



Physiological and biochemical characterization of copper-toxicity tolerance mechanism in grass species native to Pampa Biome and Atlantic Forest for use in phytoremediation

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Abstract

Orchards and vineyards account for significant copper (Cu) accumulation in the soil due to frequent Cu fungicide applications to control leaf diseases. Although grass species are distributed in these areas likely because of their physiological mechanisms to combat Cu toxicity-related stress, the aim of the present study is to identify grass species presenting biochemical-physiological responses that feature adaptive Cu toxicity tolerance mechanisms. Three grass species native to the Pampa and Atlantic Forest biomes (*Paspalum notatum*, *P. plicatum*, and *P. urvillei*) and an exotic species (*Cynodon dactylon*) were tested. Plants were cultivated in pots filled with 4 kg of typic Hapludalf soil, under two Cu availability, control, and toxicity conditions (80 mg Cu kg soil⁻¹). Photosynthetic parameters, relative growth rate, root dry matter, shoot dry matter, the activity of stress-fighting enzymes (superoxide dismutase and guaiacol peroxidase), root biometry, soluble organic carbon, soil pH, and electrical conductivity were evaluated. *P. notatum* and *P. urvillei* have physiological characteristics that allow high translocation factor and Cu accumulation in the root and shoot, and it allows their use in phytoremediation processes due to (1) greater activity of stress-fighting enzymes such as POD in the shoot; (2) to larger diameter roots, which allow greater Cu complexation in them — they are lesser sensitive to stress caused by Cu than the other species; and (3) greater soluble organic carbon exudation in the rhizosphere than species *P. plicatum* and *C. dactylon*, which can complex Cu²⁺ and reduce the presence of forms toxic to plants.

Keywords Root exudates · Phytotoxicity · Metal tolerance mechanisms · Heavy metals · Vine · Orchards

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Introduction

Fungal diseases in leaves and fruits can be observed in agricultural and fruit species, mainly when they are cultivated in humid regions and under higher air temperatures. *Monilinia fructicola* (Wint.) honey and *Cladosporium carpophilum* in peach trees (*Prunus persica*), *Venturia inaequalis* and *Colletotrichum gloeosporioides* in apple trees (*Malus domestica*), and *Plasmopara viticola* in vines (*Vitis* sp.) are some examples of these species.

Diseases reduce fruit yield and depreciate their composition; they also affect the quality of by products and their commercial value. Orchards and vineyards in different regions worldwide are subjected to frequent copper fungicide applications for disease control purposes, on a yearly basis. However, copper (Cu) in fungicides can reach soil surface through its unidirectional application on

plants; Cu flows from leaves during precipitation events or from senescent leaves that fall on the ground — these processes increase soil Cu levels over time.

Studies have indicated that acidic, sandy soils presenting low organic matter contents and that have been cultivated with 40-year-old vineyards may have more than $104.2 \text{ mg Cu kg}^{-1}$ — natural contents are close to $1.2 \text{ mg Cu kg}^{-1}$ (Giroto et al. 2014). Copper (Cu) accumulation intensity in vineyard soils changes depending on soil characteristics, application frequency, and cultivation period. Accordingly, soil Cu levels reported for different grape producing regions worldwide change — they can reach $250 \text{ mg Cu kg}^{-1}$ in Australia (Pietrzak and McPhail 2004), $372 \text{ mg Cu kg}^{-1}$ in Italy (Dell'Amico et al. 2008), and $583 \text{ mg Cu kg}^{-1}$ in Spain (Fernández-Calviño et al. 2008). Korchagin et al. (2020) have shown that Cu concentration in the soil surface layer of a centenary vineyard in Southern Brazil was close to $2000 \text{ mg Cu kg}^{-1}$, and this number represents high risk of toxicity to plants, as well as of this metal transfer to aquatic environments.

Grass species native to South America, such as *Axonopus affinis*, *Paspalum notatum*, and *Paspalum plicatulum*, grow naturally between orchards and vineyards' rows under contamination conditions. Assumingly, they use different mechanisms to both reduce absorption and minimize absorbed Cu toxicity effects (De Conti et al. 2018). De Conti et al. (2018) and Silva et al. (2020) highlighted the importance of these native grass species for phytoremediation. They reported that genus *Paspalum* can absorb and accumulate more Cu, mainly in the root system, than other native species. In addition, they also found that, because of increased soil Cu levels, native grasses cultivation mostly accounted for changing soil solution's chemical composition, for increasing organic acids' concentration and, consequently, organic carbon soluble — this process enhances Cu^{+2} complexation by decreasing the toxicity potential. However, organic acids, mainly in the rhizosphere region, can adsorb H^+ , rise solution pH and, consequently, contribute to decrease Cu^{2+} activity (Oburger et al. 2009).

Cover plant species, such as some species natives to the Pampa Biome, can be sensitive to Cu toxicity. Such a toxicity leads to reactive oxygen species (ROS) formation in the roots and shoot; therefore, it can be a tool to detect stress level in plants due to Cu excess (Huang et al. 2019). The superoxide anionic radical ($\text{O}_2^{\bullet-}$) produced by the mono-electronic reduction of oxygen, which is rapidly converted into hydrogen peroxide (H_2O_2), is one of the most representative ROS, given its spontaneous or enzymatic dismutation, which is triggered by the enzyme superoxide dismutase (SOD) (Gill and Tuteja 2010). H_2O_2 is lesser reactive than $\text{O}_2^{\bullet-}$; therefore, SOD activity is a mechanism adopted to fight oxidative stress (Gill and Tuteja 2010).

Identifying native grass species presenting tolerance mechanisms against Cu toxicity can be a strategy to mitigate contamination in these areas, since they can be grown in contaminated vineyards and orchards. Native grass species are highly adapted to the soil and climate conditions of each region; therefore, their tolerance to high soil Cu concentrations can vary. Previous studies carried out in Australia have reported a wide variety of metal-tolerant tree and grass species (Grant et al. 2002; Lamb et al. 2010). Species belonging to genera *Axonopus* and *Paspalum*, in South America, have already been reported as having soil Cu toxicity tolerance mechanisms (De Conti et al. 2019) — the best adaptations to fight toxicity are related to increased root yield and to high shoot dry matter, photosynthetic rate, as well as to larger amounts of free radical fighting enzymes.

Identifying the biochemical and physiological responses to Cu toxicity of several grass species native to the Pampa and Atlantic Forest Biomes is essential to identify species presenting greater potential to phytoremediate soils contaminated with Cu. Our hypothesis indicates that native grass species with the potential to be used for phytoremediation have one, or more, biochemical mechanisms capable of allowing greater Cu accumulation in the shoot and consequently, lesser stress, which leads to satisfactory dry matter production. The aim of the current study was to identify native grass species presenting biochemical-physiological responses that highlight adaptive Cu toxicity tolerance mechanisms capable of mitigating Cu contamination in areas such as vineyards and orchards.

