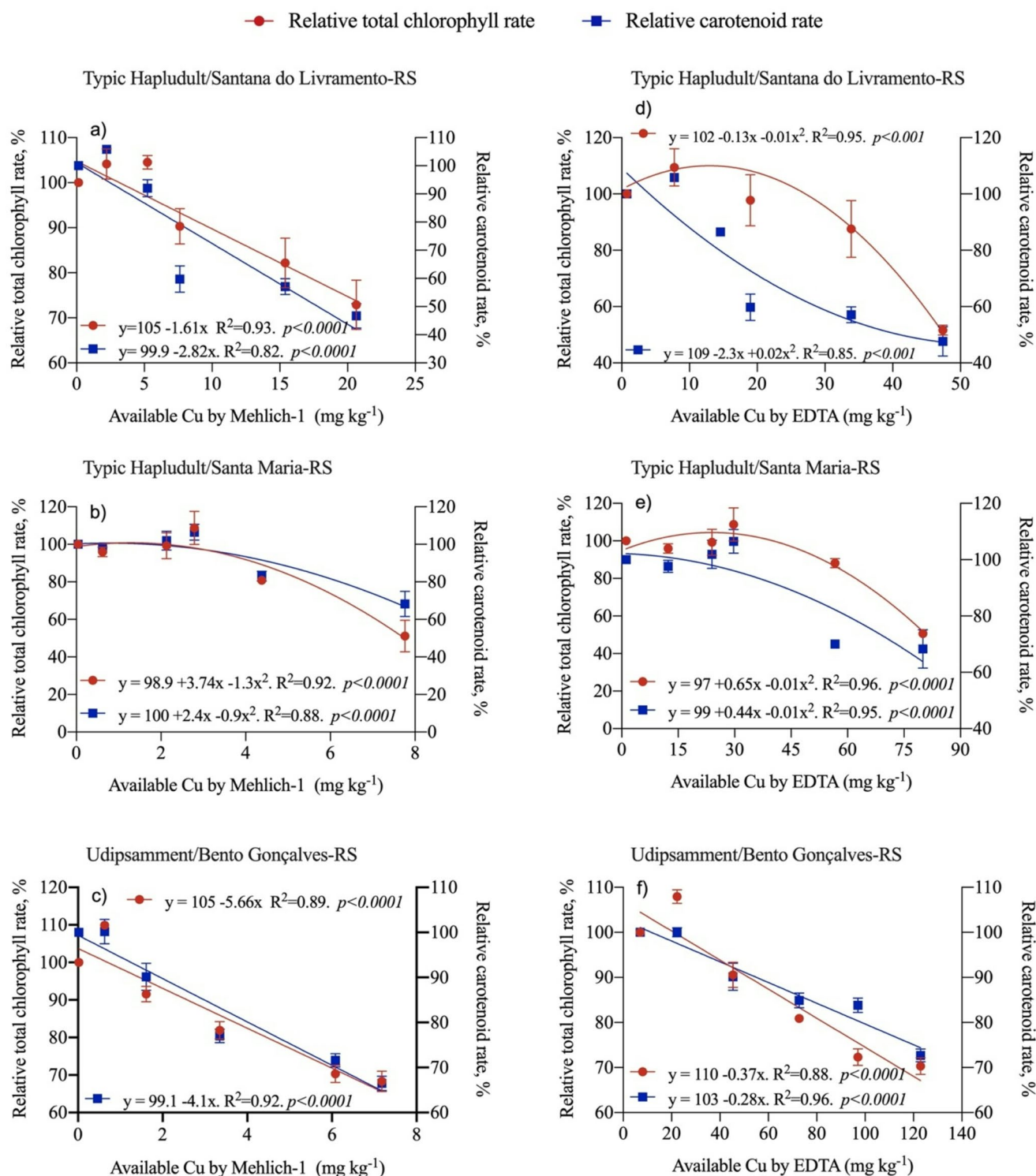


Fig. 6 Relative total chlorophyll and carotenoid contents in black oats shoots as a function of Mehlich-1 (a, b, and c) and EDTA (d, e, and f) Cu availability in three soils from Southern Brazil artificially contami-

nated with increasing Cu concentrations. The equations shown correspond to the fitted models for each photosynthetic parameter

starts to increase only after the majority of Cu adsorption sites are saturated (i.e., close to the soil maximum Cu adsorption capacity), allowing Cu to occupy spaces of electrostatic attraction in the soil. When comparing Cu availability across different soils and analytical methods, CaCl₂ was unable to extract soil Cu from lower Cu rates across all soils; however, soil Cu availability was high enough to cause plant Cu toxicity and impact plant photosynthesis and biomass production (Figs. 1, 2, 3, 4, 5 and 6). Therefore, the low Cu recovery capacity of CaCl₂ makes this method not sensitive to evaluating soil Cu availability (Fig. 1b) or plant toxicity potential. Choosing the proper analytical method to assess soil Cu availability is key in areas with high levels of this element due to agricultural and environmental impacts. Defining critical soil Cu concentration is important for assessing the risk of plant toxicity and developing management practices to mitigate adverse impacts on crop production while also evaluating the potential risk of Cu transfer from soil to aboveground and belowground water and, consequently, altering adjacent environments (Li et al. 2017).

