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VARIABILIDADE ESPACIAL DA COMUNIDADE VEGETAL E MECANISMOS DE TOLERÂNCIA DE ESPÉCIES DE PLANTAS DE COBERTURA DO SOLO PRESENTES EM VINHEDOS COM ALTOS TEORES DE COBRE

Santa Maria, RS
2021

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências do Solo, Área de Concentração em Processos Químicos e Ciclagem de Elementos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciência do Solo**.

Orientador: Prof. Dr. Gustavo Brunetto

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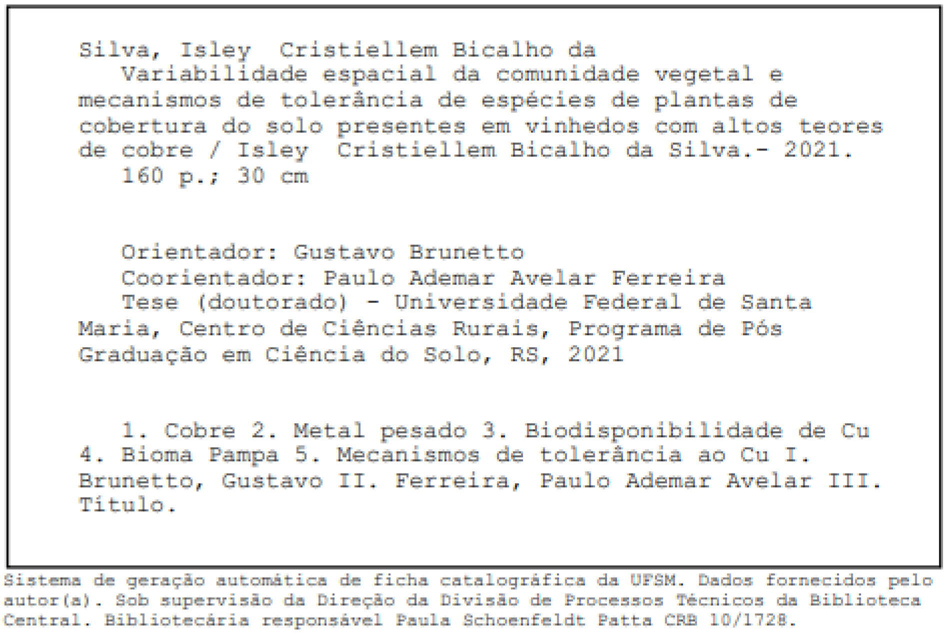

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Aprovado em 21 de janeiro de 2021:

Gustavo Brunetto

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Santa Maria – RS, Brasil
2021

Para André e Luís,
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mim uma pessoa mais corajosa.

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RESUMO

VARIABILIDADE ESPACIAL DA COMUNIDADE VEGETAL E MECANISMOS DE TOLERÂNCIA DE ESPÉCIES DE PLANTAS DE COBERTURA DO SOLO PRESENTES EM VINHEDOS COM ALTOS TEORES DE COBRE

AUTOR: Isley Cristiellem Bicalho da Silva
ORIENTADOR: Gustavo Brunetto

O bioma Pampa é um dos ecossistemas campestres mais diversos do Mundo. No Brasil, a conservação desse ecossistema tem sido negligenciada, e mais de 54% da sua área original já foi suprimida. Em contrapartida, atividades como a viticultura têm sido apontadas como formas de uso sustentável do Pampa, por possibilitar a manutenção de recursos genéticos de espécies nativas nas entrelinhas da cultura. Entretanto, o cultivo de videiras está quase sempre associado ao enriquecimento do solo com cobre (Cu), derivado de aplicações frequentes de fungicidas cúpricos. Neste caso, o excesso de Cu no solo pode causar contaminação ambiental e toxidez às plantas. E, ao longo dos anos, conduzir a degradação da diversidade de espécies vegetais nativas. Por outro lado, o excesso de Cu no solo pode favorecer o estabelecimento de plantas, nativas ou exóticas, que desenvolvam mecanismos adaptativos de tolerância ao metal. Plantas adaptadas a ambientes contaminados com Cu desempenham importante papel na manutenção da vegetação de cobertura e na diminuição da toxidez do metal nestes locais, inclusive para as próprias videiras. Neste escopo, o estudo objetivou avaliar a composição da comunidade vegetal que coabita os vinhedos e identificar mecanismos de tolerância aos elevados níveis de Cu no solo expressos por espécies vegetais que predominam estas áreas. Assim como, avaliar o potencial destas espécies em fitorremediar áreas contaminadas pelo metal. Para isso, quatro estudos foram realizados, parte a campo e parte em casa de vegetação. No estudo I foram selecionados três vinhedos, com teores crescentes de Cu disponível no solo, e uma área de campo natural, adjacente aos vinhedos. Em cada uma das áreas foi realizada a coleta do solo na camada de 0-20 cm para análise dos teores de Cu; a avaliação da composição botânica, determinação da biomassa da parte aérea e concentração de Cu na parte aérea das espécies mais frequentes de cada área. Nos estudos II e III, quatro espécies selecionadas a partir do primeiro estudo (*Cynodon dactylon*, *Axonopus affinis*, *Paspalum notatum*, e *Paspalum plicatulum*) foram cultivadas em solução nutritiva com concentrações crescentes de Cu (0,32, 15, 30 e 45 µM). No estudo II foram avaliados os impactos do aumento dos níveis de Cu sobre características específicas das plantas, como atividade fotossintética, distribuição do metal na biomassa, crescimento, morfologia radicular e estado nutricional. No estudo III foram avaliados a distribuição subcelular e as formas químicas do Cu na parte aérea e no sistema radicular das plantas; bem como as respostas do seu sistema antioxidante ao excesso de Cu. No estudo IV, as mesmas espécies foram cultivadas em solo contaminado com doses crescentes de Cu (0, 35 e 70 mg de Cu kg^{-1}). Neste estudo, foram avaliados parâmetros de crescimento e de trocas gasosas, concentração de pigmentos fotossintéticos, atividade das enzimas antioxidantes SOD e POD e o potencial fitorremediador das espécies. No estudo I, o aumento das concentrações de Cu disponível no solo de vinhedos não alterou a biodiversidade da comunidade vegetal que coabita essas áreas. Porém, a composição botânica foi modificada, e isso pode ser resultado do aumento dos teores de Cu no solo e da melhoria da sua fertilidade. As espécies *P. plicatulum* e *A. conyzoides* apresentaram os maiores valores de fator de bioacumulação de Cu na parte aérea e são candidatas potenciais a técnicas de fitorremediação. Outras herbáceas que coabitam os vinhedos, como a espécie nativa *P. notatum* e a exótica *C. dactylon*, destacaram-se pela sua ampla distribuição nas áreas estudadas. No estudo III a exposição das gramíneas *A. affinis, P. notatum, P. plicatulum* e *C. dactylon* a altas concentrações de Cu no ambiente de cultivo aumentou a concentração de espécies reativas de oxigênio (EROs) e a peroxidação lipídica das membranas celulares das plantas. Além disso, a absorção excessiva de Cu diminuiu a concentração de pigmentos fotossintéticos, comprometeu a atividade fotossintética, e alterou a morfologia radicular e o estado nutricional das plantas (Estudos II e III). Os mecanismos de tolerância ao Cu expressos pelas plantas consistiram no acúmulo do metal no sistema radicular, no aumento da atividade das enzimas antioxidantes SOD e POD para combater a produção excessiva de EROs. Além disso, nas células radiculares e das folhas, a maior parte do Cu absorvido foi retido na parede celular e nos vacúolos. Outra estratégia utilizada pelas plantas para minimizar a toxidez do Cu foi a complexação do metal com pectatos e proteínas, fosfato e com oxalatos (Estudo III). No estudo IV verificou-se que essas gramíneas são fitoestabilizadoras potenciais. No entanto, o uso das espécies nativas como fitoestabilizadoras de solo com contaminação moderada ou elevada requer a adoção de estratégias capazes de diminuir a disponibilidade de Cu no solo, de modo a minimizar os efeitos fitotóxicos do metal e favorecer o desenvolvimento da vegetação nativa. *C. dactylon* é a espécie com maior capacidade de imobilizar Cu em sua biomassa. O seu cultivo em áreas contaminadas, onde a preservação da vegetação nativa do Pampa não é prioridade, pode minimizar os processos erosivos e consequente a dispersão de poluentes.

Palavras-chave: Metal pesado**.** Biodiversidade. Pampa. Biodisponibilidade de Cu. Eficiência de uso de nutrientes. Distribuição subcelular. Fracionamento químico. Estresse oxidativo.

ABSTRACT

SPATIAL VARIABILITY OF THE PLANT COMMUNITY AND TOLERANCE MECHANISMS OF COVERAGE PLANTS SPECIES PRESENT IN VINEYARDS WITH HIGH SOIL COPPER LEVEL

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SUPERVISOR: Gustavo Brunetto

Pampa biome is one of the most diverse countryside ecosystems in the world. In Brazil, the conservation of this ecosystem has been neglected, and more than 54% of its original area has been eliminated. Nevertheless, the viticulture has been identified as sustainable activity to use Pampa areas, as it allows the maintenance of genetic resources of native species between the lines of culture. However, the cultivation of vines is frequently associated with the enrichment of the soil with copper (Cu), derived from frequent use of cupric fungicides. In this case, the soil Cu excess can cause environmental contamination and plants toxicity. Which, over the years, lead to the native plant species diversity degradation. On the other hand, the soil Cu excess can favor the establishment of plants, native or exotic, that develop adaptive tolerance mechanisms to the metal. These plants, adapted to environments contaminated with Cu, play an important role in the maintenance of vegetation and in the reduction of soil metal toxicity, including for the vines themselves. Thus, the goal of this thesis was to evaluate the composition of vegetal community that cohabits the vineyards and to identify tolerance mechanisms of plants to high soil Cu levels, expressed by vegetal species that predominate in these areas. As well as, evaluate the potential of these species to phytoremediation of Cu contaminated areas. To achieve the objective, four studies were carried out in this thesis, some of them in field and others in a greenhouse. In the first study (study I) three vineyards were selected, with increasing levels of soil available Cu, and an area of natural field, adjacent to the vineyards. In each of them, soil was sampled in the 0-20 cm topsoil layer to analyze Cu levels and the vegetation analyzed by: botanical composition, dry matter and shoot Cu concentration of the most frequent species in each area. To carried out the studies II and III, four species were selected from the first study (*Cynodon dactylon*, *Axonopus affinis*, *Paspalum notatum* and *Paspalum plicatulum*) and cultivated in nutrient solution with increasing concentrations of Cu (0.32, 15, 30 e 45 µM). In study II, the impacts of Cu levels on specific plant characteristics, such as photosynthetic activity, metal distribution in biomass, growth, root morphology and nutritional status were analyzed. In study III, the subcellular distribution and chemical forms of Cu in shoot part and in the root system of plants were evaluated; as well as the responses of your antioxidant system to Cu excess. In study IV, the same species were grown in soil contaminated with increasing rates of Cu (0, 35 and 70 mg of Cu kg^{-1}). In this study, growth and gas exchange parameters, photosynthetic pigment concentration, activity of the antioxidant enzymes SOD and POD and the phytoremediation potential of the species were evaluated. As a result, in study I, the increase of available soil Cu concentrations of vineyards did not alter the biodiversity of the plant community that lives in these areas. However, the botanical composition has been modified. This may be the result of the increase in soil Cu levels or due to increase in soil fertility. In the study II, it was observed that the species *P. plicatulum* and *A. conyzoides* presented the highest values of Cu bioaccumulation factor in shoot and have potential for phytoremediation techniques. Other herbaceous plants that cohabit the vineyards, such as the native species *P. notatum* and the exotic *C. dactylon*, stood out for their wide distribution in the studied areas. In study III, the exposure of *A. affinis*, *P.*

notatum, *P. plicatulum* and *C. dactylon* grasses to high concentrations of Cu in the growing environment increased the concentration of reactive oxygen species (ROS) and the lipid peroxidation of plant cell membranes. Moreover, excessive Cu absorption decreased the concentration of photosynthetic pigments, compromised photosynthetic activity, and altered the root morphology and nutritional status of plants (studies II and III). The Cu tolerance mechanisms expressed by plants consisted of metal accumulation in root system and the increase of antioxidant enzymes SOD and POD activity to combat the excessive production of ROS. Furthermore, in the root and leaves cells, most of the absorbed Cu was retained in the cell wall and vacuoles. Another strategy used by plants to minimize Cu toxicity was the complexation of the metal with pectates and proteins, phosphate and oxalates (study III). The study IV support that these grasses are potential phytostabilizers. Nevertheless, the use of native species as stabilizers in soil with moderate or high Cu contamination requires the adoption of strategies capable of reducing the soil Cu availability in order to minimize the phytotoxic effects of the metal and favor the development of native vegetation. Where the preservation of the Pampa native vegetation is not a priority, *C. dactylon* is the species with the greatest capacity to immobilize Cu in its biomass and can minimize the erosive processes and consequent dispersion of pollutants.