Materials and methods

Used soil and species' selection

The study was carried out in a greenhouse, at Federal University of Santa Maria (UFSM), Santa Maria County, Rio Grande do Sul State, Southern Brazil. Mean temperature in the greenhouse throughout the study was 26°C , and relative humidity was 50%. Three grass species native to the Pampa and Atlantic Forest biomes were used, namely, *Paspalum notatum* (fork grass), *Paspalum plicatulum* (mattress grass), and *Paspalum urvillei* (Roça grass), as well as an exotic species (*Cynodon dactylon* — star grass). The species were selected based on their ability to accumulate Cu — according to data from a previous study (Silva et al. 2020). Seedlings were collected from vineyards and orchards located in Santana do Livramento County, Rio Grande do Sul State, Southern Brazil ($\text{S}30^\circ46'$, $\text{W}55^\circ22'$).

Seedlings were multiplied for standardization before the experiments; In that protocol, the collected plant

species were divided into tillers, separated, and washed. The roots of all plants were standardized to 5 cm in length, and three roots and shoot were standardized to 5 cm in length. After this procedure, were transplanted to plastic trays (15-L) filled with sand (substrate), and cultivated in greenhouse under 50% shading for 3 months, based on the protocol proposed by Marques (2020a). Sand trays were irrigated three times a day, for 15 min, with 10 l of nutrient solution by using an automatic pump and timer system with complete nutrient solution (mg L^{-1}) containing 149.80 of NO_3^- , 24.80 of H_2PO_4^- , 39.27 of SO_4^{2-} , 41.31 of Mg^{2+} , 288.72 of Ca^{2+} , 234.60 of K^+ , 0.03 of Mo, 0.26 of B, 0.06 of Cu, 0.50 of Mn, 0.22 of Zn, and 4 of Fe. One month after the start of the multiplication procedure, in order to expand the plant bank and increase homogeneity among seedlings belonging to each species, again the species were divided into tillers, roots of all plants were standardized to 5 cm in length and three roots and shoot was standardized to 5 cm in length. This process was repeated once a month, for 3 months.

Treatments and experimental procedures

The soil used in the experiment was classified as typical Hapludalf (Soil Survey Staff-Soil Taxonomy, 2006). It was collected from the 0–20 cm layer in a site cultivated with native pastures located in Campanha Gaúcha region, Santana do Livramento, which is known by its low natural Cu levels. Soil physical chemical features are shown in Table 1. Soil samples were air dried, homogenized, and sieved in 2-mm mesh. Soil pH was adjusted by adding Ca carbonate (CaCO_3) and Mg carbonate (MgCO_3) at 2:1 ratio to 0.57 g kg^{-1} of soil. Subsequently, 40 mg P kg^{-1} and 100 mg K kg^{-1} were added to the soil with triple superphosphate and K chloride, respectively. Humidity was restored to 80% of field capacity (FC) and incubated for another 25 days.

The treatments counted on two Cu availability conditions: control (natural Cu concentration in the natural field) and Cu toxicity condition — 80 $\text{mg Cu kg soil}^{-1}$ (soil Cu level in approximately 40-year-old vineyards) (Giroto et al. 2014). Copper addition was performed 30 days after con-cealer application through $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution application (Reagent P.A., Vetec). Soil samples were incubated for 40 additional days in greenhouse under controlled temperature condition ($25 \pm 5^\circ\text{C}$). Distilled water was added to the samples in order to replenish the evaporated water and maintain soil water retention capacity at 80%.

The study design consisted of a root concentration system developed to separate rhizospheric from non-rhizospheric soil in 77-micron polyester screen (Supplementary material 1). Seedlings were removed from the sand and weighed for initial fresh matter determination

Table 1 Soil physical and chemical features at layer 0–0.20 m in the used typic Hapludalf soil

Soil parameters	Value
Clay (g kg^{-1})	54
Sand (g kg^{-1})	894
Silt (g kg^{-1})	52
Organic matter (g kg^{-1})	9.0
pH- H_2O (1:1)	5.2
Exchangeable Al (mg kg^{-1})	0.4
Total Cu (mg kg^{-1})	3.68
Available Cu by EDTA (mg kg^{-1})	0.7
Total Zn (mg kg^{-1})	6.26
Available Zn by EDTA (mg kg^{-1})	0.9
Available K by Mehlich-1 (mg kg^{-1})	66.4
Available P by Mehlich-1 (mg kg^{-1})	3.6
Available Fe by EDTA (mg kg^{-1})	5.9
Available Mn by EDTA (mg kg^{-1})	15.4
Exchangeable Ca (mg kg^{-1})	0.5
Exchangeable Mg (mg kg^{-1})	0.2
Exchangeable Mg (mg kg^{-1})	0.2
CECpH 7.0*, cmolc kg^{-1}	3.2

*CECpH 7.0 cation exchange capacity at pH 7.0

(FMi). They were implanted in the sampling units after they were weighed. Therefore, all plants were standardized: three roots and three fully expanded leaves. Pots filled with 4 kg of soil, with their respective treatments, distributed in bifactorial model (two soil Cu levels and four grass species) were the sampling units. The experiment followed a completely randomized design, with four repetitions.

Seven seedlings were initially implanted in the pots; only five plants were left in each pot after 7 days. The experiment started on November 3rd, 2019, and it was conducted for 75 days. In total, 15 mg N kg^{-1} of soil (in the form of urea) were applied at 25 and 50 days after grass seedling transplantation. Soil moisture was maintained at 70% of field capacity, based on daily vessel weighing.

Photosynthesis analysis

Net photosynthetic rate (A), CO_2 stomatal conductance (Gs), water use efficiency (WUE), and instantaneous carboxylation efficiency by RUBISCO [ribulose-1,5-bisphosphate-carboxylase/oxygenase (ECR)] were the evaluated parameters. These variables were assessed in a chamber, at CO_2 concentration of 400 $\mu\text{mol mol}^{-1}$, temperature ranging from 20 to 25° C, 50% \pm 5% relative humidity, and photon flux density of

1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Therefore, the association between CO_2 assimilation capacity and water loss to the environment was established due to tolerance to Cu toxicity stress.

Collection, relative growth rate determination, and biomass partitioning

Plants were removed from the experimental system on February 3rd, 2020 (after 75-day cultivation). Rhizospheric soil was separated from the roots and stored in refrigerator for soil solution extraction. Subsequently, plant roots were washed with water to remove adhered soil particles. Whole plants (i.e., roots + shoots) were weighed to find the final fresh matter (FMf). Plants' relative growth rate (RGR) was calculated through Eq. 1, based on FMi and FMf values:

$$RGR (g \text{ days}^{-1}) = \frac{FMf - FMi (g)}{\text{Experiment's conduction period (days)}} \quad (1)$$

Subsequently, plants were washed in 1 mol L^{-1} HCl solution and in distilled water. They were separated into leaves, stem, and roots. The organs were dried in oven, at 65 °C, until reaching constant mass; they were used for later leaf, stem, and root dry matter determination (LDM, SDM, and RDM).

