Potential phytoremediation of Pampa biome native and invasive grass species cohabiting vineyards contaminated with Cu in Southern Brazil

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Abstract
The objectives were (a) to evaluate whether grasses native to the Pampa biome, Axonopus affinis Chase, Paspalum notatum Flüggé and Paspalum plicatulum Michx, and the invasive grass Cynodon dactylon (L.). Pers have the potential to phytoremediate soil contaminated with Cu (0, 35 and 70 mg Cu kg\(^{-1}\)); (b) assess whether the growth of these species is compromised by the excess of Cu available in the soil; and (c) determine the impact of excess Cu on the physiological responses of the studied species. C. dactylon presented the best performance in soil contaminated with 35 mg of Cu kg\(^{-1}\). In C. dactylon, the concentrations of chlorophyll b and carotenoids increased, as did the photosynthetic rate and plant growth. Phytotoxic effects of Cu in soil contaminated with 70 mg of Cu kg\(^{-1}\) were more severe on A. affinis and led to plant death. The other species presented reduced photosynthetic and growth rates, as well as increased activity of antioxidant enzymes such as superoxide dismutase and guaiacol peroxidase. This very same Cu level has decreased photosynthetic pigment concentrations in P. notatum and P. plicatulum. On the other hand, it did not change chlorophyll a and b concentrations in C. dactylon and increased carotenoid concentrations in it. High values recorded for Cu bioaccumulation-in-grass-root factor, mainly in P. plicatulum, have indicated that the investigated plants are potential phytostabilizers. High C. dactylon biomass production—in comparison to other species—compensates for the relatively low metal concentration in its tissues by increasing metal extraction from the soil. This makes C. dactylon more efficient in the phytoremediation process than other species.

Keywords Heavy metal · Copper tolerance · Phytoremediation · Gas exchange · Antioxidant enzymes · Vineyards

Introduction
Viticulture is an activity capable of generating jobs and income in several countries worldwide (OIV, 2020). Campanha Gaúcha is one of the most promising wine-producing regions in Brazil. Nowadays, there are more than 2000 ha of vineyards installed in the region (Embrapa 2017). Besides its economic importance, viticulture implemented in Campanha Gaúcha is a strategy focusing on the sustainable use of Pampa biome, one of the most diverse rural ecosystems in the world, since it enables the preservation of native species belonging to the countryside flora between crop rows (Chomenko and Bencke, 2016). Given the advanced degradation observed in this biome, in which more than 54% of the original area has already been suppressed, it is essential encouraging economic activities that have low impact on the countryside ecosystem and that preserve its natural vegetation. Thus, the viticulture activity implemented in the region has contributed to avoid native vegetation replacement. In addition, the preservation of the local ecosystem economically benefits viticulturists by adding value to their products due to the environmental appeal generated by it (Sarmento, 2018).
However, similar to what happens in other wine-growing areas in the world (Lagomarsino et al. 2010; Soja et al. 2018; Silva et al. 2020), there is continuous and recurrent use of cupric fungicides in vineyards grown in the Pampa biome, and such a process leads to severe Cu accumulation in the soil over the years. In Brazil, the legislation has as a reference parameter for soil contamination with Cu, total contents of 200 mg Cu kg\(^{-1}\). However, factors inherent to each type of soil, such as the source material and/or physicochemical characteristics, are disregarded. In areas of the Pampa biome cultivated with grapevines, for example, the total Cu content rarely exceeds the limit established by Brazilian legislation. However, the soil is characterized by low levels of organic matter, predominantly sandy texture and low cation exchange capacity. This combination of physicochemical properties has substantial effects on increasing bioavailable Cu contents (Silva et al. 2020). This problem is of such magnitude in these areas, that in places with a history of application of copper fungicides, the concentrations of bioavailable Cu in the soil already exceed by more than 30 times the values observed in adjacent natural areas. (Girotto et al., 2016). Thus, increased Cu availability in the soil poses environmental contamination risk and can lead to severe phytotoxicity in vines, mainly in the native vegetation that has superficial root system.

The intensity of toxicity caused by excessive Cu absorption can change from plant species to plant species. In addition, it can damage plants’ photosynthetic apparatus and reduce pigment synthesis, which, consequently, leads to lower photosynthetic efficiency (Bazihizina et al. 2015; Adrees et al. 2015; Marques et al. 2018b; Houri et al. 2020). Excessive reactive oxygen species (ROS) production via Fenton and Haber–Weiss reactions results in severe oxidative damage to cell biomolecules and compromises the development of different plant species (Marques et al., 2018a, b; Schwalbert et al., 2019). Moreover, Cu toxicity can change the absorption of other nutrients (Li et al. 2019b; Saleem et al. 2020), as well as lead to chlorosis (Albarracín et al. 2010), shoot and root growth inhibition and plant death (Cambrollé et al. 2015; Schwalbert et al. 2019; Trentin et al. 2019). Thus, although the native vegetation is preserved between vineyard rows, increased Cu concentrations in the soil due to crop treatment applications can change the structure of the native plant community over time and increase species’ ability to withstand the deleterious effects of this metal. In addition to compromise the natural balance of plant communities, increased Cu and the concentration of other contaminants in the soil play fundamental role in invasive species’ establishment processes (Morgado et al. 2018).

