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Potential Phytoremediation of Pampa Biome Native And Invasive Grass Species Cohabiting Vineyards Contaminated With Cu In Southern Brazil

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Abstract

The aims of the present study are to evaluate whether the growth of Pampa biome native grass species *Axonopus affinis*, *Paspalum notatum* and *Paspalum plicatulum*, as well as of invasive grass species *Cynodon dactylon*, is compromised by excessive Cu availability in the soil (0, 35 and 70 mg of Cu kg^{-1}), to determine the impact of excessive Cu on the physiological responses of the investigated species and to assess whether these species have the potential to enable the phytoremediation of soils contaminated with Cu. *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 SOD and POD. 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.

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 2,000 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, 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, 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. This issue reaches such a magnitude in Pampa biome areas that Cu concentrations available in the soil of places with history of cupric fungicide application exceed by more than 30 times 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., 2018; 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., 2018; Schwalbert et al., 2019).

Increased incidence of native species such as *Paspalum notatum* and *Paspalum plicatulum*, as well as of invasive species such as *Cynodon dactylon*, 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, phytodegradation, 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 phytoremediation 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 collection site comprised a non-disturbed natural field (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).

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
CEC_{ph7} (cmolc.kg^{-1})	2.71
V(%)	47.45

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 three days. Subsequently, soil contamination was carried out with increasing CU doses (0, 35, and 70 mg Cu kg^{-1} of soil) - $CuSO_4.5H_2O$ was used as Cu source. After Cu addition, the soil was incubated again for 30 days, as previously described.

Producing Pampa biome native and *Cynodon dactylon* seedlings

Cynodon dactylon (Bermuda grass), *Paspalum notatum* (bahiagrass), *Paspalum plicatulum* (brownseed paspalum) and *Axonopus affinis* (common carpetgrass) were the plant species tested in the current study. 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

site soil 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 two months, for one 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 NO_3^-, 24.80 mg L^{-1} of $H_2PO_4^-$, 39.27 mg L^{-1} of SO_4^{2-}, 41.31 mg L^{-1} of Mg^{2+}, 288.72 mg L^{-1} of Ca^{2+}, 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.

Conducting the experiment in greenhouse

The investigated plant species were grown in greenhouse from August to November 2019. Fifteen treatments were evaluated, namely: four grass species (*Cynodon dactylon*, *Paspalum notatum*, *Paspalum plicatulum* 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.

Experimental units consisted in 2 liter-capacity pots. Two kilograms (2kg) of soil contaminated with Cu were 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 N_2 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 (FM_f). Plants' growth rate (GR) was determined based on variation in the fresh matter of plants per unit of time (T), in months (Equation 1).

$$GR\ (g.month^{-1}) = \frac{FM_f\ (g) - FM_i\ (g)}{T\ (months)}$$ (Equation 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 CO_2 assimilation rate (A_{net}), stomatal CO_2 conductivity (Gs), intercellular CO_2 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 CO_2 concentration of 400 µmol mol^{-1}, temperature ranging from 20°C to 25°C, relative humidity of 50 ± 5% and photon flow density of 1,500 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 H_2O_2 (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 = $[Cu_S] / [Cu_R] * 100$, wherein Cu_S (mg kg-1) is Cu concentration in the shoot and Cu_R (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 = Bt/Bc, wherein Bt (g $plant^{-1}$) is the biomass of plants grown in soils contaminated with 35 or 70 mg Cu kg^{-1} and Bc (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 = $[Cu_R] / [Cu_{So}]$, wherein Cu_R (mg kg^{-1}) is Cu concentration in the root and Cu_{So} (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).

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. plicatulum* has shown the highest Cu concentration increase in all organs. The maximum Cu concentration in *P. plicatulum* 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 and f). The other investigated species recorded increased carotenoid concentrations (Fig. 1f); *C. dactylon* recorded increased *Chl b* concentration, whereas *P. notatum* and *P. plicatulum* recorded decreased concentrations of it (Fig. 1e).

P. notatum and *P. plicatulum* plants grown in soil contaminated with 70 mg Cu kg^{-1} presented decreased photosynthetic pigment concentrations (Fig. 1d, 1e and 1f). 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 and 1f).