Key-words: Heavy metal. Biodiversity. Pampa. Cu bioavailability. Nutrient use efficiency. Subcellular distribution. Chemical fractionation. Oxidative stress.

SUMÁRIO

1. INTRODUÇÃO GERAL

A região de Pastizales del Rio de La Plata compreende uma das maiores diversidades de vegetação campestre do Mundo (KÖNIG *et al.*, 2014), ocupando uma área de aproximadamente 892.711 km², compartilhada por Brasil, Argentina e Uruguai (FONSECA *et al.*, 2013). No Brasil, esses campos correspondem ao que o IBGE denomina Bioma Pampa, o qual é restrito a metade Sul do Rio Grande do Sul (RS), abrangendo 178.243 km², o que corresponde a 63% do território estadual e a aproximadamente 2,07% do território nacional (SILVA, 2010; MMA, 2020). O Pampa apresenta fauna e flora próprias e grande biodiversidade, contando com cerca de 3000 espécies vegetais, 100 de mamíferos e quase 500 espécies de aves (MMA, 2010).

A vegetação que compõe os campos do Pampa é caracterizada fisionomicamente por gramíneas, grupo dominante nesse ecossistema. Outras famílias também apresentam alta contribuição de espécies, como as compostas (Asteraceae) e as leguminosas (Fabaceae) (BOLDRINI *et al.*, 2010). Segundo os levantamentos florísticos, nas áreas cobertas pelo Pampa é possível encontrar mais de 500 espécies de Poaceae, 600 de Asteraceae, 250 de Fabaceae entre várias espécies pertencentes a outras famílias (BOLDRINI, 2009). Espécies como grama-forquilha (*Paspalum notatum* Flüegge), grama tapete (*Axonopus affinis* Chase), capim caninha (*Andropogon lateralis* Nees) e pega-pega (*Desmodium incanum* SW) são bastante frequentes na paisagem campestre (BOLDRINI *et al.*, 2010).

Contudo, a riqueza da biodiversidade do bioma tem sido ameaçada pelas políticas que beneficiam a inserção de espécies exóticas e a conversão do campo natural em sistemas cultivados com espécies florestais, especialmente o eucalipto; e de culturas anuais, como a soja (OVERBECK *et al.*, 2009; MMA, 2015; CAUMO *et al.*, 2020; IBGE, 2020). Entre os anos de 1970 e 1996, as áreas de campo natural diminuíram de 14 para 10,5 milhões de hectares, representando uma conversão de 25% (IBGE, 2006). Em 2008, já se verificava a supressão de 54% da área original do bioma (MMA, 2012). Mais recentemente, entre os anos de 2000 e 2018, registrou-se que a área de vegetação natural campestre do Pampa sofreu redução superior a 1,56 milhões de hectares (IBGE, 2020). Atualmente mais de 80 espécies da flora estão ameaçadas de extinção (LOYOLA *et al.*, 2014).

Paralelamente a crescente degradação do Pampa gaúcho, algumas estratégias que visam o uso sustentável das áreas campestres tem sido difundidas (CHOMENKO; BENCKE, 2016). A principal proposta refere-se à adequação da pecuária extensiva, que prevê o manejo com ajuste da carga animal de acordo com a oferta de forragem (NABINGER *et al.*, 2009) ou o pastoreio em rotação dos campos nativos (QUADROS *et al.*, 2015), possibilitando o conciliamento de rentabilidade e manutenção da integridade destes ecossistemas. Outras atividades econômicas, a exemplo da fruticultura, tem sido apontadas como formas de uso sustentável do bioma (CHOMENKO; BENCKE, 2016). A fruticultura e, especialmente a viticultura, se expandiu pelo Pampa nas últimas décadas. O cultivo de videiras se estende por mais de 2000 hectares somente na Campanha Gaúcha, que corresponde a uma das principais áreas abrangidas pelo Pampa (EMBRAPA, 2017). Atualmente essa região responde pela produção de 31% dos vinhos finos elaborados no Brasil (EMBRAPA, 2020). A expansão vitícola pelos campos naturais foi motivada principalmente pelas condições edafoclimáticas da região, que são propícias ao cultivo de videiras (FLORES, 2011; MANFIO, 2018).

O cultivo de videiras ou mesmo de outras frutíferas não impede a manutenção dos recursos genéticos das espécies nativas do bioma Pampa, que são preservadas nas linhas, mas especialmente, nas entrelinhas das frutíferas (CHOMENKO; BENCKE, 2016). Além da conservação da biodiversidade, a vegetação nativa, presente nas entrelinhas, presta outros importantes serviços ecossistêmicos, como a conservação da água e do solo, sequestro de carbono e ciclagem de nutrientes (SCHOSSLER, 2016). Por fim, a conciliação da produção e qualidade das uvas com a preservação do bioma Pampa é um dos pontos relevantes para obtenção da Indicação Geográfica (IG) de vinhos e espumantes produzidos na região (SARMENTO, 2018), o que agrega maior valor aos produtos regionais.

Diante do exposto, a princípio o sistema de produção vitícola constitui uma ferramenta potencial que possibilita a geração de renda e a manutenção da vegetação campestre nativa, o que é importante para conservação do Pampa. Contudo, é necessário ponderar também os possíveis impactos negativos do estabelecimento de vinhedos sobre as áreas campestres. Sabe-se em nível mundial, que o desenvolvimento da viticultura, assim como de outras frutíferas, quase sempre implica no acúmulo significativo de cobre (Cu) no solo (LAGOMARSINO *et al.*, 2010; MATEOS-NARANJO *et al.*, 2015b; BRUNETTO *et al.*, 2016; SOJA *et al.*, 2018; IÑIGO *et al.*, 2020; KORCHAGIN *et al.*, 2020). Esse aumento deve-se as aplicações frequentes de fungicidas cúpricos foliares durante todo o ciclo de vida das videiras, para prevenir a incidência de doenças fúngicas.

Nos vinhedos localizados no bioma Pampa, com histórico de uso de fungicida cúprico, o teor total de Cu na camada de 0-20 cm do solo pode superar 90 mg kg^{-1}; enquanto que em áreas de campo natural, próximas aos vinhedos, raramente o teor excede 14 mg kg^{-1} (GIROTTO *et al.*, 2014). Aliado a isso, a textura predominantemente arenosa nas camadas superficiais do solo e os baixos teores de matéria orgânica e óxidos contribuem para que as maiores concentrações de Cu estejam na fração considerada disponível às plantas (TIECHER *et al.*, 2013). Nestes vinhedos os teores de Cu disponível, extraído por EDTA, podem ser maiores que 67,2 mg kg^{-1}, superando em mais de 30 vezes o valor encontrado nos campos naturais adjacentes (GIROTTO *et al.*, 2016); e está muito acima do que é considerado alto para a maioria das espécies vegetais, que corresponde a 0,4 mg kg^{-1}, utilizando HCl 0,1 mol L^{-1} como extrator (CQFS RS/SC, 2016). Assim, até o momento, não se sabe como o aumento do acúmulo de Cu no solo impacta a vegetação campestre, nem se conhece os mecanismos desenvolvidos pelas espécies vegetais para tolerar as altas concentrações do metal. Além disso, ainda é pouco discutido o papel dessa vegetação em alterar a biodisponibilidade de Cu no solo, o que pode, inclusive, contribuir para a diminuição do seu potencial de toxidez para as videiras.

O Cu, apesar de ser essencial para todos os seres vivos, quando presente em elevados níveis no solo, provoca efeitos tóxicos, não só às plantas, mas também para os seus consumidores diretos e indiretos (DRĂGHICI *et al.*, 2019; SHABBIR *et al.*, 2020). Nas plantas, o Cu participa dos processos fotossintéticos e respiratórios; metabolismo do C e N, e na proteção contra danos por estresse oxidativo (YRUELA, 2005, 2009; MARSCHNER, 2012; TAIZ *et al.*, 2017;). Contudo, quando sua concentração nos tecidos atinge níveis elevados, diversos distúrbios em nível morfológico, fisiológico, bioquímico e molecular são desencadeados, sendo que a intensidade desses efeitos é dependente da espécie vegetal.

Nas plantas cultivadas sob altas concentrações de Cu, observa-se alterações na arquitetura do sistema radicular das plantas, crescimento reduzido da raiz principal e aumento da densidade de raízes laterais curtas (LEQUEUX *et al.*, 2010). Ocorre o encurtamento e aumento da espessura do ápice radicular (ZHANG *et al.*, 2014; AMBROSINI *et al.*, 2015). Esses efeitos resultam em menor superfície de exploração do solo pelas raízes e menor absorção de água e nutrientes (DE VOS *et al.*, 1989; KOPSELL; KOPSELL, 2007).

Em altas concentrações nos tecidos, o Cu pode aumentar a produção de espécies reativas de oxigênio (ERO) nas células, ocasionando estresse oxidativo. Este aumento de ERO ocorre porque o Cu é encontrado em dois estados de oxidação nas plantas (YRUELA, 2005). Por meio da reação de Fenton, o ciclo redox entre Cu^{+2} e Cu^{+} catalisa a produção de radicais hidroxilo (OH^{-}) provenientes de radicais superóxido ($O_2^{\bullet -}$) e de peróxido de hidrogênio (H_2O_2), aumentando a produção de ERO (BRIAT; LEBRUN, 1999). As ERO oxidam as biomoléculas das células, e a peroxidação lipídica das membranas celulares é um dos principais efeitos observados, resultando na sua menor seletividade, com posterior ruptura e extravasamento do conteúdo celular (DE VOS *et al.*, 1989; YRUELA, 2005). A absorção excessiva de Cu, e o aumento da sua concentração no tecido, também pode reduzir a taxa fotossintética, devido a diminuição no teor de pigmentos fotossintetizantes, como clorofila e carotenoides; e a alterações na estrutura do cloroplasto e composição da membrana do tilacoide (CAMBROLLÉ *et al.*, 2013, 2015). Por fim, a toxicidade do Cu é visualmente percebida pela clorose das folhas, diminuição significativa da produção de biomassa, redução da taxa de germinação de sementes e do número de folhas, e em casos mais graves a morte das plantas (CAMBROLLÉ *et al.*, 2015).

Em face dos efeitos fitotóxicos expostos, pode-se prever que o aumento dos níveis de Cu biodisponível nos solos, como ocorre em vinhedos, ao longo dos anos oferece um risco real de degradação da comunidade vegetal nativa do bioma Pampa. Os possíveis impactos da contaminação dessas áreas com o metal consistem: (1) na diminuição da cobertura vegetal e da riqueza de espécies nativas (STRANDBERG *et al.*, 2006; VIDIC *et al.*, 2006; ROLA *et al.*, 2015) ; (2) favorecimento do desenvolvimento de algumas espécies de plantas com maior capacidade de adaptação do seu metabolismo à elevada concentração de Cu no solo (WONG, 2003; BES *et al.*, 2010; WOCH; KAPUSTA; STEFANOWICZ, 2016); e a (3) elevação do grau de invasibilidade das áreas por espécies vegetais exóticas (LU *et al.*, 2020). Pois, segundo Foster *et al.* (2002), quando a cobertura vegetal composta por plantas nativas é diminuída, ocorre maior disponibilidade de recursos, como espaço, água e nutrientes. Assim, aquelas espécies exóticas que conseguem tolerar as altas concentrações de Cu no solo, em alguns anos, podem colonizar esses espaços vagos (BES *et al.*, 2010).