The ability of native or invasive plant species to develop in soils contaminated with high Cu levels may result from the combination of different metal tolerance mechanisms (Borghi et al. 2008; Adrees et al. 2015; Mwamba et al. 2016). Thus, plants can decrease Cu absorption by releasing root exudates. This process changes soil pH and increases dissolved organic carbon concentrations in it, which, in its turn, changes the solubility, activity and distribution of Cu chemical species in the soil solution. Some plant species are capable of accumulating absorbed metals by complexing and/or compartmentalizing them in subcellular structures (Yruela 2009; Mwamba et al. 2016; Li et al. 2019a), which helps minimizing the toxic effects of these metals. On the other hand, other species trigger specific mechanisms in order to neutralize ROS generated by their exposure to high Cu levels. In these cases, there is increased activity of enzymes such as superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD), in order to minimize oxidative stress (Gill and Tuteja, 2010; Marques et al., 2018a, b; Schwalbert et al., 2019).

Increased incidence of native species such as *Paspalum notatum* Flüggé and *Paspalum plicatulum* Michx, as well as of invasive species such as *Cynodon dactylon* (L.) Pers, in older vineyards grown in the Pampa biome—which present high Cu-related pollution levels (Silva et al. 2020)—suggests that these plants have some specific mechanism capable of providing higher tolerance to this metal. Thus, the higher adaptability of these species to soil contamination conditions favors their establishment and is a strong indicative of their phytoremediation potential. Phytoremediation consists of using plants to remedy contaminated sites; it comprises five processes, namely: phytoextraction, phyto degradation, phytovolatilization, rhizofiltration and phytostabilization. Phytoextraction and phytostabilization are the main processes applied to remediate soils contaminated with heavy metals. Therefore, the aim of the current study was to (1) evaluate whether the growth of Pampa biome native grass species such as *A. affinis*, *P. notatum* and *P. plicatulum*, as well as of invasive grass species such as *C. dactylon*, is compromised by high Cu concentrations available in the soil; (2) to determine whether Cu has negative impact on the physiological responses of the investigated species, such as photosynthetic pigment concentrations, gas exchange and antioxidant enzyme activity; and (3) to evaluate whether the investigated species have the potential to be used in the phyto remediation of soils contaminated with Cu.

**Materials and methods**

**Soil featuring and preparation**

Soil samples were collected in the surface layer (0–10 cm) of a soil classified as Typic Hapludalf (Soil Survey Staff 2014). The place of soil collection was defined from the study by Silva et al. (2020) and consisted of an unanthropized natural field area (30°47′23.5″ S, 55°22′7.0″ W) adjacent to...
vineyards installed in Santana do Livramento County, Rio Grande do Sul State (RS), far Southern Brazil. Collected soil samples presented sandy texture, granulometry comprising 89.45% of sand, 4.30% of silt and 6.25% of clay. After the collection procedure was over, samples were air-dried, homogenized, sieved in 2-mm mesh and featured based on their fertility (Table 1).

Phosphorus (P) concentration in the soil was corrected based on the addition of 40 mg kg⁻¹ of P in its simple superphosphate form. Next, soil samples were divided into portions of 10 kg and packed in plastic bags, where they remained for 15 days at constant humidity corresponding to 70% of their maximum water retention capacity (MWRC)—they were revolved every 3 days. Subsequently, soil contamination was carried out with increasing Cu doses (0, 35 and 70 mg Cu kg⁻¹ of soil)—CuSO₄·5H₂O was used as Cu source. The doses of 35 and 70 mg Cu kg⁻¹ of soil were chosen, as they represent, respectively, a contamination condition that is commonly observed between the rows of older vineyards, and a future condition, in case there is no reduction or suspension of fungicide applications that contain Cu in the soil, and in areas of natural Pampa grassland, adjacent to the vineyards. In that study, the Cu bioaccumulation factor of the most frequent species in each area was also determined, providing initial information on the identification of Cu-tolerant plants with phytoremediation potential. These species were selected from the study by Silva et al. (2020), where the authors carried out a survey of the botanical composition in vineyards with increasing levels of available Cu in the soil, and in areas of natural Pampa grassland, adjacent to the vineyards. In that study, the Cu bioaccumulation factor of the most frequent species in each area was also determined, providing initial information on the identification of Cu-tolerant plants with phytoremediation potential.