The IC₂₅ and IC₅₀ values for plant biomass production were lower for Mehlich-1 than for EDTA-extractable Cu (Table 2), indicating that the critical toxicity thresholds depend on the chemical strength and selectivity of each extractant, which influence their ability to estimate the bioavailable fraction of Cu in soil. Furthermore, the critical levels are affected by soil characteristics related to Cu adsorption capacity, mainly clay and SOM contents, since pH was very similar between soils (Table 1). For EDTA, the IC₂₅ and IC₅₀ values for black oats' biomass production showed an opposite behavior to Mehlich-1 (i.e., EDTA extractable Cu concentrations and IC₂₅ and IC₅₀ increased with increased soil Cu adsorption capacity, which is directly associated with the soil clay and SOM content; Table 1), highlighting the need to calibrate method-specific soil Cu availability thresholds to prevent plant toxicity.

Several studies have shown that black oats, when grown in soils with high Cu availability, do not exhibit a significant increase in Cu concentration in the plant aboveground biomass (Giroto et al. 2014; De Conti et al. 2018; Benedet et al. 2019). In these studies, the authors reported Cu concentrations in black oats shoot biomass ranging from 8.7 to 38.3 mg Cu kg⁻¹ (Giroto et al. 2014; De Conti et al. 2018; Benedet et al. 2019). In our study, although there was an increased Cu concentration in black oats aboveground biomass with the increasing Cu rate (Fig. 2), the tissue-Cu concentrations remained within the sufficiency range (5–20 mg kg⁻¹) proposed by Marschner (2012) across all soils and Cu rate treatments. These results indicate that shoot Cu concentrations are not sensitive to increased soil Cu availability and do not serve as a reference for establishing critical levels for Cu toxicity. The relatively small increase in shoot Cu

concentration with increased Cu availability (Fig. 2) may be associated with the fact that the majority of Cu absorbed by plants is retained in the roots (Giroto et al. 2016; Guimarães et al. 2016; Ferreira et al. 2018). This happens because Cu can remain in the apoplast, possibly linked to functional groups in the cell wall, or compartmentalized in the cell vacuole (Ambrosini et al. 2015, 2018; Tiecher et al. 2017; De Conti et al. 2018). Moreover, part of the plant absorbed Cu can be complexed by P chemical species, precipitating in plant root tissues (Baldi et al. 2018a, b). These features reduce Cu mobility and transport from roots to shoots, and consequently, reduce plant Cu toxicity potential (Brunetto et al. 2016; Marastoni et al., 2019; Morsch et al. 2021). However, using plant roots' Cu concentrations to diagnose Cu toxicity potential is impractical, since the plant root sampling procedure is more complex, time-consuming, and subject to errors and greater contamination potential if not properly executed.

Plant physiological and biochemical processes, such as photosynthesis, are directly affected by increased tissue-Cu concentrations (De Conti et al. 2020a; Conti et al. 2020b; Cesco et al. 2021; Schwalbert et al. 2021). Increased Cu availability in the three soils evaluated in our experiment resulted in reduced total chlorophyll and carotenoid values (Figs. 5 and 6). Research has shown that tissue-Cu concentrations above 20 mg kg⁻¹ of Cu inhibit the plant's photosynthetic rate and reduce photosynthetic pigment levels (Ferreira et al. 2018). In our study, black oats' aboveground tissue-Cu concentrations were below this value (Fig. 2), but root-Cu concentrations were much higher and ranged from 18 to 738 mg Cu kg⁻¹ (Fig. 2). In previous studies conducted with black oats, De Conti et al. (2018) and Benedet et al. (2019) also reported that the above-ground tissue Cu concentrations remained below the critical values for the crop, while high Cu concentrations were observed in the plant root system, negatively affecting the plant's physiological processes. With the increased soil Cu availability, black oats plants showed a reduction in net photosynthesis rate and stomatal conductance (Fig. 5). The photosynthetic rate IC₂₅ and IC₅₀ values for Mehlich-1 and EDTA extractants were very close to the aboveground biomass IC₂₅ and IC₅₀ values (Table 2). Reduced photosynthetic rate is directly related to reduced plant growth and development (Ferreira et al. 2018). In our study, the reduced net photosynthesis rate can be attributed to a possible inhibition of enzymatic processes in the Calvin cycle, as Cu can reduce the synthesis or activity of enzymes in the cycle, thereby reducing the CO₂ demand and, consequently, stomatal conductance (González-Mendoza et al. 2013). Furthermore, the reduced black oats' stomatal conductance at high Cu availability (Fig. 5) may be related to a nutritional imbalance caused by the high Cu concentrations in the soil solution, which can