O estabelecimento de espécies vegetais, nativas ou exóticas, em solos contaminados, como ocorre nos vinhedos mais antigos do bioma Pampa, exige que estas desenvolvam mecanismos extra e, ou intracelulares para evitar os efeitos prejudiciais do Cu em seus tecidos. Dentre os mecanismos extracelulares, a parede celular das plantas e os exsudados radiculares tem papel fundamental (HALL, 2002; PÁL *et al.*, 2006). A parede celular das células epidérmicas radiculares apresenta proteínas em sua superfície, as quais podem servir como sítios de ligação para Cu (FU *et al.*, 2011; KRZESŁOWSKA, 2011; WANG *et al.*, 2015; RODRIGUES *et al.*, 2016), diminuindo assim a sua entrada nos compartimentos internos da célula, bem como sua translocação e acúmulo na parte aérea

A exsudação de compostos pelas raízes, por sua vez, altera as características do solo e da solução do solo. Essas alterações dependem tanto da espécie vegetal como do ambiente em que ela se desenvolve, e incluem a acidificação (HINSINGER *et al.*, 2003; WENZEL, 2009), alcalinização (BRAVIN *et al.*, 2009; ADREES *et al.*, 2015) e o aumento do conteúdo de carbono orgânico no solo, nas formas de aminoácidos e ácidos orgânicos de baixo peso molecular (ADREES *et al.*, 2015). Assim, a liberação dos compostos radiculares pode resultar na alteração da espécie química dos metais, tornando-os indisponíveis para absorção pelas plantas (CHAIGNON; QUESNOIT; HINSINGER, 2009; KABATA-PENDIAS, 2011; DE CONTI *et al.*, 2018) e aumentando ou diminuindo a mobilidade de nutrientes essenciais (CALVARUSO; TURPAULT; FREY-KLETT, 2006) e de contaminantes do solo (CERVANTES *et al.*, 2011).

Os mecanismos intracelulares são baseados principalmente na complexação do metal, na compartimentalização em estruturas subcelulares; e na ativação de sistemas antioxidantes. A desintoxicação de Cu na célula através do mecanismo de complexação envolve sua ligação com compostos celulares, como aminoácidos, ácidos orgânicos, peptídeos como as fitoquelatinas e matalotioneínas e alguns derivados de fosfatos, como os fitatos (HALL, 2002; JAN; PARRY, 2016; RODRIGUES *et al.*, 2016). A ligação dos metais com esses compostos contribui para a redução desses elementos no citosol, diminuindo sua reatividade, solubilidade e consequentemente o aparecimento de seus efeitos tóxicos nas plantas.

A compartimentalização de metais está associada ao mecanismo de complexação, de modo que, após eles serem complexados com os compostos celulares, inibindo seus efeitos tóxicos, são transportados para estruturas subcelulares como o vacúolo, para sua acumulação e consequente redução desses contaminantes no citosol (HALL, 2002). A desintoxicação a partir do mecanismo de compartimentalização consiste no acúmulo do metal contaminante em estruturas como parede celular e vacúolos, impedindo que o contaminante entre em compartimentos celulares mais sensíveis, como mitocôndrias, plastídios e núcleo (MWAMBA *et al.*, 2016; CAO *et al.*, 2018; PAN *et al.*, 2019).

Outro mecanismo de defesa desenvolvido pelas plantas para tolerância à exposição aos metais pesados, como o Cu, é o aumento de atividade de um complexo sistema antioxidante de defesa, que inclui componentes de baixa massa molecular, tais como glutationa e um sistema de enzimas antioxidantes capazes de remover ou neutralizar as ERO. Este sistema de enzimas inclui a superóxido dismutase (SOD), guaicol peroxidase (POD), ascorbato peroxidase (APX), catalase (CAT) e glutationa redutase (GR) (LOMBARDI; SEBASTIANI, 2005; VENKATESWARLU *et al.*, 2012; SINGH; PRASAD; SINGH, 2016).

Portanto, naqueles vinhedos mais antigos do Pampa, os quais apresentam altas concentração de Cu biodisponível para as plantas, a grande ocorrência de espécies nativas do bioma, como *Paspalum notatum* e *Paspalum plicatulum*; e da invasora *Cynodon dactylon*, sugere que essas plantas possuam algum mecanismo específico que proporciona maior tolerância ao metal. Neste sentido, a maior adaptabilidade dessas espécies a condição de contaminação favorece o estabelecimento delas e fornece um forte indicativo do seu potencial fitorremediador. A fitorremediação consiste no uso de plantas para remediar locais contaminados; e envolve cinco processos, fitoextração, fitodegradação, fitovolatilização, rizofiltração e fitoestabilização. Para remediação de solos contaminados com metais pesados, os processos mais utilizados são a fitoextração e a fitoestabilização.

Diante do exposto, esse trabalho de pesquisa buscou primeiramente compreender a relação entre o aumento do acúmulo de Cu nos solos, a dinâmica de espécies nativas do Pampa e a invasibilidade das áreas por espécies exóticas. Em segundo lugar, identificar os mecanismos de tolerância ao Cu das principais espécies que coabitam os vinhedos; o que ajudará a explicar a vulnerabilidade dessas plantas ao processo de contaminação e fornecerá indícios de espécies candidatas a programas de fitorremediação. Os resultados obtidos neste trabalho serão úteis para desenvolver estratégias de manejo eficientes e sustentáveis que contemplem simultaneamente a preservação das espécies nativas em vinhedos em produção e a diminuição do potencial risco de contaminação ambiental e de toxidez para as videiras.

2. HIPÓTESES

I - O incremento dos teores de Cu em solos de vinhedos altera a diversidade da comunidade vegetal presente nas entrelinhas das videiras, sendo que em vinhedos mais antigos, com maiores teores de Cu, ocorre menor riqueza de espécies nativas do bioma Pampa, somado a maior invasibilidade da área por espécies exóticas;

II - O sucesso da espécie exótica *Cynodon dactylon* na colonização de vinhedos contaminados se deve a sua menor propensão a danos no sistema radicular e no aparato fotossintético, bem como menores distúrbios nutricionais causados pela toxicidade do Cu do que as espécies nativas do bioma Pampa;

III - A tolerância da espécie exótica *C. dactylon* ao excesso de Cu no solo é por causa de sua capacidade de imobilizar e manter o metal em formas químicas estáveis no tecido e compartimentalizado em estruturas celulares menos sensíveis, como vacúolo e parede celular, diferentemente das espécies nativas *Paspalum notatum*, *Paspalum plicatulum e Axonopus affinis*;

IV - A expressiva ocorrência de espécies nativas do bioma Pampa, como *P. notatum*, *P. plicatulum e A. affinis* e da exótica *C. dactylon*, em áreas com elevado teor de Cu disponivel no solo indica que o excesso de Cu no solo não impacta de forma significativa as respostas fisiológicas e crescimento das plantas e, portanto, demonstram potencial fitorremediador de áreas contaminadas com o metal.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a composição da comunidade vegetal que coabita os vinhedos e identificar mecanismos de tolerância aos elevados níveis de Cu no solo, expressos por espécies vegetais que predominam nestas áreas. Assim como, avaliar o potencial destas espécies em fitorremediar áreas contaminadas pelo metal.

3.2 OBJETIVOS ESPECÍFICOS

I - Avaliar a variabilidade espacial na comunidade vegetal que coabita vinhedos cultivados em solos com teores crescentes de Cu no bioma Pampa;

II - Avaliar se a espécie exótica *C. dactylon* possui maior tolerância ao aumento dos níveis de Cu que as espécies nativas do bioma Pampa, *Axonopus affinis, Paspalum notatum* e *Paspalum plicatulum*;

III - Identificar o padrão de distribuição subcelular e das formas químicas do Cu nas espécies *C. dactylon, A. affinis, P. notatum* e *P. plicatulum* bem como as respostas do seu sistema antioxidante ao excesso de Cu; e analisar a influência destes mecanismos de defesa na tolerância dessas espécies ao Cu;

IV - Avaliar se o crescimento das gramíneas nativas do bioma Pampa *A. affinis*, *P. notatum* e *P. plicatulum,* e da gramínea exótica *C. dactylon* é comprometido por níveis elevados de Cu disponível no solo; determinar se há impacto negativo do Cu nas respostas fisiológicas das espécies estudadas, incluindo concentração de pigmentos fotossintéticos, trocas gasosas e atividade de enzimas antioxidantes, e avaliar se as espécies estudadas possuem potencial de fitorremediação de solo contaminado com Cu.

4. RESULTADOS

No intuito de compreender a relação entre o aumento do acúmulo de Cu nos solos de vinhedos, a dinâmica de espécies nativas do bioma Pampa, a invasibilidade das áreas por espécies exóticas e o potencial das espécies que coabitam os vinhedos em atuar como fitorremediadoras de solos contaminados com Cu, foram realizados quatro estudos:

I - Spatial variation of herbaceous cover species community in Cu contaminated vineyards in Pampa biome;

II - Tolerance of species *C. dactylon* and of Pampa biome-native grasses to Cu excess;

III - Cu toxicity in native and invasive grass species in the Pampa biome: Subcellular distribution, chemical forms and physiological effects of it;

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

4.1 ESTUDO I

Spatial variation of herbaceous cover species community in Cu contaminated vineyards in Pampa biome[1]

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Spatial variation of herbaceous cover species community in Cu contaminated vineyards in Pampa biome

Abstract

Aims Study's objective was to evaluate spatial variability of herbaceous cover species community in vineyards cultivated in soil with increasing Cu levels in Pampa Biome.

Methods Three vineyards, with increasing soil Cu available contents and a natural field area (NF) were selected. In each experimental area soil Cu content, botanical composition, cumulative aerial biomass and aerial part Cu concentration, in most frequent species were evaluated.

Results In total, 39 vascular plants species were identified, including four exotic species. Biodiversity indicators did not significantly correlate with soil Cu. However, botanical composition variation could be observed. In NF, Poaceae and Asteraceae families presented greater dry mass contribution, while this contribution decreased in higher soil Cu concentrations areas. The Cu concentration and accumulation in plant aerial part were higher in older vineyards; as plant aerial part accumulated, in average, 13.8 mg Cu m^{-2}. Among species found in experimental fields, *Ageratum conyzoides,* a species known to form Cu tolerant populations, occurred in most areas, especially in vineyards, presenting higher aerial Cu concentrations, with a mean of 126.47 mg $kg-^{1}$.

Conclusions Soil enrichment with Cu did not alter the vegetation's biodiversity, but may have contributed to the botanical composition modification. The native species, *P. plicatulum* and *A. conyzoides*, presented a high bio-accumulation factor and are potential candidates for phytoremediation techniques

Keywords: Biodiversity, Floristic Composition, Invasiveness, Heavy metals

Introduction

Pasture is one of world's major terrestrial ecosystem with a wealth of flora and fauna, including many endemic species; being considered large carbon sinks (Bond and Parr 2010). However, worldwide, intensive agriculture has replaced 41% of this ecosystem; and more than 13.5% were urbanized and either degraded (Sui et al. 2013). In Brazil, more than 54% native subtropical pastures, known as Pampa Biome, were converted into areas under annual crops and forest species (Overbeck et al. 2009). This conversion has been accompanied by vegetal biodiversity decrease; according to a floristic survey carried out by (Loyola et al. 2014) more than 80 Pampa Biome native species are threatened with extinction.

Therefore, sustainable use of these pastures remaining areas is essential for biodiversity maintenance, which includes developing of economic activities, harmoniously coexisting with rural ecosystem. In Brazil, vines cultivation has been pointed out as a low impact activity on Pampa Biome, as it allows rural vegetation maintenance between the vine rows, as vineyard soil is usually managed with strip grass and consequently flora biodiversity conservation (Chomenko and Bencke 2016). In the last decades, more than 2000 ha of vineyards were installed on Pampa Biome native fields (Embrapa 2017). In these areas, vines are predominantly trained as in an espalier trellis system allowing light entrance between the lines of crop as well as rural vegetation development, which is mostly heliophilous.