Plants were collected in a vineyard grown for 35 years in Santana do Livramento County (30°46′36″ S, 55°22′03″ W), Southern Brazil. Bioavailable Cu content at the 0–20-cm layer of the collection soil site was 40.38 mg kg⁻¹ (extracted through EDTA), and the index of Cu pollution in the soil was classified as high (Silva et al. 2020). Collected species were grown in hydroponic sand culture system, based on the protocol suggested by Marques et al. (2020). Vegetative propagation of the investigated species was carried out every 2 months, for 1 year, in order to expand the plant bank and increase homogeneity between seedlings deriving from each species. During the propagation period, seedlings were irrigated twice a day with complete nutrient solution comprising 149.80 mg L⁻¹ of NO₃⁻, 24.80 mg L⁻¹ of H₂PO₄⁻, 39.27 mg L⁻¹ of SO₄²⁻, 41.31 mg L⁻¹ of Mg²⁺, 288.72 mg L⁻¹ of Ca²⁺, 234.60 mg L⁻¹ of K⁺, 0.03 mg L⁻¹ of Mo, 0.26 mg L⁻¹ of B, 0.06 mg L⁻¹ of Cu, 0.50 mg L⁻¹ of Mn, 0.22 mg L⁻¹ of Zn and 4 mg L⁻¹ of Fe.

**Producing Pampa biome native and Cynodon dactylon seedlings**

*Cynodon dactylon* (Bermuda grass), *Paspalum notatum* (bahiagrass), *Paspalum plicatum* (brownseed paspalum), and *Axonopus affinis* (common carpetgrass) were the plant species tested in the current study. These species were selected from the study by Silva et al. (2020), where the authors carried out a survey of the botanical composition in vineyards with increasing levels of available Cu in the soil, and in areas of natural Pampa grassland, adjacent to the vineyards. In that study, the Cu bioaccumulation factor of the most frequent species in each area was also determined, providing initial information on the identification of Cu-tolerant plants with phytoremediation potential. These species were selected from the study by Silva et al. (2020), where the authors carried out a survey of the botanical composition in vineyards with increasing levels of available Cu in the soil, and in areas of natural Pampa grassland, adjacent to the vineyards. In that study, the Cu bioaccumulation factor of the most frequent species in each area was also determined, providing initial information on the identification of Cu-tolerant plants with phytoremediation potential.

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**Conducting the experiment in greenhouse**

The investigated plant species were grown in greenhouse from August to November 2019. Throughout the whole study, the average temperature in the greenhouse was 26 °C, and the relative humidity was 50%.

Fifteen treatments were evaluated, namely: four grass species (*Cynodon dactylon*, *Paspalum notatum*, *Paspalum plicatum* and *Axonopus affinis*) cultivated in soil contaminated with three Cu doses (0, 35 and 70 mg Cu kg⁻¹ of soil), whereas the other treatments, which were used as negative control, comprised soil contaminated with the same Cu doses, although without plant cultivation. Treatments have followed a completely randomized design (CRD), with five repetitions.

Table 1 Chemical features of the 0.0–0.10 m topsoil layer in a Typic Hapludalf soil under natural grassland

| pH H₂O (1:1) | 5.33 |
|-------------|------|
| Soil organic carbon (%) | 0.54 |
| Available P by Mehlich-1 (mg kg⁻¹) | 2.97 |
| Available K by Mehlich-1 (mg kg⁻¹) | 232.88 |
| Exchangeable Ca (cmolc.kg⁻¹) | 0.44 |
| Exchangeable Mg (cmolc.kg⁻¹) | 0.25 |
| Available Cu by Mehlich-1 (mg kg⁻¹) | 0.81 |
| Available Fe by Mehlich-1 (mg kg⁻¹) | 22.35 |
| Available Mn by Mehlich-1 (mg kg⁻¹) | 24.06 |
| Available Zn by Mehlich-1 (mg kg⁻¹) | 1.06 |
| Exchangeable Al (cmolc kg⁻¹) | 0.20 |
| H + Al (cmolc kg⁻¹) | 2.02 |
| CECef (cmolc kg⁻¹) | 1.49 |
| m (%) | 13.46 |
| CECpH7 (cmolc kg⁻¹) | 2.71 |
| V (%) | 47.45 |
Experimental units consisted in 2-L capacity pots. Two kilograms (2 kg) of soil contaminated with Cu was added to all pots in August 2019. Pots were moistened with distilled water at 70% MWRC. Subsequently, three vigorous and healthy seedlings from each species were weighed to find the initial fresh matter (FMi) and transplanted to different experimental units, based on the adopted treatment. Pots were irrigated on a regular basis to keep water content close to 70% MWRC throughout the experiment. Water availability in the soil was monitored by weighing the pots—distilled water was replenished whenever necessary.

Random leaf samples from each plant were collected at 90 days after transplanting (DAT). They were stored in liquid N2 right away and subsequently placed in ultra-freezer, at −80 °C, until laboratory analysis time (photosynthetic pigment concentration and activity of enzymes such as superoxide dismutase (SOD) and guaiacol peroxidase (POD)).

Assessments

Plant growth

Plant shoot was cut close to the soil at 90 DAT. Roots were manually separated from the soil, washed in running water, then in EDTA (0.002 mol L−1) and, soon after that, in distilled water again. Shoots and roots were weighed to find the fresh matter accumulated in the growing period (FMf). Plants’ growth rate (GR) was determined based on variation in the fresh matter of plants per unit of time (T), in months (Eq. 1).

\[ GR \left( g \text{ month}^{-1} \right) = \frac{FM_f(g) - FM_i(g)}{T(\text{months})} \]  

(1)

Plant shoots were separated into leaves and stem. Leaves, stem and roots were dried in forced air circulation oven, at 65 °C, until they reached constant mass in order to find their dry matter (LDM, SDM and RDM).