Gas exchange

All investigated species grown in soil contaminated with 35 mg Cu kg^{-1} (Fig 2a and 2b) recorded increased net photosynthetic rate (A) and stomatal conductance (Gs); species *P. plicatulum* recorded the maximum value for these parameters. Intercellular CO_2 concentration (Ci) in species *A. affinis* has decreased under this very same condition (Fig. 2c), whereas Rubisco's carboxylation efficiency (A/Ci) has increased (Fig 2d). Species *P. plicatulum* and *P.notatum* presented increased Ci,as well as decreased A/Ci, at 35 mg Cu kg^{-1} (Fig. 2c and 2d). 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. plicatulum* grown in this very same soil presented increased Ci (Fig. 2a, 2b, 2c and 2d). Species *P. plicatulum* 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. plicatulum* was higher in plants grown in soil contaminated with 70 mg Cu kg^{-1} (Fig. 2e and f). *P. plicatulum* 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 (Fig. 2f). *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 Figs. 3a, 3b, 3c and 3d. Plant growth rate, as well as LDM, SDM and RDM production in species *A. affinis*, *P. notatum* and *P. Plicatulum*, have 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 1,369.45 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, 4e and 4f.

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).

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**	**70**	**35**	**70**
TF	0.06	0.12	0.07	0.13	0.10	0.08	0.09
TI	0.60	1.07	0.13	0.65	0.15	0.33	0.11
BCF	6.54	3.93	3.84	3.50	3.61	8.26	12.76

Discussion

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 (Krzesłowska 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).

Although the absorbed Cu is mainly retained in the root system (Fig. 1c), the increased Cu concentration observed in shoot organs was enough to damage the photosynthetic apparatus of the investigated plant species (Fig. 1d, 1e and 1f). 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 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, 1e and 1f). 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 mesophile 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 FSII reaction center inhibition due to Cu insertion in pheophytin (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. 3e and 3f). 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 ($O2^{\bullet-}$) 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_2O_2 that was generated by SOD and additionally converted into H_2O 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 and f; Fig. 2a, b, c and d). This process resulted in reduced CO_2 fixation rate (Cambrollé et al. 2015) and contributed to decrease 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} has 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).

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. plicatulum*, in association with their high BCF value, enable 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.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Isley Cristiellem Bicalho da Silva has planned the study, conducted and monitored the experiment, interpreted the collected data and wrote the manuscript.

Camila Peligrinotti Tarouco, Vanessa Marques Soares and Raíssa Schwalbert have analyzed leaf gas exchanges, tissue nutritional status, photosynthetic pigment and enzyme activity, as well as revised and edited the manuscript.

André Somavilla, Edicarla Trentin and Paulo Ademar Avelar Ferreira performed the statistical analyses, generated tables and figures, and edited the manuscript.

Fernando Luiz Ferreira Quadros, Fernando Teixeira Nicoloso and Gustavo Brunetto 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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Figures