Despite of that, viticulture development almost always implies Cu accumulation in soil, due to frequent copper fungicides applications (Brunetto et al. 2016), a widely reported fact in vineyards located in several Countries, such as Portugal (Fernández-Calviño et al. 2010), France (Fründ et al. 2007) and Italy (Lagomarsino et al. 2010). The Cu accumulates predominantly in soil most superficial layer, due to absence in soil rotation and Cu adsorption affinity to organic compounds, Fe and Mn oxides and clay minerals (Pietrzak and Mcphail 2004; Komárek et al. 2010). In older vineyards installed on Pampa, available Cu concentration, extracted by EDTA, in the 0-5 cm soil layer is 98.0 mg.kg^{-1}, while in adjacent natural fields this value is close to 1.0 mg.kg^{-1} (Miotto et al. 2017).

This is a preoccupying scenario since, although Cu is an essential element for plants, when in excess it leads to phytotoxicity. For most plant species, available Cu concentrations, extracted by HCl, above 0.4 mg kg^{-1}, are already considered high. Plants excessive Cu absorption leads to oxidative stress, which can result in chlorosis, necrosis, decreased photosynthetic rates, changes in other nutrients absorption, as well as both growth and death inhibition (Yruela 2009; Cambrollé et al. 2015; Tiecher et al. 2017).

Thus, bioavailable Cu accumulation in oldest Pampa vineyards soil may represent a degradation risk of biome native plant community. Normally, increased metals concentration in soil exerts a selective pressure on original vegetation (Woch et al. 2016). Most sensitive species to contaminant can be eliminated from native plant community, whereas species with greater adaptation capacity metabolism exhibit higher metals tolerance and are favored. Richness reduction in native species and vegetation cover are frequently reported in floristic surveys from Cu contaminated areas (Vidic et al. 2006; Bes et al. 2010). Developing of certain plants, mainly perennial species from Poaceae family, is also observed in contaminated areas (Shu et al. 2005; Bes et al. 2010).

Another implication of the raise of soil contaminants is the increase invasiveness. Native plants less tolerant to contamination tend to decrease their biomass production, lowering soil cover and resulting in greater resources availability, as space, water and nutrients. Therefore, if exotic species can tolerate contamination levels, they can colonize these empty spaces, and may even suppress native vegetation development (Foster et al. 2002; Morgado et al. 2018).

Study of herbaceous vegetation dynamics in vineyards, as well as invasiveness degree, is essential in order to evaluate if high Cu levels in soil represent a risk to Pampa native vegetation biodiversity. In addition, studies like this can provide initial information on Cu tolerant plants with phytoremediation potential. Study's hypothesis is that Cu increase in soil alters herbaceous community diversity in vineyard; being old vineyards, with higher Cu levels in soil, characterized by a lower Pampa Biome native species richness, added to greater area invasiveness by exotic species. Study aimed to (1) evaluate herbaceous community spatial variability of vineyards cultivated in soils with increasing Cu levels in Pampa Biome (2) identify plant species with potential value for phytoremediation programs of Cu contaminated soils.

Material and methods

Study area and management history

Study was conducted in vineyards located in the city of Santana do Livramento, Rio Grande do Sul state (RS), in Brazil extreme south. Vineyards relief was slightly undulated (Streck et al. 2002) and soil classified as Typical Hapludalf (Soil Survey Staff, 2006). Region weather is subtropical, classified as Cfa by Köppen classification, characterized by mild temperatures and rains with little variation throughout the year. Annual rainfall average is 1600 mm, being August the lowest rainfall month, with 93 mm average, and October the rainiest month, with 148 mm average precipitation. Average annual temperature is 17.6 °C. In the hottest month of January, average temperature is 23.8 °C, while July is the coldest month, with 11.9 °C average temperature (Embrapa 2012).

Vineyard was trained as in an espalier trellis system. Vegetation present in the alleys between vine rows was submitted to approximately five annual mowing, carried out to 10 cm height, and residues deposited on soil surface. Plants growing on vine row were desiccated with glyphosate or glufosinate ammonium, at 2 L ha^{-1}, using directed jet "pens".

In December 2016, three vineyards were selected: vineyards 1 (VN1), 2 (VN2) and 3 (VN3), with increasing soil Cu concentration, a natural field area (NF), adjacent to the vineyards was used as reference. The three chosen vineyards were Cabernet Sauvignon (*Vitis vinifera*), grafted on SO4 rootstock (*Vitis berlandieri x Vitis riparia*). Further vineyards information is presented in Table 1.

Assessments

The four experimental areas NF, VN1, VN2 and VN3 were evaluated in two seasons, April and November 2017, thus contemplating the fall-winter and spring-summer periods. In each experimental area four 350 m^2 plots (replicates) were demarcated, and distributed as a function of soil slope.

Botanical Composition

Botanical composition sampling was carried out according to BOTANAL method (Tothill 1992). In April, two 0.25 m^2 frames were defined in each plot, and used as sample units, where floristic composition was described through component species listing. By visual estimation, percentage contribution of each species for aerial biomass was determined.

Above-ground biomass contained in each sampling unit was cut and reserved for dry matter (DM) determination and tissue Cu concentration. In addition, aerial part from most frequent species was separately collected for further total tissue nutrient concentration analysis. In November, same botanical composition evaluation procedures were realized, using three 0.25 m^2 sample unit per plot. The highest sampling intensity obtained in second survey is due to higher temperatures and longer days common at this time of year, which favors a larger species number development and a higher growth rate. Soil samples were also collected, in 0-0.20 m layer, with use of a soil probe, at each botanical composition evaluation unit side. Soil was then air dried and reserved for chemical analysis.

Vegetation dynamics

Species dynamics was evaluated through three components: richness, diversity and equitability. Species richness was estimated by total species number in each plot, in a 0.50 m^2 area in first survey; and 0.75 m^2 in the second. Species diversity was estimated by Shannon index (H '), calculated by Equation 1:

$$H' = -\sum_{i-1}^{S} \frac{ni}{N} \ln \frac{ni}{N} \qquad \text{(Equation 1)}$$

where: S is sampled species number; ni is dry matter from same species plants; N is total dry matter from species and ln is the natural logarithm.

Equitability was estimated through Pielou index (J) (Equation 2):

$$J = \frac{H'}{SR} \qquad \text{(Equation 2)}$$

where: H' is Shannon diversity index; SR is species richness.

In addition, the floristic similarity between the experimental units was determined. Floristic similarity was determined by cluster analysis, using the Euclidean distance as similarity measure between the groups, and the Ward method for the elaboration of the dendrogram.

Dry matter and tissue nutrient concentration

Plant material collected in each sample unit, as well as above ground part from most frequent species collected, were dried in a forced air circulation oven, at 65 °C average temperature until constant mass, for dry matter obtainment. Subsequently, these materials were milled in a Wiley-type mill and subjected to nitro-perchloric digestion (Embrapa 2000). Total Cu concentration was determined in the acid extract using the atomic absorption spectrometer (EAA; Varian SpectrAA-600, Australia).

Soil Chemical analysis

Collected soil in 0-0.2 m layer was prepared and submitted to chemical analyzes as pH-H_2O (1: 1 ratio) (Tedesco et al. 1995); Soil organic carbon (SOC) was determined using an elemental analyzer (Flash model EA 1112, Thermo Finnigan); Pseudo-total Cu content (Cu_{pT}), determined by EPA method 3051A, with addition of 9 mL HNO_3 and 3 mL HCl (USEPA 2007). The Cu available content was extracted by EDTA (Chaignon et al. 2003) and by Mehlich-1 (Tedesco et al. 1995). Phosphorus (P), iron (Fe), zinc (Zn) and manganese (Mn) available concentrations and exchangeable K were extracted using Mehlich-1 solution (Tedesco et al. 1995). Exchangeable concentrations of calcium (Ca) and magnesium (Mg) were extracted using

KCl (1*M)*. The P determination was carried out using spectrophotometer (1105, Bel Photonics) while flame photometer (DM 62, Digimed) was used for K determination. The Cu, Fe, Mn, Zn, Ca and Mg concentration determinations in all extracts were performed using EAA (Varian SpectrAA-600, Australia).

Level of soil pollution by Cu and Bioaccumulation factor

Soil pollution level by Cu was classified in each of evaluated areas by pollution index (PI) determination, according to Chen et al. (2005) and (Usman et al. 2012). The PI was defined as the ratio between pseudo-total Cu concentration in soil of each area and natural soil Cu content. Natural soil Cu is the element concentration with no anthropic addition. In the NF this concentration corresponded to 11 mg kg^{-1} (Althaus et al. 2013, 2018; FEPAM 2014). PI was calculated and classified as low ($PI \leq 1$), medium ($1 < PI < 3$) or high ($PI \geq 3$).

Above-ground part bioaccumulation factor (BF_{AP}) is an index reflecting plant capacity to accumulate a certain metal in relation to its soil concentration. The BF_{AP} was calculated, using equation 3, for most frequent plant species that appeared in studied areas.

$$\mathrm{BF} = \frac{[\mathrm{Cu}]\text{plant aerial part}}{[\mathrm{Cu}]\text{pseudo}-\text{total}} \qquad \text{(Equation 3)}$$

Statistical Analysis

The variables regarding soil characteristics, vegetation dynamics (Richness, Shannon Index and Pielou Index), production of dry matter and Cu concentration within the plant tissue were submitted to the nonparametric test of randomization with 5% probability, for that the MULTIV (Pillar 1997) software was used. The floristic similarity among the experimental units was determined from the cluster analysis, which was processed by the R studio software, pyclust package. The distribution of the main component species of vegetation from the sample units was determined by the Principal Component Anlysis (PCA), using the R studio software. Finally, with the goal of analyzing the characteristics of soil and vegetation together, the Multivariate Principal component analysis was carried out.

Results

Soil chemical analysis

Soil Cu_{pT} in VN1, VN2 and VN3 was 2.6, 7.3, and 17.9 times higher than that observed in NF, respectively (Table 2). In VN2 and VN3, more than 70% of Cu_{pT} was considered potentially available to plants. Soil pH remained between 5.51 and 6.17, and SOC content was considered low in all areas. Available P was "very low" (≤ 7.0 mg kg^{-1}) for NF and "Medium" (14.1 - 21.0 mg kg^{-1}) for VN1, VN2 and VN3 (Table 2) (CQFS RS/SC 2016). Exchangeable K, Ca, Mg and available Zn concentrations were higher in VN2 and VN3, than VN1 and NF. The lowest available Mn concentration was observed in vineyards soils. Soil available Fe concentration was not affected by experimental area. The PIs by Cu in areas ranged from 0.28 to 5.05 (Table 2).

Biodiversity descriptors

In total, 39 vascular plants species were distributed in 12 families (Tables S1 and S2). In all evaluated areas, total species number was lower in April than in November. Maximum species number in the two evaluated periods occurred in VN3, followed by VN2. The VN1 presented the lowest total species number in sampling periods, 15 in April and 18 in November.

A slight increase, from 10 to 12, in total families number, was observed from April to November (Tables S1 and S2). In VN1, the largest and the smallest families number occurred in November and April, respectively. In April, families with highest representative species number were Poaceae (11 species) and Asteraceae (7 species), with occasional variations occurring in each area. In November, these families also had a higher occurrence, but species number in Poaceae family was lower than Asteraceae in NF and VN1 areas. In addition, during this period, Fabaceae and Rubiaceae families increased their representativeness, by 4 and 3 species, respectively (Tables S1 and S2).

Mean species richness pattern was similar in all experimental areas, with approximately 10 species in April and 13 in November (Table 3). Shannon diversity index (H') and Pielou (J) equitability index, in April, were higher in VN2; in November these indexes were not significantly affected by investigated areas. In plots from all experimental fields, H' ranged from 0.78 to 1.84 in April, and from 1.04 to 1.54 in November, being considered low in both assessment periods. The natural field vegetation is the one that presents the biggest singularity among the assessed areas, while the VN2 and VN3 vineyard vegetation were the most similar in both sampling periods (Fig. 1).