Copper (Cu) concentration and accumulation in plant tissues

Leaf, stem and root dry matter was ground and subjected to nitro-perchloric digestion to determine total Cu levels in them, based on the methodology by Embrapa (1999). Total Cu concentrations were read in atomic absorption spectrophotometer (EAA; Varian SpectrAA-600, Australia). The total amount of Cu extracted from the soil by plants was calculated by multiplying Cu concentration in the leaves, stem and roots by the dry matter of these organs.

Photosynthetic pigment concentrations

Samples were prepared to determine the photosynthetic pigment concentrations in leaves, based on the methodology by Hiscox and Israelstam (1979). Tissue samples (0.05 g) were incubated with dimethylsulfoxide (DMSO) at 65 °C, until full pigment removal. The supernatant extract was subjected to absorbance reading in spectrophotometer, at wavelengths of 663, 645 and 470 nm. Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid concentrations were estimated based on the equation by Lichtenthaler (1987); results were expressed as mg g−1 FM (fresh matter).

Gas exchange

Gas exchange parameters in the last fully expanded leaf of each plant were quantified with the aid of Photosynthesis Analyzer—IRGA (Li-6400, Li-COR Inc., Neb., USA). Net CO2 assimilation rate (Anet), stomatal CO2 conductivity (Gs), intercellular CO2 concentration (Ci) and instant carboxylation efficiency (A/Ci) (based on ribulose-1,5-bisphosphate-carboxylase/oxygenase) were the herein evaluated parameters. These variables were determined in chamber, at CO2 concentration of 400 µmol mol−1, temperature ranging from 20 to 25 °C, relative humidity of 50 ± 5% and photon flow density of 1500 µmol m−2 s−1.

Enzyme activity

The activity of enzymes such as superoxide dismutase (SOD) and guaiacol peroxidase (POD) was determined in leaf samples that had been previously frozen and macerated with liquid nitrogen. Leaf samples (1.0 g) were homogenized in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8) added with 1 mM EDTA and 1% Triton X-100. The homogenate was centrifuged at 13,000 g, at 4 °C, for 20 min. Supernatant was used for enzyme activity and protein content assays, based on Zhu et al. (2004). Superoxide dismutase (SOD) activity was assessed based on the spectrophotometric method described by Giannopolitis and Ries (1977). One SOD unit was defined as the number of enzymes inhibiting nitroblue tetrazolium (NBT) at 50% photoreduction. Guaiacol peroxidase (POD) enzyme activity in leaves was determined based on the methodology by Zeraik et al. (2008). The reaction mix comprised 1.0 mL of potassium phosphate buffer (100 mM (pH 6.5), 1.0 mL of guaiacol (15 mM) and 1.0 mL of H2O2 (3 mM)). After the homogenization process was over, the solution was added with 50 µL of plant extract. Guaiacol oxidation into tetraguaiacol was measured through absorbance increase at 470 nm.
Copper (Cu) concentration in the soil and indices

The soil of each pot was removed and homogenized at plant removal time (90 DAT). Next, soil samples were collected to determine Cu concentrations available in them (extracted through Mehlich-1).

Some indices were calculated to evaluate species’ tolerance to Cu and their ability to accumulate it. Among them, one finds: translocation factor (TF), which indicates plants’ ability to translocate metals from the roots to the shoot—it is calculated as: \( TF = \frac{[Cu_S]}{[Cu_R]} * 100 \), wherein \( Cu_S (\text{mg kg}^{-1}) \) is Cu concentration in the shoot and \( Cu_R (\text{mg kg}^{-1}) \) is Cu concentration in the root; Tolerance Index (TI), which is based on biomass production and was used to assess the tolerance of all four investigated species to each Cu concentration—it was calculated as: \( TI = \frac{Bt}{Bc} \), wherein \( Bt (\text{g plant}^{-1}) \) is the biomass of plants grown in soils contaminated with 35 or 70 mg Cu kg\(^{-1}\) and \( Bc (\text{g plant}^{-1}) \) is the biomass of control plants; Bioconcentration Factor (BCF), which corresponds to the root Cu/soil Cu ratio and is calculated as: \( BCF = \frac{[Cu_R]}{[Cu_{So}]} \), wherein \( Cu_R (\text{mg kg}^{-1}) \) is Cu concentration in the root and \( Cu_{So} (\text{mg kg}^{-1}) \) is soil Cu concentration available to plants.

Statistical analysis

The experiment has followed a completely randomized design (CRD), with 5 repetitions. Twelve (12) treatments resulting from grass species cultivation at each Cu concentration (Cu was used as contaminant agent) were taken into consideration at the time to analyze plant variables. Fifteen (15) treatments resulting from soil cultivated with plants and from the negative control without plant were taken into consideration at the time to analyze Cu content in the soil. First, results were subjected to variance normality and homogeneity tests such as the Lilliefors and Shapiro–Wilk tests. Once the variance normality and homogeneity assumptions were confirmed, data were subjected to analysis of variance. Means recorded for treatments showing significant effect in the \( F \) test \( (p \leq 0.05) \) were compared to each other through Scott-Knott test \( (p \leq 0.05) \). All analyses were performed in the SISVAR software (Ferreira, 2011).