Physiological stress analysis

The last two fully expanded leaves were removed from five plants per sample unit at plant harvesting time (after 75-day cultivation). Leaves were frozen in liquid N_2 and, subsequently, preserved in ultrafreezer, at -80 °C, for superoxide dismutase (SOD) and guaiacol peroxidase (POD) determination.

SOD enzyme (enzyme units (U mg^{-1} protein) activity was estimated through the spectrophotometric method (Gianopolitis and Ries 1977). Potassium phosphate (50 mM, pH 7.8), methionine (13 mM), riboflavin (2 μM), nitroblue tetrazolium (75 μM), ethylene diaminetetraacid (0.1 mM), and enzyme extract (100 μL) solution was used for such a purpose. Formosanblue photochemically produced from nitroblue tetrazolium was monitored through absorbance increase by 560 nm. One SOD unit was defined as the amount of enzyme required to inhibit nitroblue-tetrazolium photoreduction by 50% (Beauchamp and Fridovich 1971).

Guaiacol was used as substrate to evaluate POD enzyme (U mg^{-1} protein) activity. Potassium phosphate (100 mM, pH 6.5), guaiacol (15 mM), and hydrogen peroxide (3 mM) solution were used. Enzyme activity was measured through guaiacol oxidation into tetraguaiacol; it recorded absorbance

at 470 nm. Molar extinction coefficient of 26.6 $\text{mM}^{-1} \text{cm}^{-1}$ was used to calculate enzyme activity.

Protein was determined based on the method by Bradford (1976) by using albumin, in all enzymatic assays.

Root biometrics

Roots were separated from the shoot, sieved in 1-mm mesh, washed in distilled water and stored in freezer at 4 °C, at plant harvesting time (after 75-day cultivation). Roots were thawed, suspended in 0.5 cm water slide on 30 \times 40 cm^2 clear acrylic tray, and scanned at 600 dpi in scanner EPSON Expression 11,000, equipped with additional TPU light to determine total root length, diameter, and volume (Marques et al. 2020a, b). WinRHIZO© Pro 2007 software (Regent Instruments, Quebec, Canada) was used to analyze the digitalized images. Subsequently, each root sample was dried for 72 h, at 65 °C, and weighed in order to obtain root dry matter (RDM).

Soil solution and soil and tissue Cu concentration

Rhizospheric soil samples were thawed and weighed to assess chemical changes in soil solution, which was extracted through the centrifuge method, as described by Somavilla et al. (2017). Then, soluble organic carbon (SOC), pH, and electrical conductivity (EC) were determined.

In total, 1 ml of solution was subjected to digestion for 4 h, at 60° C, in sulfochrome mixture of $\text{K}_2\text{Cr}_2\text{O}_7$ 1.25 mol L^{-1} , with 80 mL of concentrated H_2SO_4 , at sample:mix ratio of 1:1 for SOC analysis. Reading was performed based on the colorimetry method, at 580 nm. Factor of 1.35 was used in comparison to the glucose curve, since this procedure is correlated ($r=0.997$) to the method described by Moore (1985). The pH was determined in pH meter. R-TEC-7-MP conductivity was determined in digital conductivity meter model TEC-4MP.

Shoot and root DM were ground in Wiley mill for Cu concentration determination. Then, 0.2 g of DM was digested in 3.0 ml of HNO_3 + 1 ml of HClO_4 (Tedesco et al. 1995). Samples' digestion was carried out in digestion block heated to 130 °C, for 4 h. Copper (Cu) concentration in the extracts was analyzed in atomic absorption spectrophotometer (EAA; Varian SpectrAA-600, Australia). Translocation factor (TF) was determined based on Cu concentration in shoot and root DM. Plants' ability to accumulate a certain metal in the shoot, in relation to its concentration in the roots, was calculated through Eq. (2) (Ali et al. 2013):

$$TF = \frac{\text{Cu concentration in the shoots}}{\text{Cu concentration in roots}} \quad (2)$$

Cu accumulated in both the root (CuRac) and shoot (CuSac) was determined by multiplying Cu concentration in tissue by its DM.

Statistical analysis

Variables were subjected to analysis of variance in RStudio software—Easynova package. Differences between means were compared through Tukey test at 5%, based on bifactorial model, species (4) × treatments (2), with four repetitions, in case treatment effects were significant at 5% probability level, in the *F* test.

Principal component analysis was performed in MULTIV software in order to assess the association between TF, CuRac and CuSac, and the assessed variables (Pillar 2001).

Results

Biomass growth and partition

There was interaction between factors in all variables. *P. notatum* presented the highest relative growth rate (RGR) among the assessed species under natural soil Cu availability conditions (Table 2). However, all species showed the same RGR pattern under toxicity conditions, and they did not statistically differ from each other. All species had their RGR reduced in Cu-contaminated soil.

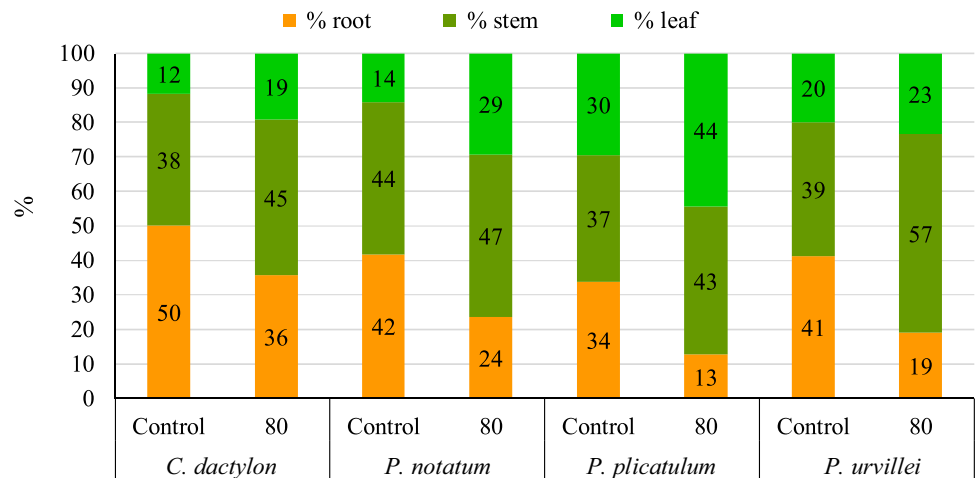
All species recorded reduced root partition rate in total dry matter, and it resulted in increased leaf and stem participation (Fig. 1). Roots' contribution to total dry matter in species *C. dactylon*, *P. notatum*, *P. plicatulum*, and *P. urvillei* decreased by 15, 18.2, 21.2, and 22.1%, respectively. Species *P. notatum* and *P. plicatulum* recorded greater leaf contribution to total dry matter due to toxicity condition; it increased leaf contribution to total dry matter by 15%.