Botanical Composition Dynamics

Perennial species had a higher occurrence in all experimental fields for both sampling periods (Tables S1 and S2). Species as *Ageratum conyzoides*, *Paspalum notatum* and *Cynodon dactylon* were among the top five species in several areas during the two surveys (Table 4).

Species belonging to Poaceae and Asteraceae families dominated NF, contributing to more than 90% biomass production. In vineyards, Poaceae family contribution decreased, while families as Fabaceae and Rubiaceae began to occupy a greater proportion of these experimental fields (Fig. 2 a, b).

Exotic species had little contribution in NF, but in vineyards, their participation increased. In April, *C. dactylon* grass, an exotic species, was found exclusively in vineyards. In VN2 and VN3, this species contributed to more than 15% on biomass of inter-rows (Table 5). In November, four exotic species were identified, and *Lolium multiflorum* and *C. dactylon* stood out for their wide coverage in vineyards (Table 5). In this period, *L. multiflorum* was the most abundant species in the three vineyards, contributing 49, 29 and 34% of biomass in VN1, VN2 and VN3, respectively.

Principal Component Analysis (PCA) indicated botanical composition dynamics among experimental fields, according to main species dry mass contribution (Fig. 3 a, b). In April, data sorting diagram (Fig. 3 a) showing most of the botanical composition variation between areas (66.40%) was synthesized on the first two axes (44.10% on x-axis and 22.30% on y-axis). Species with highest x-axis correlations were *Cynodon dactylon* (0.94), *Ageratum conyzoides* (0.93) and *Paspalum notatum (*-0.93), while species with highest y-axis correlations were *Cyperus brevifolius* (-0.93). *Paspalum urvillei* (-0,86) and *Paspalum plicatulum* (-0.75) The NF areas differed from others by wide mass contribution species as *Axonopus argentinus* and *Vernonia nudiflora*. Species as *C. dactylon, Richardia brasilienses* and *A. conyzoides* were associated to VN2 and VN3 (Fig. 3 a, Table 4).

In November, most of botanical composition variability, explained by the two main axes (42.10% in x-axis and 34.00% in y-axis), indicated that the highest x-axis correlation species were *L. multiflorum* (-0.94), *Cyperus brevifolius* (-0.90) e *Richardia brasilienses* (-0.90). Species with higher y-axis correlations were *D. incanum* (-0.93), *C. dactylon* (-0.89) and *V. nudiflora* (0.83). Similar to that observed in April, NF vegetation was singular, presenting a great contribution of species as *A. argetinus, V. nudiflora* and *Eragrostis plana* (Fig. 3 b and Table 4). Vineyards vegetation was similar, especially in VN2 and VN3, where *L. multiflorum* and *C. dactylon* gave the greatest contribution (Fig. 1, Fig. 3, Table 4 and Table 5).

Cumulative biomass, Cu concentration and accumulation in aerial part in fall-winter period

Cumulative biomass, Cu concentration and accumulation in aerial part of vegetation presents in alleys between vine rows varied significantly among experimental fields, with highest values observed in VN2 and VN3 (Fig. 4 a, b, c). In April, the species with the highest accumulated biomass was *A. argentinus*, which produced 843 kg ha^{-1} in NF.

The Cu found in vegetation biomass from the vineyard alleys depended not only on soil Cu concentration, but also on species composing plant community. *A. conizoydes* and *P. plicatulum*, collected in VN2, presented the highest Cu concentrations in aerial part, 127 mg kg^{-1} and 118 mg kg^{-1} respectively (Table 6), while the lowest occurred in *P. notatum* collected in NF. *A. conizoydes* and *P. plicatulum* showed the higher bioaccumulation factor (BF).

The PCA (Fig. 5) indicated that pH values and nutrient contents were positively correlated to available Cu contents, extracted by Mehlich-1. The NF had low levels of soil available nutrients, except for Mn, as well as lowest pH values. On the other hand, VN2 and VN3 soils presented the highest Cu, Zn, P, Ca and Mg and the lowest Mn concentrations. Biodiversity indicators, richness and Shannon and Pielou indexes did not significantly correlate to soil Cu available, extracted by Mehlich-1. Dry matter production correlated positively with soil Cu, Cu_{pT}, P, Ca, K and Zn levels. Dry matter contribution of species belonging to Poaceae and Asteraceae families presented a negative correlation to soil Cu, P, K, Zn and Mg.

Discussion

In the long term, Copper fungicides application in vineyards, enriched soil Cu levels. Based on soil pollution index, values observed in VN2 and VN3 indicate moderate and high soil Cu contamination, respectively (Table 2). In experimental areas, soil is characterized by low organic matter content, predominantly sandy texture and low cation exchange capacity (Tables 1 and 2). This soil physical and chemical properties combination had substantial effects on Cu content and availability to plants in inter-rows (Table 2).

Predominant species belonged to Poaceae, Asteraceae, Fabaceae and Rubiaceae families (Fig. 2). These are also dominant families characteristic to Pampa Biome flora (Boldrini 2009). However, in NF, Poaceae and Asteraceae families together, contributed to more than 90% of biomass in the area, in both April and November. Especially in VN2 and VN3, these family biomass contribution was significantly lower than in NF, indicating a great alteration in botanical composition in these experimental sites. This result is in contrast to what is commonly reported in the literature. In general, in soil with high heavy metals levels, Poaceae and Asteracea families predominate, whereas with low metals levels, Fabaceae, Rosaceae, as well as other families, contribution increases (Bes et al. 2010; Gutiérrez-Ginés et al. 2015; Xiao et al. 2018).

Soil Cu content probably is not the only factor explaining spatial variability of plant communities in evaluated areas. Possibly, soil fertility improvement (Table 2) was one of determining factors for botanical composition variation observed in the evaluated areas. Greater nutrients availability, as occurs in vineyards, may benefit species with greater capacity to respond to soil fertility. In general, P, K and Ca supply favors Fabaceae species establishment (Carlen et al. 1998; Gaujour et al. 2012).

Although botanical composition has changed, biodiversity indicators, richness, Shannon and Pielou indexes, suggest that increasing Cu levels does not affect plant diversity. Possibly, in areas containing higher soil Cu levels, such as VN2 and VN3, plants and Cu interaction is balanced by other factors, to reduce Cu phytotoxic effects. In these areas, along with soil Cu, also Zn, Ca and P concentrations were high, with pH values close to 6.0 (Table 2). Tiecher et al. (2016), evaluating Cu and Zn interaction in soil and its effects on plants, verified that even low Zn application rates in Cu contaminated soil, potentiated plant development. According to the authors, Zn may have contributed to reduce Cu^{+2} activity in soil solution, decreasing its phytotoxicity to plants.

Exchangeable Ca increase in inter-rows, in turn, may have improved plant metal tolerance. The Ca^{+2} and Cu^{+2} are similar ionic forms elements, interacting antagonistically; so it is common to observe lower Cu accumulation in plants growing in Ca-rich environments (Maksymiec and Baszyński 1998; Min et al. 2013; Ambrosini et al. 2015). In addition, Ca, when bound to phospholipids, stabilizes lipid bilayers and thus provides structural integrity to cell membranes (Hepler 2005), minimizing excessive Cu absorption damage. Min et al. (2013) verified that Ca application to Cu contaminated medium was effective in minimizing Cu phytotoxic effects in *Elodea canadians*; excessive Cu accumulation in plant was reduced, and there was a decrease in photosynthetic pigments degradation and soluble proteins. In addition, Ca application promoted proline synthesis, counteracting oxidative damages and reversing nutritional imbalance induced by Cu in plants.

High P levels in these soils may also have minimized toxic Cu effects. Increases in soil P content, due to long vineyard fertilization period, may reduce Cu availability in soil solution due to Cu insoluble phosphates formation (Ayati and Madsen 2000; Cao et al. 2003). Phosphate anion can bind to reactive soil particles surface, as oxides, increasing negative charges (Barrow 1999; Pérez-Novo et al. 2009), in turn, increasing the potential for ternary oxide complexes phosphate-metal formation (McBride 1994). These, in this way, decrease Cu availability in soil solution and, consequently, its phytotoxicity. In addition, phosphate can form complexes with Cu in plant's root system, reducing Cu mobility and transport to shoot (Zambrosi et al. 2013).

Decreased Cu toxicity following P application was reported by Guimarães et al. (2016) in black oat (*Avena strigosa* Schreb.) plants, through root system anatomy parameters and plants dry matter production.

Plant species exhibit substantial different capacity of heavy metal absorption and translocation to aerial part (Singh et al. 2010; Khan et al. 2015); and, depending on area contamination level, the same plant presents significant differences in Cu concentration in its aerial part (Table 6). This large variation in Cu content in the same species, as observed for *P. notatum*, *P. plicatulum* and *A. conyzoides*, reflects the adaptive potential of these species to tolerate varying levels of soil Cu and helps to explain these species wide distribution in evaluated areas and in both sampling periods (Table 4).

Despite none of collected plant species exhibited Cu hyperaccumulators characteristics, *P. plicatulum* and *A. conyzoides* species achieved maximum of 118 and 127 mg Cu kg^{-1} concentrations of dry matter, respectively. These values are higher than the normally considered toxic concentrations in mature leaf tissue, which is 20 to 100 mg Cu kg^{-1} dry matter (Kabata-Pendias 2011). De Conti et al. (2020) also verified *P. plicatulum* greater potential to absorb and accumulate Cu in its biomass when compared to other Pampa biome native species, as *P. notatum* and *A. affinis.* Also, authors verified that, with increase soil Cu levels, this species cultivation was the great responsible in altering soil solution; increasing soluble organic carbon concentration and favoring Cu^{+2} complexation, thus reducing element most phytotoxic form. The *A. conyzoides* species, in turn, is often reported as metal tolerant (Sharma and Pant 2018), including Cu. However, it should be taken into account that, due to *A. conyzoides* leaves long duration characteristic, the high observed Cu concentrations can also be attributed to Cu fungicide residues applied in previous months.

Although plant species with higher BF, *A. conyzoides*, *P. plicatulum* and *P. notatum*, are Pampa biome native, it is fundamental to adopt management strategies favoring a greater contribution of these species in crop interlines, simultaneously controlling invasive species advance, especially the perennial grass *C. dactylon*. These native plant species higher biomass production associated to their respective BF values will enable them to effectively contribute to soil cover, minimizing erosive processes and consequent pollutant dispersion; also being able to immobilize larger amounts of Cu in their biomass. Thus, available Cu concentration in soil will be reduced, creating more suitable conditions for other native species, which are more sensitive to Cu, later to thrive in these areas.

The present study provided description about vegetation dynamics that cohabits vineyards with high soil Cu levels. Thus, Cu availability in soil varies according to soil characteristics and plant absorption rate, and may be altered as a function of its interaction, antagonistic or synergistic, with other elements present in the soil. It remains essential the conduction of experiments evaluating Cu individual contribution on development and establishment of these plants.

Conclusion

Vegetal community biodiversity that cohabits vineyards did not change with increase available Cu levels in soil. However, botanical composition in the studied areas appeared modified, and this may be due to soil Cu level increase as well as its fertility improvement.

The *P. plicatulum* and *A. conyzoides* species, which are among Pampa biome most representative, presented highest BF values and are potential candidates for phytoremediation techniques.

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Tables

Table 1 Vineyards 1 (VN1), 2 (VN2) and 3 (VN3) characterization.