Finally, a principal component analysis (PCA) was carried out to evidence the association of plant variables and Cu factor with plants species. The PCA was done by “FactoMineR” package (Le et al. 2008) in R program (R Core Team, 2019). Additionally to the PCA analysis, a one-way analysis of similarities (ANOSIM) test was performed, in a PAST 4.10 software, to evidence difference between plants species.

Results

Cu concentration in plants

Data about species \( A. affinis \) grown at Cu concentration of 70 mg Cu kg\(^{-1}\) were not presented in the current study due to the death of plants subjected to this treatment. Copper concentration in tissues of all four grass species has increased as soil contamination with Cu also increased (Fig. 1a, b and c). All investigated species recorded higher Cu concentration increase in the roots than in the shoot. Species \( P. plicatum \) has shown the highest Cu concentration increase in all organs. The maximum Cu concentration in \( P. plicatum \) leaves, stems and roots reached 32.75 mg kg\(^{-1}\), 133.73 mg kg\(^{-1}\) and 813.55 mg kg\(^{-1}\), respectively, in plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\).

Photosynthetic pigments

Soil contamination with 35 mg Cu kg\(^{-1}\) did not change Chl a concentration in any of the analyzed species (Fig. 1d). Chlorophyll b and carotenoid concentrations in species \( A. affinis \) have also remained unchanged under this contamination condition (Fig. 1e, f). The other investigated species recorded increased carotenoid concentrations (Fig. 1f); \( C. dactylon \) recorded increased Chl b concentration, whereas \( P. notatum \) and \( P. plicatum \) recorded decreased concentrations of it (Fig. 1e).

\( P. notatum \) and \( P. plicatum \) plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\) presented decreased photosynthetic pigment concentrations (Fig. 1d, e and f). Chlorophyll b concentration in \( C. dactylon \) plants remained unchanged in comparison to that of the control treatment (0 mg Cu kg\(^{-1}\)); this species has also recorded increased carotenoid concentrations (Fig. 1e, f).

Gas exchange

All investigated species grown in soil contaminated with 35 mg Cu kg\(^{-1}\) (Fig. 2a, b) recorded increased net photosynthetic rate (A) and stomatal conductance (Gs); species \( P. plicatum \) recorded the maximum value for these parameters. Intercellular CO\(_2\) concentration (Ci) in species \( A. affinis \) has decreased under this very same condition (Fig. 2e), whereas Rubisco’s carboxylation efficiency (A/Ci) has increased (Fig. 2d). Species \( P. plicatum \) and \( P. notatum \) presented increased Ci, as well as decreased A/Ci, at 35 mg Cu kg\(^{-1}\) (Fig. 2c, d). Ci values recorded for species \( C. dactylon \) did not change in plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\) (Fig. 2c), whereas A/Ci values have increased.
Plants grown in soil contaminated with 70 mg Cu kg$^{-1}$ recorded significant decrease in A, Gs and A/Ci. On the other hand, species *C. dactylon*, *P. notatum* and *P. plicatum* grown in this very same soil presented increased Ci (Fig. 2a, b, c and d). Species *P. plicatum* underwent the greatest changes in all gas exchange parameters. *C. dactylon* grown in soil contaminated with 70 mg Cu kg$^{-1}$ was the species that reached the highest A, Gs and A/Ci values.

**Enzyme activity**

SOD and POD enzyme activity in species *C. dactylon*, *P. notatum* and *P. plicatum* was higher in plants grown in soil contaminated with 70 mg Cu kg$^{-1}$ (Fig. 2e, f). *P. plicatum* recorded the highest SOD enzyme activity increase, in comparison to the control treatment (Fig. 2e), whereas *P. notatum* recorded the highest POD enzyme activity increase.
A. affinis plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\) presented increased SOD enzyme activity (Fig. 2e), although POD enzyme activity in them remained unchanged under this very same condition (Fig. 2f).

**Copper increase and accumulation in plants**

The changes in plant growth rate and dry matter yield in C. dactylon, A. affinis, P. notatum and P. plicatulum plants were observed after they were exposed to soil contaminated with Cu, as shown in Fig. 3a, b, c and d. Plant growth rate, as well as LDM, SDM and RDM production in species A. affinis, P. notatum and P. plicatulum, has significantly decreased in plants grown in soil contaminated with 35 and 70 mg Cu kg\(^{-1}\). P. plicatulum grown in soil contaminated with 35 mg Cu kg\(^{-1}\) was the grass species presenting the most affected plant growth; growth rate, LDM, SDM and RDM in this species have decreased by 71.80%, 59.95%, 66.91% and 72.09%, respectively, in comparison to those of plants grown in soil contaminated with 0 mg Cu kg\(^{-1}\). Species A. affinis grown in soil contaminated with 70 mg Cu kg\(^{-1}\) presented the most severe phytotoxic damage, which led to plant death. Leaf dry matter (LDM) production in C. dactylon plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\)
remained unchanged. However, their SMD production has slightly decreased by 7.08%, and their RDM production has increased by 25.19%, under that very same condition. The herein observed RDM production increase has led to increased plant growth rate in *C. dactylon* plants grown in soil contaminated with intermediate Cu concentrations. Thus, no visible Cu toxicity symptom was harmful to *C. dactylon* plant growth. However, similar to what was observed for native grass species, *C. dactylon* plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\) recorded significantly decreased plant growth rate and, consequently, decreased LDM, SDM and RDM production. Despite the sharp decrease in the growth of *C. dactylon* plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\), their growth rate, as well as their LDM, SDM and RDM production, remained higher than values observed for the other grass species grown under that very same condition.