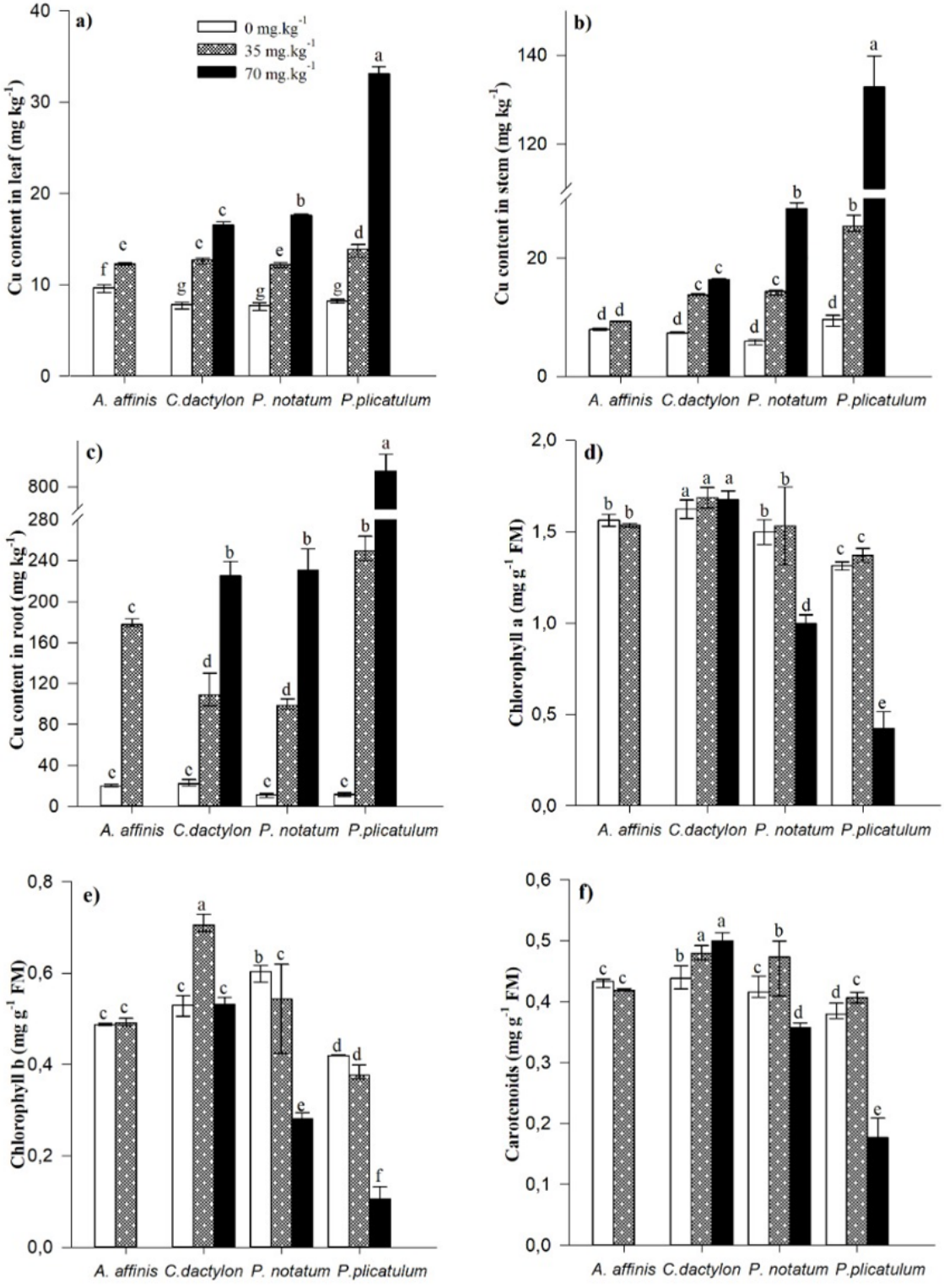

Figure 1

Copper concentration in leaves (a), stem (b) and roots (c); Chlorophyll a (d), Chlorophyll b (e) and carotenoid (f) concentration in 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 ($p<0.05$).