	VN1	VN2	VN3
Location	S30°46'41", W55°22’34”	S30°47'44", W55°21’56”	S30°46'36", W55°22'03"
Implementation year	2004	1998	1981
Vineyard density (plants ha^{-1})	2778	2525	1429
Spacing (m)	3.0 x 1.20	3.30 x 1.20	3.50 x 2.0
Planted area (ha)	4.73	5.30	6.45
Applied Nitrogen (kg N ha^{-1} $year^{-1}$)	45	45	45
Applied Phosphorus (kg P_2O_5 ha^{-1} $year^{-1}$)	45	45	45
Applied Potassium (kg K_2O ha^{-1} $year^{-1}$)	45	45	45
Leaf Cu applied (kg Cu ha^{-1} $year^{-1}$)	2.11	6.41	10.51
Clay (g kg^{-1})	90	120	120
Soil organic matter (g kg^{-1})	8.00	8.00	11.00
pH (H_2O)	5.70	5.50	5.70
Available Cu (extracted by Mehlich-1) (mg dm^{-3})	4.25	19.42	40.38
CEC ($pH_{7,0}$) ($cmol_c$ kg^{-1})	3.90	4.10	4.80

Table 2 Soil chemical attributes collected 0.00-0.20 m layer from natural field area (NF) and in the three studied vineyards (VN1, VN2 and VN3).

Parameters	Units	NF	VN1	VN2	VN3
Cu (EPA- 3051A)		3.10d⁽¹⁾	8.20c	22.56b	55.57a
Cu EDTA	mg kg^{-1}	1.43d	3.50c	17.86b	40.38a
Cu Mehlich-1	mg kg^{-1}	1.05d	3.14c	14.57b	34.89a
P	mg kg^{-1}	3.23c	14.52b	17.95ab	20.08a
K	mg kg^{-1}	154.00b	159.50ab	187.00a	195.25a
Ca	$cmol_c$ kg^{-1}	0.78d	1.95c	2.14b	2.45a
Mg	$cmol_c$ kg^{-1}	0.21c	0.35a	0.27b	0.38a
Zn Mehlich-1	mg kg^{-1}	2.1d	4.78c	8.20b	12.21a
Fe Mehlich-1	mg kg^{-1}	36.41ns	30.85	33.84	35.67
Mn Mehlich-1	mg kg^{-1}	52.58a	45.45b	39.73b	40.53b
pH in water	(1:1 relation)	5.51b	6.17a	5.87a	5.84a
COS	%	0.54b	0.54b	0.58b	0.68a
Cu pollution index		0.28	0.74	2.05	5.05

(1) Means followed by same letter in line are not statistically significant different by randomization test at 5% probability

Table 3 Richness, Shannon diversity index (H ') and Pielou equitability index (J) in natural field areas and in vineyards (VN1, VN2 and VN3) with increasing soil Cu levels in April and November

	Richness (Species number)		Diversity Index Shannon (H’)		Equitability index Pielou (J)	
	April ^{ns}	November ^{ns}	April	November ^{ns}	April	November ^{ns}
NF	10.25	12.50	1.08b	1.33	0.11b	0.11
VN1	8.50	12.00	1.29ab	1.32	0.15ab	0.11
VN2	9.75	11.75	1.58a	1.43	0.16a	0.12
VN3	10.5	13.75	1.49ab	1.42	0.14 ab	0.11

Means followed by same letter in line are not statistically significant different by randomization test at 5% probability

Table 4 Contribution, in biomass percentage, of main species from each area in the two evaluation periods

	NF		VN1		VN2		VN3	
	Species	%	Species	%	Species	%	Species	%
April	*Axonopus argentinus*	42.70	*Paspalum notatum*	42.90	*Paspalum plicatulum*	19.10	*Desmodium incanum*	17.90
	Paspalum notatum	29.70	*Richardia brasiliensis*	20.40	*Cynodon dactylon*	16.79	*Cynodon dactylon*	18.50
	Paspalum plicatulum	12.60	*Ageratum conyzoides*	15.70	*Richardia brasiliensis*	12.70	*Ageratum conyzoides*	17.70
	Vernonia nudiflora	9.30	*Cynodon dactylon*	8.57	*Ageratum conyzoides*	12.50	*Richardia brasiliensis*	11.90
			Desmodium barbatum	5.90	*Desmodium barbatum*	11.30	*Paspalum plicatulum*	9.40
November	*Paspalum notatum*	48.30	*Lolium multiflorum*	49.03	*Lolium multiflorum*	28.72	*Lolium multiflorum*	34.22
	Axonopus argentinus	23.20	*Paspalum notatum*	27.00	*Paspalum plicatulum*	20.30	*Cynodon dactylon*	20.93
	Vernonia nudiflora	12.00	*Ageratum conyzoides*	7.80	*Cynodon dactylon.*	19.89	*Desmodium incanum*	14.10
	Eragrostis plana Nees	6.22	*Cyperus brevifolius*	3.50	*Desmodium incanum*	11.90	*Paspalum notatum*	10.60
	Ageratum conyzoides	3.50	*Desmodium incanum*	3.40	*Ageratum conyzoides*	5.80	*Ageratum conyzoides*	9.40

Table 5- Contribution, in biomass percentage, of Pampa biome exotic species in fall-winter (April) and spring summer (November) periods. NF: Natural field, VN1: Vineyard 1, VN2: Vineyard 2, VN3: Vineyard 3.

	April				November			
	NF	VN1	VN2	VN3	NF	VN1	VN2	VN3
C. dactylon	0.00c	8.57b	16.79a	18.50a	0.07b	0.00c	19.89a	20.93a
L. multiflorum	–	–	–	–	0.00d	49.03a	28.72c	34.22b
E. plana	–	–	–	–	6.22a	0.00b	0.00b	0.00b
V. sativa	–	–	–	–	0.00b	0.00b	0.27a	0.23a

Means followed by same letter in line are not statistically significant different by randomization test at 5% probability

Table 6 Cu content (mg kg^{-1}) in aerial part tissue from most frequent species in experimental areas in April. NF: Natural field, VN1: Vineyard 1, VN2: Vineyard 2, VN3: Vineyard 3.

	NF	**VN1**	**VN2**	**VN3**
P. notatum	12.14c	34.14b	84.29a	
R. brasiliensis		30.87b	63.24a	33.74b
A. conyzoides		22.67c	126.74a	42.04b
D. barbatum		24.64b	42.71a	
P.plicatulum	14.89c		117.64a	51.17b
C. dactylon			49.04a	32.74a

Means followed by same letter in line are not statistically significant different by randomization test at 5% probability.

Figures

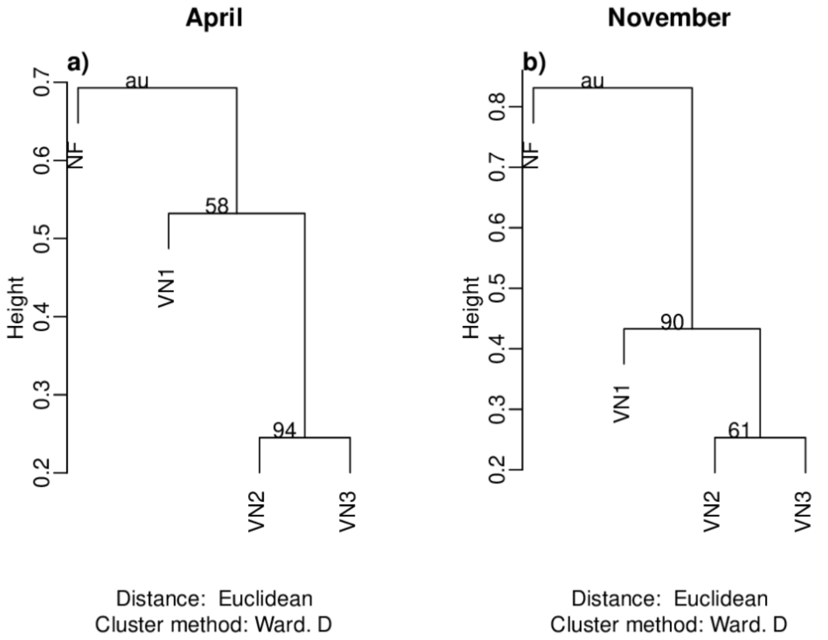

Fig.1 Dendrogram of floristic similarity with Euclidean distance between natural field (NF) and vineyards (VN1, VN2 and VN3) with increasing soil Cu levels, in the months of (a) April and (b) November. Elaborated through the Ward method. Values at branches are AU p-values (approximately unbiased p-value)

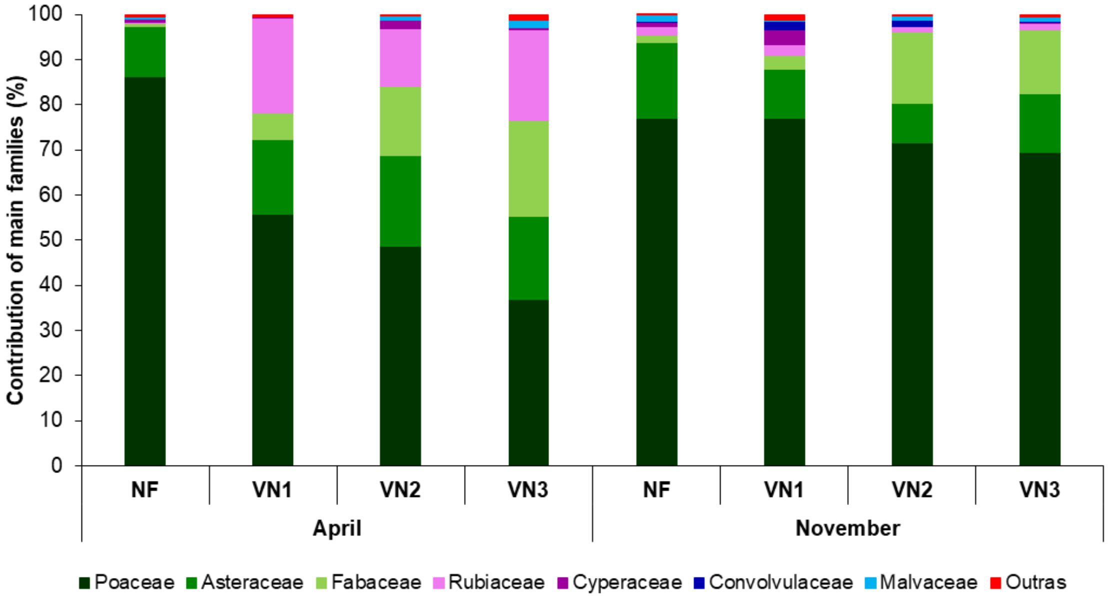

Fig.2 Contribution, in biomass percentage, of main families in fall-winter (April) and spring-summer (November). NF: Natural field, VN1: Vineyard 1, VN2: Vineyard 2, VN3: Vineyard 3.