inhibit the absorption of plant essential ions that are important for plant leaf turgor and essential for the photosynthetic process (Alaoui-Sossé; et al., 2004).

We observed a lower chlorophyll concentration in black oat leaves grown in soil with a high Cu concentration (Fig. 6), which may be associated with the fact that excess Cu inhibits photosynthetic pigment synthesis, such as chlorophylls and carotenoids, and/or alters their structure. This may also occur because the addition of Cu addition to the soil can increase K availability, and K^+ can compete with several cations for absorption sites in the plasma membrane, mainly with Ca^{2+} and Mg^{2+} (Ambrosini et al. 2016). Thus, a decrease in tissue-Mg concentration may occur, which may stimulate the degradation of chloroplasts' internal structure, through the replacement of the central Mg ion by Cu in chlorophyll molecules, contributing to a decrease in the synthesis and concentration of pigments in the leaves (Ciscato et al. 1997; Küpper et al. 2002). Furthermore, Cu can also exert its toxicity by interfering with electron transport, respiration, and ATP production, among other key plant physiological processes (Yruea 2005). Hence, increased tissue-Cu concentration not only inhibits the synthesis of photosynthetic pigments but also interferes with other plant nutrient functions, culminating in reduced photosynthesis and overall plant development.

5 Conclusions

Our working hypothesis was that critical Cu toxicity concentrations would be greater for EDTA, followed by Mehlich-1 and $CaCl_2$, due to increased extraction power of the analytical method and increased soil Cu solubility. Overall, the three analytical methods showed different Cu extraction capacities and critical IC_{25} and IC_{50} concentrations across three diverse soils, which supports our hypothesis and may be strongly related to the characteristics of each soil combined with the chemical properties of each analytical method. Our results suggest the need for method-specific critical Cu phytotoxicity calibration, accompanied by soil properties associated with Cu adsorption capacity, such as clay or soil organic matter content, following a similar approach to Mehlich-1 P availability interpretation in Southern Brazil (CQFS-RS/SC 2016). The Cu phytotoxicity threshold based on Cu concentrations extracted with EDTA and Mehlich-1 for plant biomass production, photosynthetic rate, stomatal conductance, total chlorophyll, and carotenoids, allowed us to more realistically estimate comparable toxicity values between three diverse soils based on an inhibitory concentration of 25 and 50% (IC_{25} and IC_{50}). Thus, our results suggest that, in the future, the definition of critical phytotoxicity Cu and other trace metals should be

based on extractable concentrations. Additionally, the IC_{25} and IC_{50} values for aboveground biomass productions and photosynthetic rate were consistent and relatively within a close range for black oats, suggesting that plant photosynthetic rate may be an effective non-destructive method to assess metal toxicity. While our research brings new insights into the definition of critical Cu toxicity levels for two widely used soil testing methods (EDTA and Mehlich-1), further research is needed to validate the proposed critical Cu toxicity levels and investigate the applicability of the IC_{25} and IC_{50} approach to diverse crops grown in soils with high Cu availability.

Author contributions Paulo Ademar Avelar Ferreira: study design, experiment conduction, laboratory analysis, data collection, interpretation of results, and writing and editing the article. Cesar Cella, Daniela Basso Facco, and Adriele Tassinari: experiment conduction, data collection, laboratory analysis, interpretation of results, and writing and editing the article. Camila Peligrinotti Tarouco and Fernando Teixeira Nicoloso: study design, plant bio-chemical analysis, photosynthetic analysis, experiment conduction, and writing and editing the article. Fábio Joel Kochem Mallmann, Gerson Laerson Drescher, Leandro Souza da Silva, and Gustavo Brunetto: resources, study design, results interpretation, and writing and editing the article.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614). The authors are grateful to Brazilian Federal Agency for Support and Evaluation of Graduate Education- CAPES (Finance Code 001), Brazilian National Council for Scientific and Technological Development – CNPq, and Foundation for Research Support of the State of Rio Grande do Sul – FAPERGS for the scholarships provided and the financial resources made available for this study.

Data Availability Data will be made available upon request.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests All authors declare no financial or non-financial competing interests.

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