*C. dactylon* grown in soil contaminated with 35 and 70 mg Cu kg\(^{-1}\) was the species that accumulated the highest Cu amount. *C. dactylon* plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\) have accumulated 1369.45 µg of Cu in their biomass (66.96 µg in leaves, 105.97 µg in the stem and 1,196.52 µg in roots), whereas plants grown in soil contaminated with 70 mg kg\(^{-1}\) have accumulated 348.19 µg of Cu (8.07 µg in leaves, 13.98 µg in the stem and 326.14 µg in roots), as shown in Fig. 4d, e and f.

### Soil Cu concentration and indices

Copper content in the soil at the end of the experiment was consistent with the applied concentrations of it (Fig. 5). After the cultivation period was over, soils planted with *A. affinis* subjected to copper concentration of 35 mg Cu kg\(^{-1}\) recorded the lowest Cu concentration in them (27.42 mg kg\(^{-1}\)); they were followed by soils planted with *P. notatum*, *C. dactylon* and *P. plicatulum*, respectively. Soils planted with *C. dactylon* plants subjected to copper concentration of 70 mg Cu kg\(^{-1}\) recorded the lowest Cu concentration in them (59.38 mg kg\(^{-1}\)); they were followed by soils planted with *P. notatum* and *P. plicatulum*.

All species grown in soil contaminated with 35 and 70 mg Cu kg\(^{-1}\) presented low translocation factor (TF). Maximum TF values were observed for *P. notatum* plants grown under these two contamination conditions (Table 2). *C. dactylon* plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\) stood out in tolerance index (TI), which reached values higher than 1. All species grown in soil contaminated with 70 mg Cu kg\(^{-1}\) have shown low TI values. *P. plicatulum* plants...
subjected to both contamination levels recorded the highest bioconcentration factor (BCF).

Fig. 4 Cu accumulation in the leaves (a), stem (b) and roots (c) of A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with 0, 35 and 70 mg kg⁻¹ of Cu. Equal letters mean that values did not differ from each other in the Scott-Knott test \((p < 0.05)\)

Fig. 5 Mean Cu content in the soil after the cultivation of species A. affinis, C. dactylon, P. notatum and P. plicatulum, and in non-cultivated soil (without plant), based on the applied Cu concentrations. Equal letters mean that values did not differ from each other in the Scott-Knott test \((p < 0.05)\)

Principal component analysis and ANOSIM test

In the PCA (Fig. 6), 62% of the variance was explained by the first two components. In these components, the plants species were separated by Cu concentration, with emphasis on P. plicatulum and Cu accumulation, dry matter production and indices, with emphasis to C. dactylon. By ANOSIM test (Table 3), it was possible to evidence significant differences between plants species, principally C. dactylon versus native species, which reinforces the significant difference in plant species responses to Cu levels.

Discussion

In this study, the effects caused by high levels of soil Cu on the development of plants, whether native or invasive, are evident. The growth of these plants in contaminated soil with 70 mg Cu kg⁻¹ inhibited growth and biomass production. This development reduction was mainly linked to the effects caused by Cu excess on the physiological activities of plant cells with consequences for photosynthetic efficiency, carbon immobilization and cell growth. We verified, however, that the intensity of the effects caused by the high concentrations of Cu in the soil over the development of the plants is strongly associated with the plant species. This is because each species adopts different strategies to minimize the toxic effects of Cu.
The low Cu translocation rate recorded for grass shoot (Fig. 1a, b and c) likely resulted from Cu retention in root apoplast (Lequeux et al. 2010). Previous studies have suggested that cell walls are highly capable of binding to heavy metals, mainly to Al and Cu, and that they can act as barrier to prevent these metals from entering the cytoplasm of plant cells (Krzelewskis 2011; Torasa et al. 2019). This high binding ability is attributed to Cu sorption by cell wall components such as lignin, pectin, as well as some polysaccharides and proteins (Colzi et al. 2011; Torasa et al. 2019). Thus, Cu retention in the root system has been described as the main process to limit metal accumulation in leaves, which consequently minimizes Cu effects on plants’ shoot, mainly on their photosynthetic apparatus (Marques et al. 2018b; Li et al. 2019b).