Table 2 Relative growth rate (RGR), root dry matter (RDM), and shoot dry matter (SDM) production by grass species based on Cu conditions

Species	Treatment	RGR (g day ⁻¹)	RDM (g)	SDM (g)
<i>C. dactylon</i>	Control	1.3 ± 0.3 bA*	16.9 ± 16.0 aA	12.0 ± 2.0 aA
	80	0.3 ± 0.1 aB	2.0 ± 1.2 aB	3.1 ± 0.7 aB
<i>P. notatum</i>	Control	1.6 ± 0.1 aA	13.6 ± 2.8 aA	18.7 ± 1.7 aA
	80	0.4 ± 0.1 aB	2.2 ± 1.1 aB	6.5 ± 1.0 aB
<i>P. plicatulum</i>	Control	1.2 ± 0.2 bA	9.2 ± 3.4 aA	17.3 ± 1.5 aA
	80	0.4 ± 0.1 aB	0.8 ± 0.5 aB	5.5 ± 0.3 aA
<i>P. urvillei</i>	Control	1.1 ± 0.1 bA	8.3 ± 1.0 aA	11.8 ± 0.7 aA
	80	0.6 ± 0.1 aB	2.3 ± 0.8 aB	9.3 ± 2.2 aA

*Means ± standard deviation. The same lowercase letters in the column did not statistically differ ($P < 0.05$) in species subjected to the same treatment. The same capital letters in the column did not statistically differ ($P < 0.05$) between treatments applied to the same species

Fig. 1 Root, stem and leaf distribution rate in the dry matter of grass species based on Cu conditions



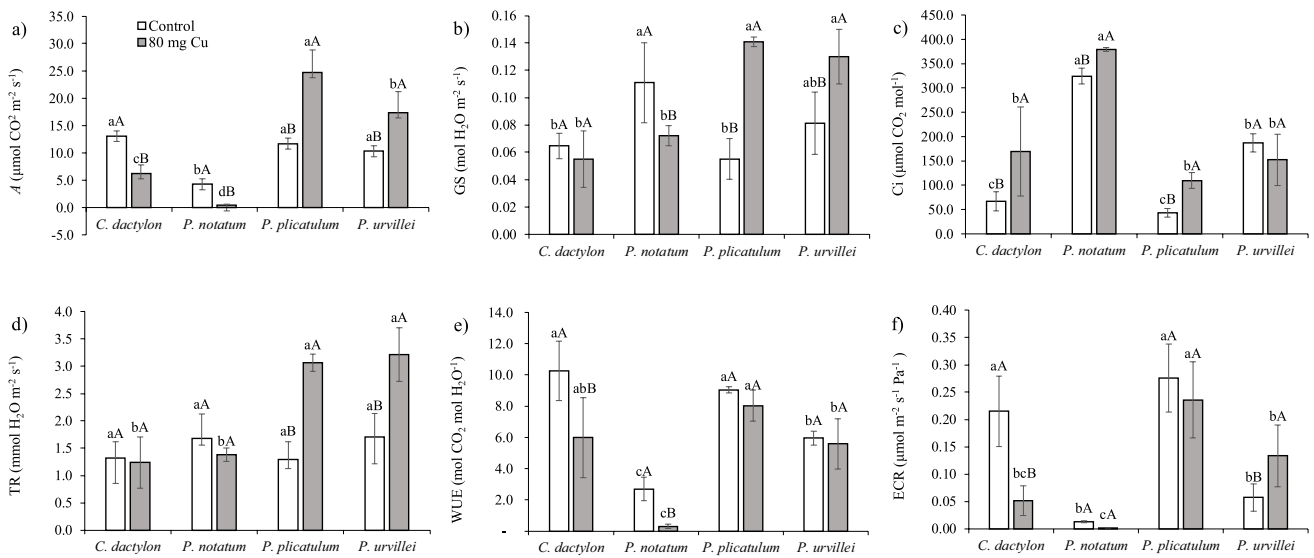


Fig. 2 Photosynthetic parameters of grass species’ net photosynthetic rate (A), CO₂ stomatal conductance (GS), internal CO₂ (Ci) concentration, transpiration rate (TR), water use efficiency (WUE), and RUBISCO’s instantaneous carboxylation efficiency (ECR) based on

soil Cu conditions. Same lowercase letter did not statistically differ ($P < 0.05$) in species subjected to the same treatment. Same capital letter did not statistically differ ($P < 0.05$) between treatments applied to the same species

Photosynthesis analysis

The net photosynthetic rate (A) recorded for *C. dactylon*, *P. plicatulum*, and *P. urvillei* was the highest under natural Soil Cu availability conditions (Fig. 2a). However, *P. plicatulum* and *P. urvillei* presented higher A under high Cu availability — it increased by 112% and 69% in comparison to the control condition, respectively. CO₂ (Gs) stomatal conductance showed higher values in *P. urvillei* and *P. notatum* (Fig. 2b); however, *P. plicatulum* and *P. urvillei* presented the highest Gs values — approximately 113% higher than that of *C. dactylon* and *P. notatum* — when plants were cultivated under Cu toxicity conditions.

P. notatum presented the lowest A values and the highest Ci values, either under natural soil Cu concentration

conditions or at natural toxicity levels when the internal CO₂ (Ci) concentration was taken into account. This finding results from the low A values, since lower CO₂ consumption by the photosynthetic process tends to increase intercellular CO₂ concentration — this process is evidenced by RUBISCO’s lower instantaneous carboxylation efficiency (ECR).

The species did not show differences in transpiration rate (TR) under natural Cu availability conditions (Fig. 2d). However, *P. plicatulum* and *P. urvillei* presented the highest TR values under Cu toxicity conditions. Water use efficiency (WUE) by the assessed species recorded different responses. On the one hand *C. dactylon* and *P. notatum* reduced WUE, but, on the other, *P. urvillei* and *P. plicatulum* maintained the same WUE, without reduction in it due to toxicity. RUBISCO’s instantaneous carboxylation efficiency for *P.*

Table 3 Root parameters of the assessed grass species based on Cu conditions

Species	Treatment	Length (cm pot ⁻¹)	Surface area (cm ² pot ⁻¹)	Mean diameter (mm)	Root volume (cm ³ pot ⁻¹)
<i>C. dactylon</i>	Control	16,620.8 ± 1050.8 aA*	2836.9 ± 1931.8 aA	0.54 ± 0.12 bA	39.5 ± 28.7 aA
	80	1493.3 ± 1044.7 aB	239.6 ± 151.6 bB	0.54 ± 0.07 bA	3.1 ± 1.8 bB
<i>P. notatum</i>	Control	6660.1 ± 998.6 bA	1566.6 ± 112.8 bA	0.75 ± 0.06 aA	29.5 ± 1.0 bA
	80	787.2 ± 720.9 bB	181.5 ± 147.1 bB	0.77 ± 0.08 aA	3.3 ± 2.4 bB
<i>P. plicatulum</i>	Control	6968.8 ± 2546.6 bA	1288.3 ± 472.4 bA	0.59 ± 0.05 abB	19.1 ± 7.4 bA
	80	627.2 ± 459.8 bB	128.2 ± 84.7 bB	0.67 ± 0.07 abA	2.1 ± 1.2 bB
<i>P. urvillei</i>	Control	8649.8 ± 2246.7 bA	1626.9 ± 336.5 bA	0.61 ± 0.09 abB	24.8 ± 7.1 bA
	80	1873.3 ± 1411.3 aB	409.2 ± 297.8 aB	0.70 ± 0.09 aA	7.1 ± 5.0 aB

* Means ± standard deviation. The same lowercase letters in the column did not statistically differ ($P < 0.05$) in species subjected to the same treatment. The same capital letters in the column did not statistically differ ($P < 0.05$) between treatments applied to the same species

plicatulum did not decrease under toxicity conditions (80 mg of Cu kg soil⁻¹); it accounted for the highest ECR values among the assessed species.