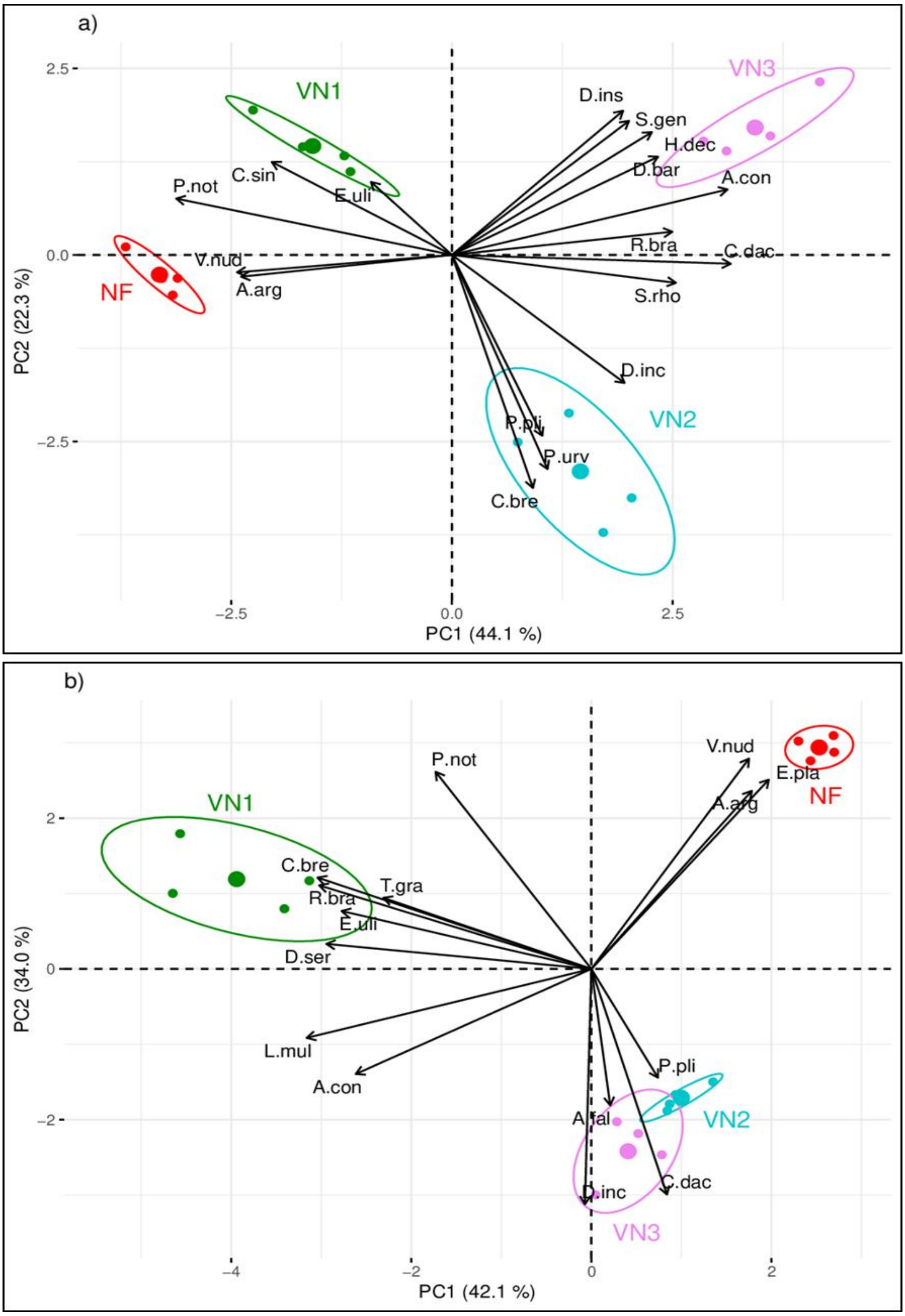

Fig. 3 Principal Component Analysis (PCA) of component species from natural field and vineyard. (a) Data from April 2017 survey and (b) November 2017 survey. A.arg: *Axonopus Argentinus*, V.nud: *Vernonia nudiflora*, P. not: *Paspalum notatum*, P. pli: *Paspalum plicatulum*, P.urv: *Paspalum urvillei*. D. bar: *Desmodium barbatum*, C. bre.: *Cyperus brevifolius*; C.dac: *Cynodon dactylon*, A.con: *Ageratum conyzoides*, S. gen: *Setaria geniculata*; E. pla: *Eragrostis plana*; A.fal: *Aeschynomene falcata*; L.mul: *Lolium multiflorum*; D.ser: *Dichondra sericea*; D. ins: *Digitaria insulares,* R.bra: *Richardia brasiliensis,* E.uli.: *Eustachys ulignosa*, C. sin.: *Chaptalia sinuata*; T.gra: *Tibouchina gracilis*; D. inc.: *Desmodium incanum*. NF: Natural field, VN1: Vineyard 1, VN2: Vineyard 2, VN3: Vineyard 3.

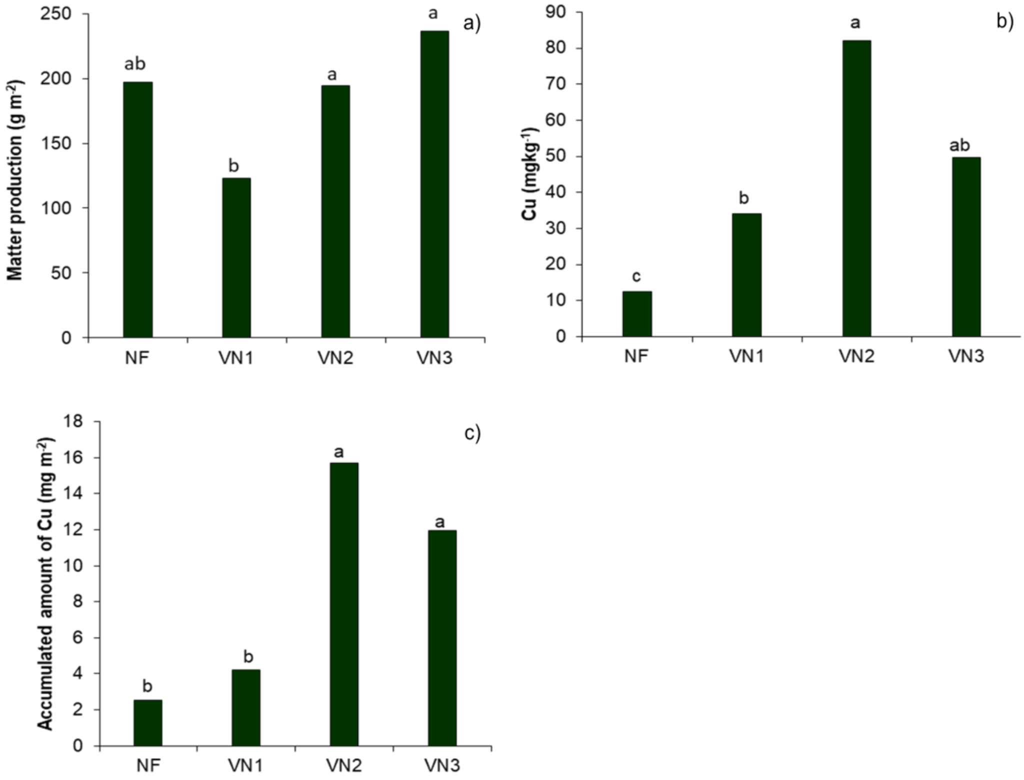

Fig 4 Dry matter production (a), tissue Cu concentration (b) and Cu aerial part (c) accumulated amount in plant community from natural field areas and vineyards in April 2017. NF: Field natural, VN1: Vineyard 1, VN2: Vineyard 2, VN3: Vineyard 3.

Means followed by same letter are not statistically significant different by randomization test at 5% probability.

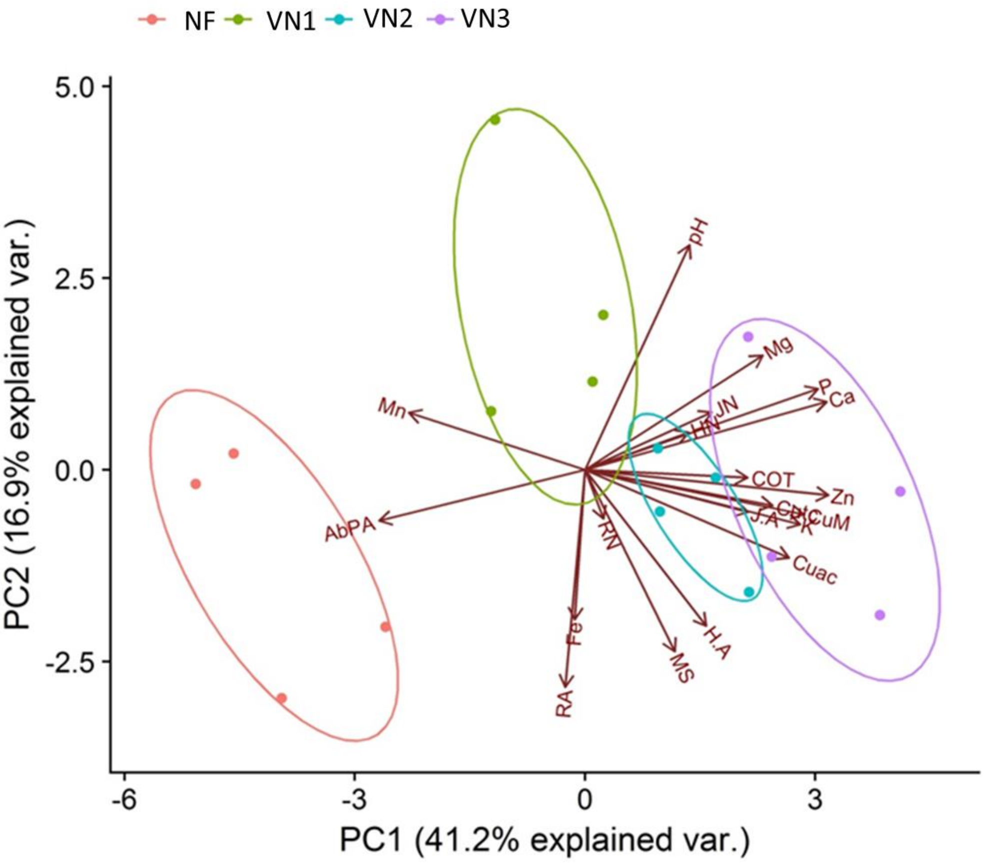

Fig. 5 Principal Component Analysis (PCA). Cu_M, Zn, Fe, Mn and P correspond to the concentrations of Cu, Zn, Fe, Mn and P available in the soil. Ca, Mg and K correspond to elements exchangeable concentrations in soil. MS is dry mass production from aerial part, Cu_{ac} is the Cu accumulated amount in aerial part. AbPA is the contribution, in dry mass, from Poaceae and Asteraceae families belonging species. R is species richness, H is Shannon index, A and N indicate sampling periods, April and November. The ACP explains 59.1% of total variance. NF, VN1, VN2 and VN3 correspond to natural field areas and vineyards 1, 2 and 3.

Supplementary material

Supplementary Table S1 Plant species occurrence recorded in April, in natural field (NF) and vineyards (VN1, VN2 and VN3) with increasing soil Cu levels

Family	**Species**	NF	**VN1**	**VN2**	**VN3**	**N/E**	**P/A**[1]
Asteraceae	*Ageratum conyzoides* L.	*	*	*	*	N	A
	Bidens pilosa L.				*	N	A
	Chaptalia sinuata (DC.) Baker	*	*			N	P
	Chevreulia acuminata L.	*	*	*	*	N	P
	Conyza bonariensis (L.) Cronquist			*		N	A
	Eupatorium ascendens				*	N	P
	Vernonia nudiflora Less.	*	*			N	P
Commelinaceae	*Commelina benghalensis* TrCigana		*	*	*	N	A
Convolvulaceae	*Dichondra macrocalyx* Meisn.				*	N	P
	Dichondra sericea Sw.	*				N	P
Cyperaceae	*Cyperus brevifolius*	*	*	*	*	N	P
Fabaceae	*Desmodium barbatum*	*	*	*	*	N	P
	Desmodium incanum	*		*	*	N	P
Hypoxidaceae	*Hypoxis decumbens L.*				*	N	P
Malvaceae	*Sida rhombifolia* L.	*		*	*	N	P
Oxalidaceae	*Oxalis brasiliensis G.* Lodd.	*		*	*	N	P
Poaceae	*Setaria geniculata*	*			*	N	A
	Axonopus affinis Chase				*	N	P
	Axonopus argentinus	*				N	P
	Cynodon dactylon		*	*	*	E	P
	Dichanthelium sabulorum		*			N	P
	Digitaria insularis (L.)		*	*	*	N	A
	Eustachys uliginosa (Hack.) Herter		*	*		N	P
	Paspalum notatum	*	*	*	*	N	P
	Paspalum plicatulum Michx.	*	*	*	*	N	P
	Paspalum urvillei Steud.			*	*	N	P
	Piptochaetium montevidense	*	*		*	N	P
Rubiaceae	*Relbunium richardianum*			*	*	N	P
	Richardia brasiliensis Gomes	*	*	*	*	N	P

N/E: Pampa Biome Native/Exotic; P/A: Perennial/Annual

[1] Depending on environmental conditions, perennial species may behave as annuals, and vice versa.