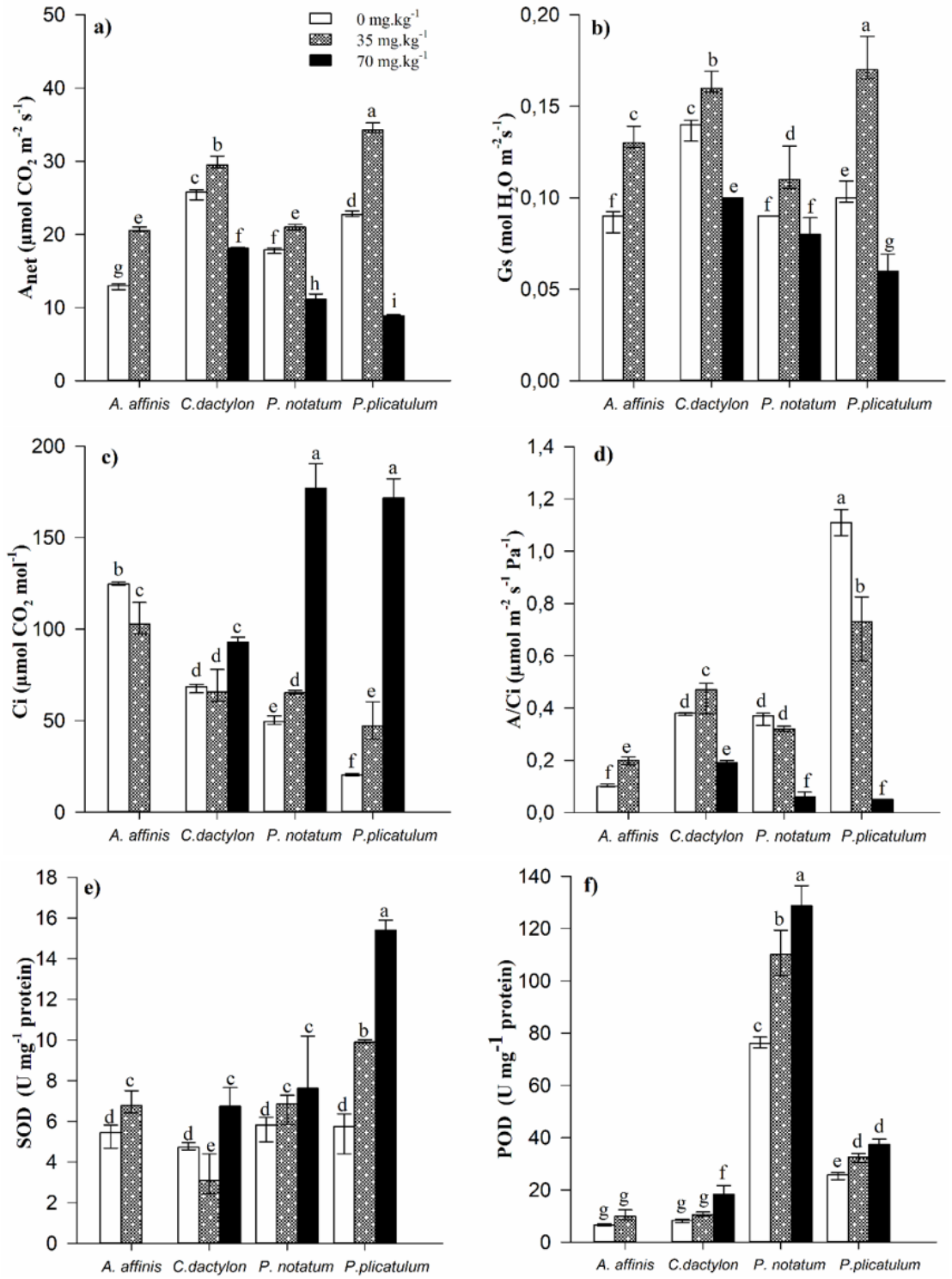

Figure 2

Net photosynthetic rate (a), Stomatal conductance (b), Intercellular CO2 concentration (c), Instant carboxylation efficiency (d); and SOD (e) and POD (f) enzyme activity in the leaves 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 ($p < 0.05$).