Table 2: Translocation factor (TF), tolerance index (TI) and bioconcentration factor (BCF) of A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with Cu

| Species        | A. affinis | C. dactylon | P. notatum | P. plicatulum |
|----------------|------------|-------------|------------|--------------|
| mg kg⁻¹ of Cu  | 35         | 35          | 70         | 35           |
| TF             | 0.06d      | 0.12a       | 0.07d      | 0.13a        |
| TI             | 0.60c      | 1.07a       | 0.13c      | 0.65b        |
| BCF            | 6.54c      | 3.93d       | 3.84d      | 3.50d        |

Equal letters on the line do not differ from each other in the Scott-Knott test (p<0.05)

Fig. 6: A principal component analyses of all studied variables. CuLeaf leaf Cu concentration, CuStem stem Cu concentration, CuRoot root Cu concentration, Chla chlorophyll A, Chlb chlorophyll B, Carot carotenoids, Anet net CO₂ assimilation rate, Gs stomatal CO₂ conductivity, Ci intercellular CO₂ concentration, A_CI instant carboxylation efficiency, SOD SOD enzyme activity, POD POD enzyme activity, GR plants’ growth rate, LDM leaf dry matter, SDM stem dry matter, RDM root dry matter, CuAcL leaf Cu accumulation, CuAcSt stem Cu accumulation, CuAcR root Cu accumulation, TF translocation factor, BCF bioconcentration factor, TI tolerance index.

Table 3: Differentiation of A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with Cu by ANOSIM test. Significance values (p) of the test are shown

| Species        | A. affinis | C. dactylon | P. notatum | P. plicatulum |
|----------------|------------|-------------|------------|--------------|
| A. affinis     | 0.0001     | 0.0149      | 0.0263     |              |
| C. dactylon    | 0.0149     | 0.0136      | 0.0004     |              |
| P. notatum     | 0.0263     | 0.0004      | 0.0664     |              |
| P. plicatulum  | 0.0136     | 0.0149      | 0.0004     |              |

The lower photosynthetic pigment concentration observed in P. notatum and P. plicatulum plants grown in soil contaminated with 70 mg Cu kg⁻¹ has indicated that Cu excess in leaves may have inhibited the synthesis of the chlorophyll precursor—i.e., the δ-aminolevulinic acid—as well as the protochlorophyllide reductase activity—this enzyme accounts for catalyzing the reductive chlorophyllide formation from protochlorophyllide during chlorophyll biosynthesis (Stiborová et al. 1987; Petit et al. 2012). Decreased pigment content in plants subjected to heavy metal-based treatments can also be caused by other factors such as oxidative pigment breakdown, thylakoid membrane destruction and defective nutrient absorption (Pan et al. 2019).

Unlike P. notatum and P. plicatulum, Chl a and b concentrations in C. dactylon leaves did not change in plants grown in soil contaminated with 70 mg Cu kg⁻¹ (in comparison to the control treatment), although the carotenoid concentration in them has increased. According to Borghi et al. (2008), chlorophyll concentrations in the most tolerant plants can increase or do not undergo significant changes under heavy metal-associated stress conditions. Besides absorbing light as accessory pigments in light collection complexes, carotenoids also act as photoprotective agents in the photochemical apparatus and avoid photooxidative damage to chlorophyll molecules by eliminating reactive oxygen species (Houri et al., 2020; Oliva et al., 2010). Thus, increased carotenoid concentrations observed in C. dactylon leaves may play key role in protecting the photosynthetic apparatus, and it...
enables maintaining chlorophyll concentrations in response to Cu excess.

Therefore, the significantly decreased photosynthetic pigment concentration observed for *P. notatum* and *P. plicatulum* plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\) was one of the factors leading to decreased photosynthetic rate (A) in these plants (Fig. 1d, e and f). Non-stomatal limitation was another factor that contributed to decrease the photosynthetic rate in these species, as well as in species *C. dactylon*, which were grown in soil contaminated with 70 mg Cu kg\(^{-1}\). Intercellular CO\(_2\) concentration (Ci) in the mesophyll has increased in these plants, although Gs has significantly decreased in them; this outcome has indicated that decreased A resulted from lower CO\(_2\) assimilation by carboxylative enzymes, such as Rubisco.

According to Mateos-Naranjo et al. (2015), Cu toxicity is associated with decreased rubisco enzyme concentration and/or activity. Based on Simova-Stoilova et al. (2002), rubisco content has decreased in barley leaves subjected to toxic Cu concentrations. This decrease was likely associated with oxidative stress resulting from ROS formation due to Cu toxicity. Another possible explanation for the non-stomatal limitation observed in these plants lies on increased fluorescence (Zhang et al. 2013), which may have decreased photosynthesis in plants subjected to the highest Cu concentrations. Finally, decreased A in plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\) may have resulted from the denaturation of antenna complex proteins or from direct PSII reaction center inhibition due to Cu insertion in photosynthetic (Yruela 2009) and to the subsequent compromise of PSII electron donation (Bazihizina et al. 2015).