Root biometrics

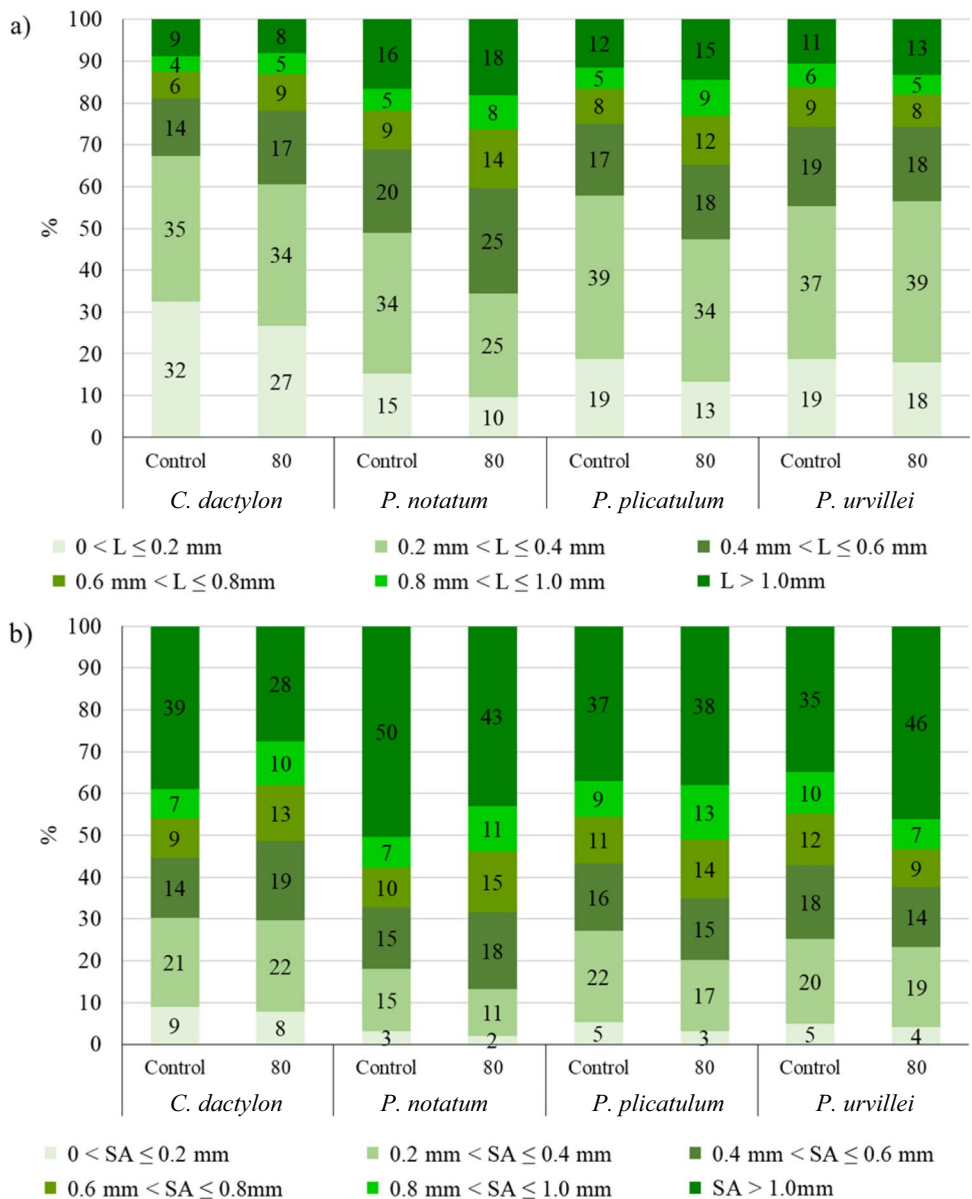
C. dactylon recorded the highest root length values in the control group, among all the assessed species (Table 3). However, *C. dactylon* root length was close to that of *P. urvillei* under Cu toxicity conditions -this species recorded the lowest root length decrease, 78%, respectively, in comparison to the control.

C. dactylon recorded the greatest root surface area (Table 3) among the assessed species under natural Cu concentration conditions. However, *P. urvillei* was the species

accounting for the highest root surface area values under toxic soil Cu concentrations. *C. dactylon* root mean diameter was always smaller, regardless of soil Cu concentration — this species always showed thinner roots than the other species, on average, as well as no root diameter changes. Species *P. plicatulum*, *P. notatum*, and *P. urvillei* produced larger roots under toxic conditions.

Root volume decreased due to Cu concentration increase in all species (Table 3). However, the volume of roots produced in each plant presented different patterns depending on soil Cu availability. *C. dactylon* recorded the highest root volume under natural Cu availability conditions (Control). However, *P. urvillei* was the species accounting for the highest root volume under Cu toxicity conditions.

Fig. 3 Root length (L) (a) and surface area (SA) (b) in different root diameter classes recorded for grass species based on Cu conditions



Species *C. dactylon* showed the highest total length rate among fine roots — 32% of root lengths were shorter than 0.2 mm under the control treatment (Fig. 3a). This species showed 26.7% of root lengths in roots < 0.2 mm (in diameter), even under toxic conditions. Species *P. urvillei* recorded the smallest root length variation rate among the thinnest class of roots (between 0 and 0.2 mm): 18.7% in the control, and 17.9% under toxicity condition.

Root surface area rate (between 0 and 0.2 mm, in diameter) decreased due to Cu concentration increase in all species (Fig. 3b). However, *P. urvillei* recorded the highest rate of roots greater than 1 mm in diameter under Cu treatment. *P. notatum* and *P. urvillei* recorded a very close rate of roots > 1 in diameter; *P. notatum* accounted for the highest rate (50%) of roots > 1 mm, in diameter, in the control group.

Physiological stress analysis

Peroxidase activity (POD) under natural soil Cu concentration conditions was the highest in *P. notatum*. However, *P. plicatulum* showed POD activity increase in tissue at toxic soil Cu concentration (Table 4). *P. urvillei* was the only species that did not show POD activity modification between natural and Cu toxicity conditions. Differently, *P. notatum* is the species with high activity in natural conditions. On the other hand, the species *C. dactylon* and *P. plicatulum* showed increased POD activity under stress conditions.