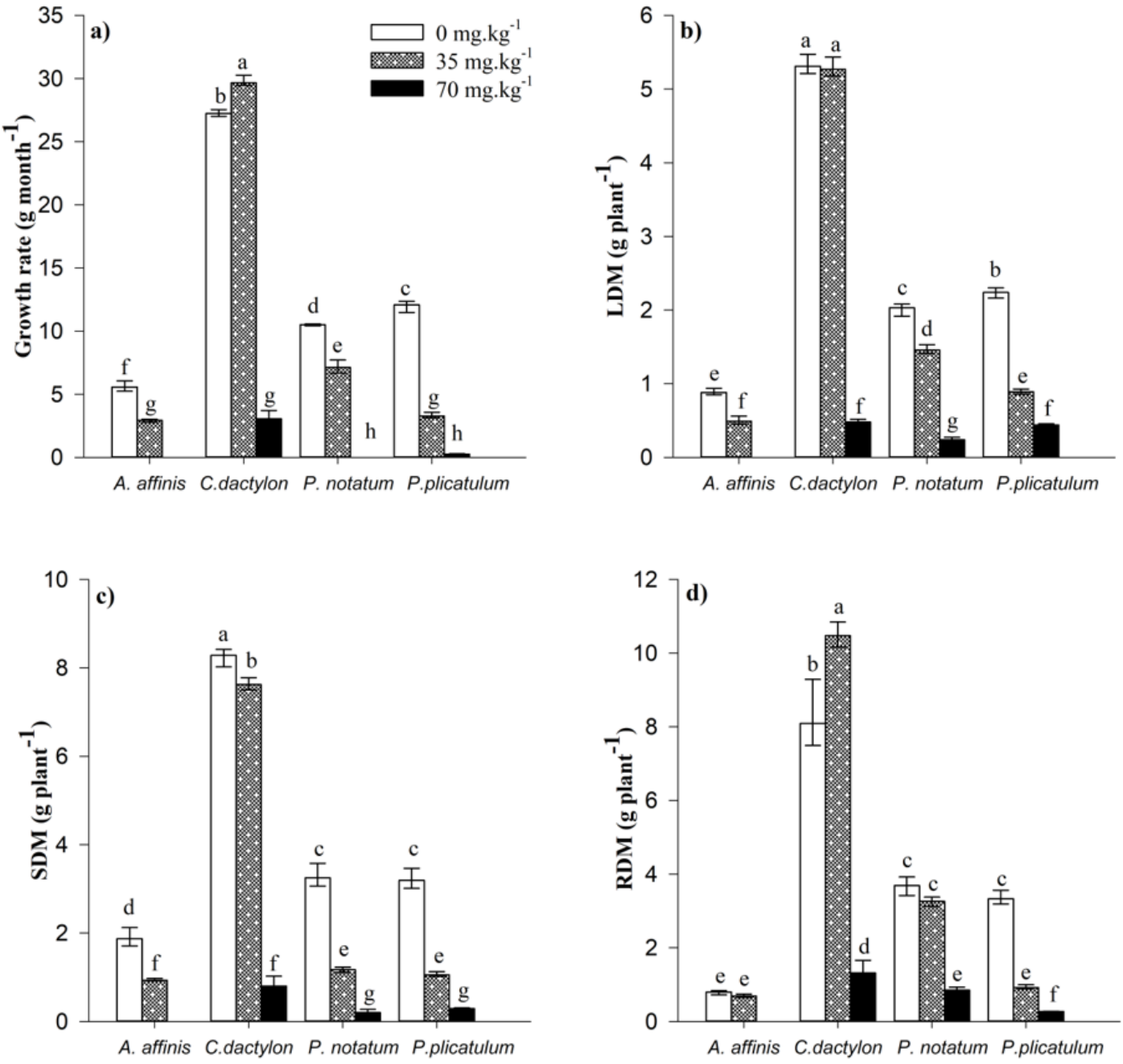

Figure 3

Growth rate (a), and leaf (b), stem (c) and root (d) dry matter production 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 ($p < 0.05$).

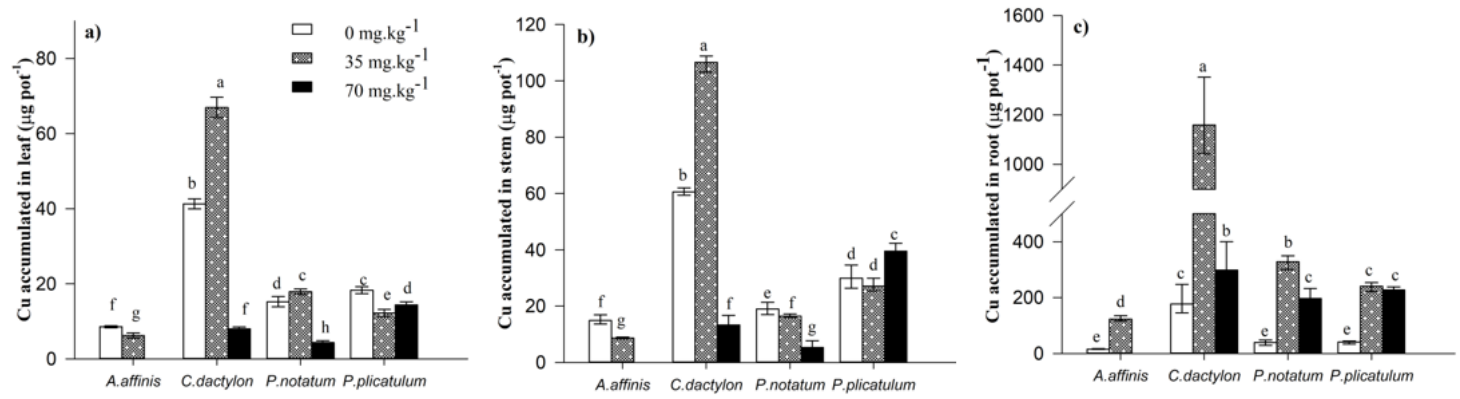

Figure 4

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 ($p < 0.05$).