Plants grown in soil contaminated with 35 and 70 mg Cu kg\(^{-1}\) have shown increased SOD and POD enzyme activity (Fig. 3a, f). The increased activity of these enzymes was necessary to mitigate the excessive ROS production caused by high Cu concentrations in plant tissues. Increased SOD activity observed in the investigated species can be attributed to superoxide radicals (O2\(^{-}\)) accumulation induced by increased Cu concentrations. SOD acts in the first line of defense against oxidative stress in order to catalyze the superoxide anion (first ROS formed) dismutation (Gill and Tuteja 2010). The gradual increase in POD activity—which was mainly observed in *C. dactylon*, *P. notatum* and *P. plicatulum* leaves—was necessary to remove the H\(_2\)O\(_2\) that was generated by SOD and additionally converted into H\(_2\)O and O\(_2\) in order to mitigate cell damages.

Cu excess in plant tissues, mainly in plants grown in soil contaminated with 70 mg Cu kg\(^{-1}\), has compromised the proper functioning of the photosynthetic apparatus (Fig. 1d, f; Fig. 2a, b, c and d). This process resulted in reduced CO\(_2\) fixation rate (Cambrollé et al. 2015) and contributed to decreased plant growth rate, as well as LDM, SDM, RDM production (Fig. 3a, b, c and d). The lower growth and development of plants grown in soil contaminated with Cu may also result from Cu effect on cell elongation and division inhibition (Ambrosini et al. 2015), as well as from lower water absorption and imbalances in the absorption of other nutrients (Saleem et al. 2020).

However, increased photosynthetic rate observed for *A. affinis*, *P. notatum* and *P. plicatulum* plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\) did not increase the growth of these plants (Figs. 2 and 3). This outcome may be associated with the adoption of Cu excess-tolerance mechanisms that have used photosynthetic products at the expense of plant growth (Bazihizina et al. 2015). Among them, one finds the synthesis of metal binders to sequester the excess of Cu in cell cytoplasm and/or Cu compartmentalization in the vacuole. This process is based on the principle that organisms are capable of mobilizing their energy reserves to withstand stress conditions, such as detoxification processes, with consequent costs to biological functions such as growth (Calow, 1991).

The translocation factor (TF) has shown that all four grass species analyzed in the current study presented low Cu translocation, regardless of the soil contamination level; therefore, they do not have the potential be used for Cu phytoextraction purposes (Table 2). On the other hand, high bioaccumulation factor (BCF) values recorded for the investigated grass species, mainly for *P. plicatulum*, have indicated that these plants are potential phytostabilizers (BCF > 1), at both soil contamination levels (Table 2). Although *C. dactylon* recorded TF and BCF value lower than that observed for other grass species such as *P. plicatulum* and *A. affinis*, this plant may be more efficient in phytoremediation processes than the other species, since plant biomass production is significantly correlated to successful phytoremediation processes (Luo et al. 2016). *C. dactylon* biomass production at both soil contamination levels has exceeded values observed for the other species. Thus, the high biomass production ability of plants compensates for the relatively low Cu concentration in tissues, and the extent of metal removal from the soil can be even greater (Fig. 4a, b and c).

The high growth rate and biomass production of *C. dactylon* plants grown in soil contaminated with 35 mg Cu kg\(^{-1}\) have evidenced its greater ability to tolerate this contamination condition in comparison to the other investigated species. This factor enabled high TI in *C. dactylon* plants grown under this contamination condition (Table 2). Furthermore, all these variables were decisive to, simultaneously, reinforce the better performance of *C. dactylon*, in soil contaminated with Cu, compared to the other analyzed species (Fig. 6).

In light of the foregoing, it is worth emphasizing the importance of monitoring the incidence of invasive species such as *C. dactylon* in areas where the maintenance of
Pampa biome native plant biodiversity is prioritized, despite the high Cu levels in the soil. The high growth rate of *C. dactylon* plants, as well as their higher tolerance to Cu, may be a greater competitive advantage of this species over native species. Invasive species can colonize vacant spaces in areas subjected to this condition and even suppress the development of Pampa biome native species (Foster et al. 2002; Morgado et al. 2018). Therefore, it is also essential adopting strategies capable of reducing the Cu availability in the soil in these areas in order to minimize the phytotoxic effects of this metal and favor native vegetation development. The higher biomass production observed for native species, such as *P. plicatum*, in association with their high BCF value, enables them to effectively contribute to the phytostabilization of contaminated soils. However, the use of species *C. dactylon* as cover plant in areas contaminated with Cu, where native species preservation is not the priority, appears to be a viable strategy to phytostabilize the contaminated area, since this species is capable of minimizing erosive processes and the consequent dispersion of pollutants, as well as of immobilizing larger amounts of Cu in its biomass.

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**Author contribution** ICBS has planned the study, conducted and monitored the experiment, interpreted the collected data and wrote the manuscript. CPT, VMS and RS have analyzed leaf gas exchanges, monitored the experiment, interpreted the collected data and wrote the manuscript. AS, ET and PAAF performed the statistical analyses, generated tables and figures, and edited the manuscript. FLFQ, FTN and GB have planned the experiment, as well as edited and thoroughly revised the manuscript. All authors have critically revised the manuscript and approved its final version.

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**Data availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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