The highest superoxide dismutase (SOD) activity values were recorded for species *P. notatum* and *P. plicatulum*, mainly under soil Cu toxicity conditions (Table 4). Species *P. urvillei* also showed high SOD activity under natural soil Cu concentration conditions. Overall, there was no SOD activity increase due to high soil Cu concentration in these species.

Soil chemical attributes

Soluble organic carbon (SOC) concentration under natural soil Cu conditions was higher in the three species belonging to genus *Paspalum* (Table 5). *P. notatum* presented the highest soil solution SOC values under soil Cu toxicity conditions. SOC concentrations decreased in all species under soil Cu toxicity conditions.

The aforementioned finding may have resulted from lower root production, which led to less soil rhizodeposition; it may have also resulted from lower carbon assimilation determined by a rhizospheric soil pH value decreased in all species due to Cu concentration increase. Mean soil pH value among species was 6.4 under natural soil Cu availability conditions; this number is 30% higher than that recorded for soil presenting high Cu concentration (mean pH at 4.9).

Solution electrical conductivity (EC) values increased in the soil presenting higher Cu concentration, mainly in species *P. plicatulum* and *P. urvillei*. *P. urvillei* was the species

Table 4 Peroxidase (POD) and superoxide dismutase (SOD) activity in leaves from grass species assessed based on Cu conditions

Species	Treatment	POD (U mg ⁻¹ protein)	SOD (U mg ⁻¹ protein)
<i>C. dactylon</i>	Control	14.8 ± 2.9 cB*	383.3 ± 32.7 bA
	80	19.1 ± 1.7 cA	389.1 ± 19.0 bA
<i>P. notatum</i>	Control	125.1 ± 14.2 aA	590.8 ± 109.2 abA
	80	87.7 ± 16.7 aB	552.9 ± 7.6 abA
<i>P. plicatulum</i>	Control	70.1 ± 4.8 bB	751.9 ± 99.8 aA
	80	97.7 ± 2.5 aA	740.1 ± 71.1 aA
<i>P. urvillei</i>	Control	35.3 ± 7.3 cA	608.2 ± 54.5 aA
	80	58.7 ± 3.5 bA	515.1 ± 81.5 bA

*Means ± standard deviation. The same lowercase letters in the column did not statistically differ (*P* < 0.05) in species subjected to the same treatment. The same capital letters in the column did not statistically differ (*P* < 0.05) between treatments applied to the same species

Table 5 Soluble organic carbon (SOC) concentrations, pH values, and electrical conductivity (EC) in the soil solution, based on grass species cultivation and Cu concentration

Species	Treatment	SOC (ppm)	pH	EC (µS)
<i>C. dactylon</i>	Control	47.3 ± 36.2 bA*	6.5 ± 0.5 aA	504.9 ± 217.3 aA
	80	13.8 ± 5.3 bB	4.8 ± 0.4 aB	888.3 ± 202.1 bA
<i>P. notatum</i>	Control	90.0 ± 13.5 aA	6.5 ± 0.1 aA	251.8 ± 85.1 bA
	80	47.3 ± 3.5 aB	5.2 ± 0.2 aB	456.9 ± 305.2 bA
<i>P. plicatulum</i>	Control	79.2 ± 28.1 abA	5.9 ± 0.4 aA	193.9 ± 32.1 bB
	80	12.8 ± 7.4 bB	5.0 ± 0.8 aB	600.3 ± 405.3 bA
<i>P. urvillei</i>	Control	69.5 ± 9.2 abA	6.8 ± 0.3 aA	484.2 ± 74.6 aB
	80	19.9 ± 9.1 bB	4.6 ± 0.3 aB	3332.7 ± 5274.2 aA

*Means ± standard deviation. The same lowercase letters in the column did not statistically differ (*P* < 0.05) in species subjected to the same treatment. The same capital letters in the column did not statistically differ (*P* < 0.05) between treatments applied to the same species

Table 6 Shoot (CuS), roots (CuR) Cu concentrations, and translocation factor (TF) in grass species cultivated under different soil Cu concentrations

Species	Treatment	CuS (mg kg ⁻¹)	CuR (mg kg ⁻¹)	CuSac (g)	CuRac (g)	TF
<i>C. dactylon</i>	Control	17.2 ± 11.4 bB*	20.7 ± 4.6 aB	0.22 ± 0.19 aA	0.30 ± 0.22 aB	81.3 ± 40.0 bA
	80	74.8 ± 25.7 bA	463.1 ± 24.3 cA	0.23 ± 0.09 cA	0.92 ± 0.57 bA	16.3 ± 6.5 bB
<i>P. notatum</i>	Control	17.1 ± 6.7 bB	30.6 ± 3.5 aB	0.32 ± 0.14 aB	0.41 ± 0.05 aB	57.0 ± 23.3 bA
	80	215.0 ± 134.1 aA	809.5 ± 98.0 bA	1.30 ± 0.60 aA	1.68 ± 0.81 aA	25.6 ± 13.1 aB
<i>P. plicatulum</i>	Control	11.7 ± 1.2 bB	16.6 ± 1.0 aB	0.20 ± 0.03 aB	0.15 ± 0.06 aB	70.6 ± 8.3 bA
	80	170.7 ± 16.4 abA	1583.8 ± 406.9 aA	0.93 ± 0.05 bA	1.19 ± 0.50 bA	11.2 ± 3.0 bB
<i>P. urvillei</i>	Control	27.9 ± 27.0 aB	20.8 ± 4.4 aB	0.32 ± 0.28 aB	0.18 ± 0.06 aB	120.8 ± 87.0 aA
	80	151.02 ± 54.1 abA	909.96 ± 235.0 bA	1.31 ± 0.08 aA	1.93 ± 0.52 aA	16.53 ± 2.8 bB

*Means ± standard deviation. The same lowercase letters in the column did not statistically differ ($P < 0.05$) in species subjected to the same treatment. The same capital letters in the column did not statistically differ ($P < 0.05$) between treatments applied to the same species

accounting for the highest electrical conductivity values under the highest soil Cu concentration.