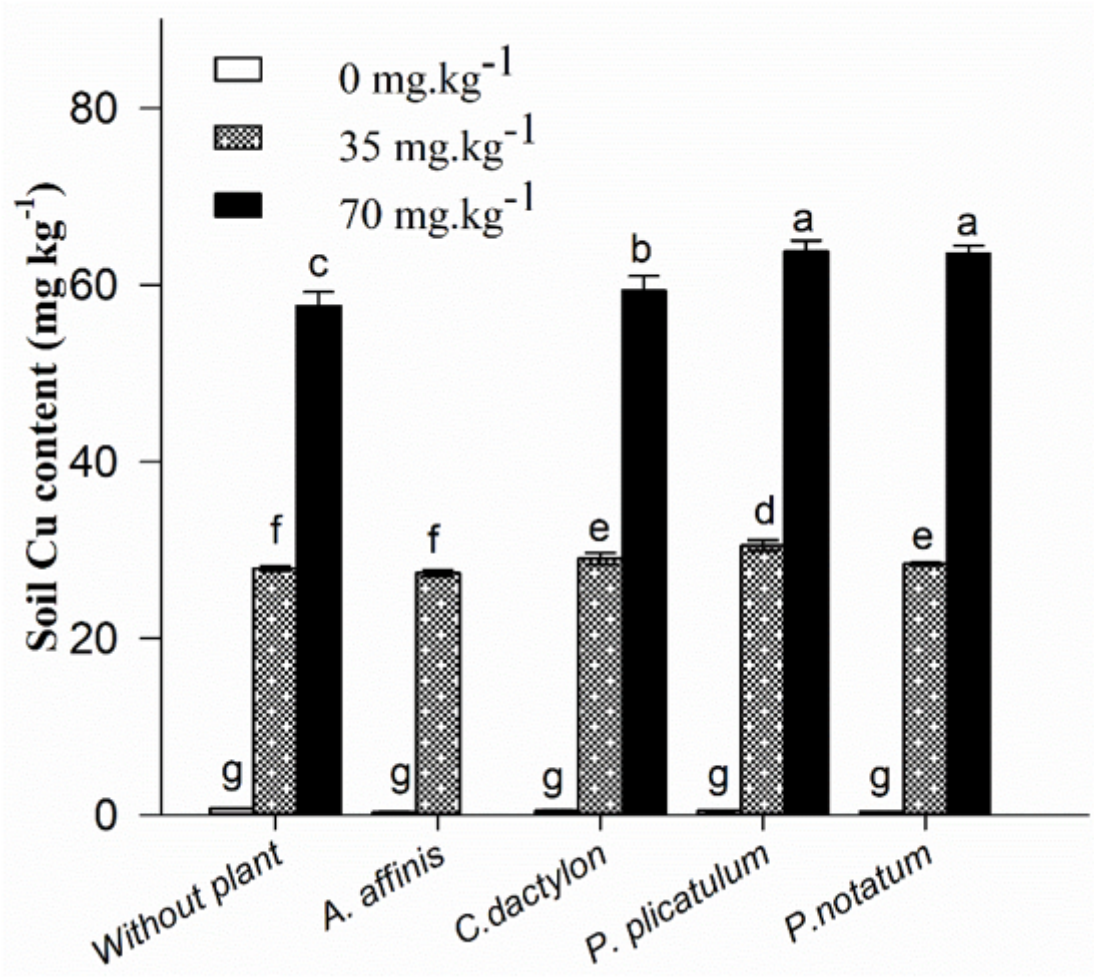

Figure 5

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 ($p < 0.05$).