Cu concentration in tissue

Shoot Cu concentration (CuS) was higher in all species under Cu toxicity condition (Table 6). Overall, species belonging to genus *Paspalum*, mainly *P. notatum*, presented

higher CuS concentrations. Similarly, root Cu concentration (CuR) was high in all species, under Cu toxicity condition. Species *P. plicatulum* recorded the highest CuR values. *P. notatum* accounted for the highest translation factor (TF) value under Cu toxicity condition. This species was the species with the highest potential to absorb and accumulate Cu in tissue; this finding is indicative of its potential to be used in phytoremediation processes. *P. notatum* was even

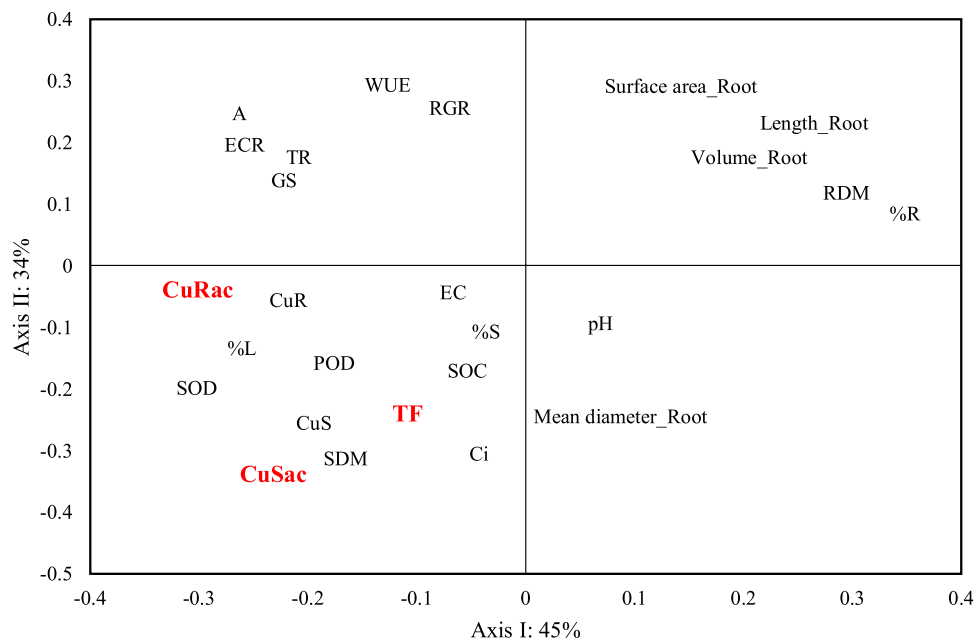


Fig. 4 Principal component analysis (PCA) applied to translocation factor (TF), Cu accumulated in root (CuRac), Cu accumulated in the shoot (CuSac), root Cu concentration (CuR), shoot Cu concentration (CuS), soluble organic carbon (SOC), soil solution pH (pH), electrical conductivity (EC), peroxidase activity (POD), superoxide dismutase (SOD) activity, root length, root surface area, mean root diameter, root volume, relative growth rate (RGR), root dry mat-

ter production (RDM), shoot dry matter production (SDM), root dry matter rate (%R), leaf dry matter rate (%L), stem dry matter rate (%S), rubisco instantaneous carboxylation efficiency (ECR), water use efficiency (WUE), transpiration rate (TR), intercellular CO₂ concentration (Ci), stomatal conductance (GS), and net assimilation rate (A) in native grass species based on concentration of 80 mg Cu kg soil.⁻¹

better than *C. dactylon*, which is an exotic species featured by inhabiting contaminated areas.

Cu accumulated in both the root (CuRac) and shoot (CuSac) was higher in all species under Cu toxicity condition, except for *C. dactylon*, which showed the same CuSac under both conditions (Table 6). *P. notatum* and *P. urvillei* showed the highest CuRac and CuSac values under copper toxicity condition. Physiological features likely contributing to higher TF are shown in Fig. 4. Principal component analysis (PCA) indicated that the Cu translocation factor (TF) was associated with plants recording higher CuR and CuS concentrations. Furthermore, plants accounting for higher SOD and POD activity recorded higher TF values. It likely happened due to their greater capacity to tolerate high soil Cu concentrations because of physiological stress reduction, which led to higher RDM and SDM production.

Discussion

Morphological responses under toxicity conditions

High RGR in plants belonging to genus Paspalum, mainly in *P. notatum*, can be attributed to its great nutrient-use efficiency, such as P, which allows greater dry matter production per absorbed nutrient unit (Marques et al. 2020a, b). Furthermore, this genus has good shoot (Oliveira et al. 2018) and root area production capacity per dry matter unit (Marques et al. 2020a, b). This feature ensures leaf surface production for light capture, and roots for nutrient absorption. This feature also resulted in high dry matter production by *P. urvillei*.

Growth reduction, similar to what is observed in the root system of species accounting for the highest Cu accumulation in the roots, is not necessarily a negative fact. Heavy metals' complexation/immobilization processes under the aforementioned conditions are remarkable on root surface (Barceló and Poschenrieder 2002); they cause complexed Cu^{2+} increase. This is a positive aspect to reduce potential heavy metals' toxicity in plant species, during phytoremediation processes, mainly in production areas (Barceló and Poschenrieder 2002).

Complexation processes in- and outside the roots limit the translocation of the free Cu^{2+} fraction available to the shoot. Furthermore, when these plants are intercropped with fruit plants, fruit trees reduce their metal in soils contaminated with Cu. Thus, the improved development of roots from these grass species in intercropping system could be a cultivation strategy for Cu-contaminated soils (De Conti et al. 2018). Copper (Cu) accumulation in roots is a tolerance mechanism to prevent and/or reduce the translocation of excess Cu to the shoot, since it would

cause significant damage to important plant physiological processes (De Conti et al. 2018).

Biochemical responses under toxicity conditions

Grass species belonging to genus Paspalum, mainly *Paspalum plicatulum*, showed high photosynthetic rate (Fig. 2a), and this finding is indicative of high CO_2 assimilation capacity per leaf dry matter unit (Marques et al. 2020a, b). Based on photosynthetic parameters, *P. urvillei* was the least affected species or the species presenting the best response to Cu treatment. Therefore, it resulted in the smallest RGR reduction for this species (45%) in comparison to that recorded under control conditions.

De Conti et al. (2018) reported that the net photosynthetic rate (A) significantly decreased due to increased concentration of Cu added to soil cultivated with vines (monoculture). On the other hand, whenever vines were intercropped with species belonging to genus Paspalum, A decrease only happened at very high soil Cu concentrations (Conti et al. 2018). This outcome showed that these native species have mechanisms to fight toxicity and that they can benefit from physiological processes such as photosynthesis and intercropping systems (De Conti et al. 2018).

High POD and SOD activity in *P. notatum* and *P. plicatulum* in Cu-contaminated soils represented response to fight metal toxicity by these grass species. SOD activity — which is one of the most effective intracellular antioxidant enzymes observed in all aerobic organisms and subcellular compartments in these species — is an oxidative response to abiotic or biotic stress. This enzyme provides the first defense line against the toxic effects of high reactive oxygen species' levels (Gill and Tuteja 2010). SOD removes $\text{O}_2^{\bullet-}$ and catalyzes dismutation — one $\text{O}_2^{\bullet-}$ was reduced to H_2O_2 and another was oxidized to O_2 . Accordingly, the enzyme removes $\text{O}_2^{\bullet-}$ and, therefore, reduces the risk of OH^\bullet formation inside the cell. However, SOD was not efficient in reducing stress caused by Cu, since it was not activated and there was growth decrease; thus, this enzyme is not featured as efficient mechanism in these species.

On the other hand, POD, from the group of oxidoreductases, can catalyze a large number of oxidative reactions in plants by using H_2O_2 as substrate, or, in some cases, by using oxygen as hydrogen acceptor. The produced H_2O_2 is efficiently removed by enzymes such as POD, and it reduces plant stress. We observed that POD action can be an important mechanism to fight stress, since it makes plants able to grow and tolerate these conditions. This process results in plants capable of accomplishing phytoremediation processes, such as *P. plicatulum* and *C. dactylon*, which

showed increased POD activity due to increased soil Cu concentration.

On the other hand, the fact that *P. urvillei* did not show POD and SOD activity decrease between natural and Cu toxicity conditions points out that this species has defense mechanisms against oxidative stress and that such mechanisms allow high dry matter and RGR production, even in soils contaminated with Cu. These mechanisms can be related to complex enzymatic and non-enzymatic antioxidant defense systems that work together to protect plant cells from oxidative damage by eliminating ROS (Gill and Tuteja 2010).

Changes in soil attributes

Soluble organic carbon (SOC) exudation by native grass species certainly contributes to maintain/increase Cu^{2+} content complexed to carboxylic groups (-COO-) (Brunetto et al. 2014). Another mechanism that may be related to tolerance and needs to be studied is the possibility of exudation of organic acids in the rhizosphere that reduce Cu absorption. Plant roots release a very wide variety of organic compounds that change the rhizosphere's chemical features. For example, changes in pH and the presence of organic binders can increase stable Cu^{2+} complexation in soil solution and, consequently, reduce its bioavailability. These processes favor plant growth, since Cu uptake by roots mostly happens in the free form (De Conti et al. 2018).

In addition to concentration, it should be noticed that plants can also change SOC composition through soluble organic compounds' exudation from the root, such as organic acids and phenolic compounds, as described for malic acid in response to high Cu levels (Nian et al. 2002). This process decreases contaminants' phytotoxic potential (Kim et al. 2010). These compounds' metal complexation property and their release into the rhizosphere have also been described as an attempt by plants to ensure balanced absorption in cases of nutrient scarcity or to limit the availability of toxic elements (Jones 1998; Malta et al. 2016). For example, Cu interaction with SOC functional groups likely account for Cu^{2+} availability decrease in soils treated with biochar and organic compounds (Oustriere et al. 2016).

Interestingly, high soil Cu concentrations led to lower pH in comparison to the control. This phenomenon is strongly related to root concentration effect on the control and to its effect on rhizospheric soil. Custos et al. (2020) highlighted that this process is the effect of absorption by plants — simulations have suggested that the nutrient absorption process is mainly caused by nitrogen, which has strong influence over rhizosphere's pH. Nitrate is approximately 5 to 400 times more absorbed than other ions in aerated soils without ammonium fertilization. Concentration reduction in the rhizosphere is compensated by root H^+ absorption or by equivalent OH^- exudation. Thus, rhizosphere pH of

non-legume plants in non-flooded soils can oftentimes be more alkaline than that in soil layers farther from the roots (Custos et al. 2020).

Interactions of factors allowing tolerance to toxicity

Photosynthetic variables were not necessarily related to TF, but it is related to dry matter increase and production. Although features related to dry matter production are important, *P. notatum*, which is the species accounting for the highest TF in copper-treated plants, did not present higher values, mainly for A, GS, and TR. This pattern was not consistent in all species.

The highest stem rate (%S) — 57% — is an interesting feature that may have contributed to the highest **CuSac** recorded for *P. urvillei*. This finding may highlight the most adapted plants to this toxicity condition. Stem is a plant fraction that has good Cu retention capacity, but such a feature leads to less Cu translocation to the leaves, a fact that results in less damage to the photosynthetic apparatus, which, in its turn, leads to higher CO_2 assimilation rates.

The highest values of A led to greater ECR, as in the *P. plicatulum*. However, higher TR results in lower carbon concentration (Ci), which can estimate higher ECR. Therefore, higher ECR does not necessarily result in more carbon converted to dry matter. Thus, although the species has greater ECR in the condition of toxicity, it produces the same dry matter as the other species in the condition of toxicity. This is because part of the fixed carbon can be converted to stress-reducing compounds, as exudates by the roots like organic acids, used to generate energy in cellular respiration for synthesis of stress-reducing compounds such as antioxidants. Thus, part of the carbon is not used for the production of dry matter and is directed to for the synthesis of stress-reducing compounds (Souri et al. 2019; Hagemeyer 2004).

Plants' root system showed significant association between TF and root diameter. Overall, plants recording the highest TF were the ones accounting for the largest root diameter, such as *P. notatum*. Even without Cu toxicity conditions, these plants presented larger root diameters. This finding is evidenced by the fact that *P. notatum* is the species accounting for the highest root length and surface area rates in the thickest root classes cultivated in Cu-contaminated soils. On the other hand, these variables showed negative association with CuR and CuS, due to root length and volume reduction under Cu toxicity conditions.

Under toxicity conditions, De Conti et al. (2018) highlighted the growth and toxicity reduction capacity by species belonging to genus *Paspalum*, mainly due to intercropping, when it comes to Cu toxicity, since it reduces the negative impact of Cu toxicity (mainly, on leaf and stem dry matter). These results, along with those observed in the current study, point towards the need of integrating the cultivation

of native plants, such as *P. notatum*, to Cu-contaminated soils' phytoremediation, in order to ensure plants' proper development, mainly of young fruit trees.

P. notatum consortium can reduce Cu²⁺ chemical form, if Cu²⁺ is considered the form preferentially absorbed by plants and microorganisms. The consequences of complexed Cu chemical species (Cu-SOC) increase in soil solution lies on reducing fruit plants' bioavailability and potential toxicity. Part of this process is assumingly related to greater root exudation in this species, which releases greater SOC amounts into the rhizosphere (Table 5).

According to De Conti et al. (2018), young vines intercropping with *P. plicatulum* was efficient in promoting plant growth in soils with moderate and low Cu contamination levels by reducing Cu bioavailability. They also showed that Cu phytotoxic effects on root morphology in young vines were reduced by intercropping with these grass species at 40 mg Cu kg⁻¹. This outcome indicates that native grass species maintenance, such as those belonging to genus *Paspalum*, in soils contaminated with Cu based on the effective phytoremediation strategy.

Conclusions

The growth patterns that allow the use of native grasses in phytoremediation are high TF, high CuSac, and high CuRac. These patterns occur due to (1) increased activity of stress-fighting enzymes such as POD, (2) larger roots for greater Cu complexation, which makes roots less sensitive to stress caused by Cu, and (3) greater soluble organic carbon exudation in the rhizosphere, since it can complex Cu²⁺ and reduce the presence of toxic forms in plants. Native grass species do not only have a specific mechanism to reduce Cu toxicity, but a set of physiological features that allow greater dry matter production and higher CuSac and CuRac. *P. notatum* and *P. urvillei* have the greatest potential for phytoremediation in Cu-contaminated areas.

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Data availability The datasets generated and analyzed during the current study are not publicly available due do not belong to a public database but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication All authors agree to publish the manuscript in this form.

Competing interests The authors declare competing interests.

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