Supplementary Table S2 Plant species occurrence recorded in November, in natural field (NF) and vineyards (VN1, VN2 and VN3) with increasing soil Cu levels

Family	**Species**	NF	**VN1**	**VN2**	**VN3**	**N/E**	**P/A**
Apiaceae	*Apium leptophyllum (Pers.)*	*	*	*	*	N	A
Asteraceae	*Ageratum conyzoides L.*	*	*	*	*	N	A
	Baccharis trimera (Less.) DC.			*		N	P
	Chaptalia sinuata (DC.) Baker			*		N	P
	Chevreulia acuminata Less.	*	*	*	*	N	P
	Conyza bonariensis (L.) Cronquist	*	*	*	*	N	A
	Elephantopus mollis Kunth				*	N	A
	Eupatorium ascendens	*			*	N	P
	Soliva pterosperma (Juss.) Less.	*			*	N	A
	Vernonia nudiflora Less.	*	*	*		N	P
Commelinaceae	*Commelina benghalensis TrCigana*		*	*	*	N	A
Convolvulaceae	*Dichondra sericea Sw.*	*	*	*	*	N	P
Cyperaceae	*Cyperus brevifolius*	*	*	*	*	N	P
Fabaceae	*Aeschynomene falcata (Poir.) DC*				*	N	P
	Desmodium barbatum		*			N	P
	Desmodium incanum (Sw.) DC	*	*	*	*	N	P
	Vicia sativa Guss.			*	*	E	A
Hypoxidaceae	*Hypoxis decumbens L.*		*		*	N	P
Malvaceae	*Sida rhombifolia L.*	*	*	*	*	N	P
Melastomataceae	*Tibouchina gracilis*	*	*			N	P
Oxalidaceae	*Oxalis brasiliensis G. Lodd.*		*	*	*	N	P
Poaceae	*Axonopus argentinus*	*				N	P
	Cynodon dactylon			*	*	E	P
	Dichanthelium sabulorum	*		*		N	P
	Eragrostis plana Nees	*				E	P
	Eustachys ulignosa (Hack.) Herter		*	*	*	N	P
	Lolium multiflorum Lam.		*	*	*	E	A
	Paspalum notatum	*	*	*	*	N	P
	Paspalum plicatulum Michx.			*		N	P
	Paspalum urvillei Steud.			*		N	P
	Setaria geniculata	*				N	A
Rubiaceae	*Borreria acuminata*				*	N	A
	Relbunium richardianum	*		*	*	N	P
	Richardia brasiliensis Gomes	*	*	*	*	N	P

N/E: Pampa Biome Native/Exotic; P/A: Perennial/Annual

[1] Depending on environmental conditions, perennial species may behave as annuals, and vice versa.

4.2 ESTUDO II

Tolerance of species *C. dactylon* and of Pampa biome-native grasses to Cu excess [2]

Isley C. B. Silva; João Pedro Jung dos Santos; Letícia Morsch; Camila Peligrinotti Tarouco; Anderson Cesar Ramos Marques; Lincon Oliveira Stefanello da Silva; André Somavilla; Fernando Luiz Ferreira Quadros; Fernando Teixeira Nicoloso; Paulo Ademar Avelar Ferreira; Gustavo Brunetto

Abstract

Soil contamination by Cu in vineyards grown in Pampa biome can change the growth and development of native and invasive herbaceous plants who cohabit these sites. The way these features change determines plants’ tolerance to Cu contamination and it can assure greater competitive advantage and favor plant establishment in vineyards. The aim of the current study is to investigate whether invasive species *Cynodon dactylon* has higher tolerance to increased Cu levels than Pampa biome-native species such as *Axonopus affinis, Paspalum notatum and Paspalum plicatulum*. The native and invasive species were grown in Hoagland nutrient solution added with 0.32, 15, 30 and 45 μM of Cu for 15 days. Specific plant features, such as photosynthetic activity, Cu content and distribution in plant organs, plant growth, root morphology and nutrient use efficiency were assessed at the end of this period. Increased Cu concentrations affected the development of native and invasive species in the Pampa biome. However, *C. dactylon* roots, even the ones subjected to excessive Cu concentrations, have shown longer length and bigger surface area than those of native species. *C. dactylon* also stood out for maintaining high nutrient use efficiency and root (organ where Cu accumulation prevailed) dry matter production, even at high Cu concentrations. Species *A. affinis* stood out for its significant ability to maintain photosynthetic activity, although it was not enough to maintain high plant growth rates. All these responses have indicated that *C. dactylon* has higher tolerance to Cu than Pampa biome-native species, a fact that may be contributing to its establishment in vineyards contaminated with Cu in Southernmost Brazil.

Keywords: Heavy metal; Copper tolerance; Nutrient use efficiency; WinRHIZO; Gas exchange; Vineyards.

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[2] Artigo elaborado de acordo com as normas de formatação da revista Journal of Plant Research

Introduction

Invasive plants are capable of proliferating rapidly outside their original ecosystems (Prabakaran et al., 2019; Richardson and Van Wilgen, 2004). The introduction of plant species in exotic environments can significantly change the composition and structure of their native vegetation, as well as their ecosystem processes, and harm the environment, the economy and human health (Gulezian et al., 2012; Monaco and Genovesi, 2014). The expansion and colonization of invasive plants in new areas are often associated with anthropic disturbances, mainly when it comes to soil contamination with heavy metals (Hess et al., 2019; Morgado et al., 2018; Prabakaran et al., 2019).

The accumulation of heavy metals in bioavailable forms in the soil tends to have negative impact on the biomass production of native plants lesser tolerant to contamination; consequently, it reduces soil cover and increases resource availability in it (e.g., space, water and nutrients). The native floristic community opening under toxic conditions allows invasive plants to enter it; consequently, invasive plants tolerant to different contamination levels can colonize vacant spaces and even suppress the development of native vegetation (Foster et al., 2002; Morgado et al., 2018). Floodplain contamination with heavy metals in Sydney (Australia) - due to urban development and the consequent increase in the number of floods in the area - was one of the main causes of the invasion of exotic plants in the woods of Lane Cove Basin (Riley and Banks, 1996). According to Gulezian et al. (2012), invasive species *Conium maculatum* has shown higher tolerance to contaminants such as Arsenic (As), Lead (Pb) and Cadmium (Cd) than native species such as *Desmodium canadense* and *Echinacea purpurea*. The higher tolerance of invasive species to high metal contamination levels has favored its establishment along the roads of Chicago and close to sludge drying beds.

Increased copper (Cu) levels bioavailable in vineyard soils in Southernmost Brazil have been suggested as one of the factors favoring invasion processes by exotic species, mainly by *Cynodon dactylon* (Silva et al., 2020). Copper concentrations bioavailable in the 0-20-cm soil layer of the oldest vineyards exceeds by more than 30 times the values observed in natural fields adjacent to vineyards in Pampa biome*; C. dactylon* contributes to 20% of all biomass produced between crop rows, on average (Silva et al., 2020). The successful establishment of invasive species in these areas is worrisome, since the vegetation between vineyards' rows initially comprised herbaceous species native to the Pampa biome. The maintenance of this vegetation between vineyards' rows can help preserving Pampa biome-native species - since nowadays this biome is at advanced degradation stage (Chomenko and Bencke, 2016) – and, at the same time, it helps protecting the soil from erosive processes and favors nutrient cycling.

Several features capable of influencing invasive plants' success are often investigated in the literature (Allred et al., 2010; Holzmueller and Jose, 2009; Wang et al., 2018), although, oftentimes, they are generalizations. Studies focused on featuring specific communities, such as vegetation coexisting in vineyards contaminated with Cu, remain scarce. Therefore, they are necessary to help better understand the differences and similarities in Cu-tolerance mechanisms developed by invasive and native plants. Such an understanding is essential to enable developing strategies capable of controlling the infestation of these areas by already established invasive plants and of improving the biotic resistance of plant communities not yet invaded by exotic plants.

Thus, the aim of the current study was to investigate the impacts of increasing Cu levels on specific features such as metal distribution in biomass, growth, photosynthetic activity, root morphology and nutritional status of native and invasive plants cohabiting vineyards in the Pampa biome. It is known that stress induced by Cu excess changes several physiological processes in plants due to increased production of reactive oxygen species (ROSs), which, in their turn, decrease carbon assimilation and, consequently, inhibit plant development and directly affect its ability to establish in the areas (Marques et al., 2018; McDowell, 2002; Trentin et al., 2019).

In addition, stress caused by high Cu concentrations can lead to a series of impacts on plants' root system, such as decreased cellular proliferation in the meristem (Lequeux et al., 2010), damage to cell integrity in the transition zone (Minkina et al., 2020), cell death (Tamás et al., 2017), reactive oxygen species formation (Macar et al., 2020; Tamás et al., 2017) and changes in hormonal homeostasis (Demecsová and Tamás, 2019; Lequeux et al., 2010). All these changes lead to root architecture remodeling, which is featured by primary root growth inhibition and simultaneous lateral root formation (Lequeux et al., 2010; Marques et al., 2018). Therefore, changes in the root system can significantly affect plants' ability to absorb water and nutrients. In addition, excessive Cu concentrations in the root growth environment compete with other cations for absorption and transport in plants, which indicates the presence of common transporters and helps explaining changes in the absorption and translocation of other nutrients (Sağlam et al., 2015; Vatansever et al., 2017).

Therefore, the way plant species respond to these features can be a determining factor for their ability to tolerate Cu and, consequently, it can contribute (or not) to their establishment in soils contaminated with this metal. The aim of the current study was to investigate whether invasive species *C. dactylon* has higher tolerance to increased Cu levels than Pampa biome-native species such as *Axonopus affinis*, *Paspalum notatum* and *Paspalum plicatulum*. The current hypothesis is that the successful colonization of contaminated vineyards by invasive species *C. dactylon* results from its lower propensity to present damage in the root system and photosynthetic apparatus, as well as lesser nutritional disturbances caused by Cu toxicity than that observed in Pampa biome-native species.

Materials and methods

Study site

The study was carried out in greenhouse environment 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 26ºC and relative humidity was 50%.

Seedlings of the herein investigated species - *Cynodon dactylon* (Bermuda grass), *Paspalum notatum* (bahiagrass), *Paspalum plicatulum* (brownseed paspalum) and *Axonopus affinis* (common carpetgrass) - were collected in a vineyard located in Santana do Livramento County (30° 46’ 36” S; 55° 22’ 03” W), Southern Brazil. The Cu content bioavailable in the 0-20-cm soil layer in the vineyard where plants were collected was 40.38 mg kg^{-1} (Extracted by EDTA); Cu pollution index in the soil was classified as high (Silva et al., 2020). Collected species were grown in sand-based hydroponic system, based on the protocol suggested by Marques et al. (2020). They were subjected to vegetative propagation every two months, for one year, in order to expand the plant bank and increase homogeneity among seedlings belonging to each species. Seedlings were irrigated twice a day with full 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.

Experimental treatments and procedures

Seedlings used in the current study were selected, washed, standardized by species (leaving three roots and three fully expanded leaves) and transferred to 5-L pots filled with Hoagland and Arnon’s (1950) nutrient solution (50% strength) for 15-day acclimation. Eight seedlings were inserted and fixed in each pot with the aid of a plastic support (Fig. S1).

After the acclimation period was over, three plants from each pot were randomly selected, marked and weighed to find the initial fresh matter (FM_i). The nutrient solution was supplemented with different Cu concentrations using $CuSO_4.5H_2O$ as metal source. Sixteen treatments were evaluated; they followed a 4x4 factorial arrangement with four grass species

(*C. dactylon*, *P. notatum*, *P. plicatulum* and *A. affinis*) and four Cu concentrations (0.32, 15, 30 and 45 µM). Treatments have followed a completely randomized design (CRD) with three repetitions. Nutrient solution was continuously aerated with the aid of an air pump throughout the experiment and fully replaced every three days. The pH was adjusted to 5.5 ± 0.2, at all solution changing times, using 1 M NaOH or 1 M HCl.

Relative biomass growth and partition rate

Three plants previously marked in the experimental units were collected and weighed to find the final fresh matter (FM_f), after 15 days of exposure to Cu. Plant growth rate (GR) was calculated based on FM_i and FM_f values, through equation 1:

$$\mathrm{GR(g\ day-1)} = \frac{\mathrm{FMf-FMi(g)}}{\mathrm{Experiment\ duration\ (days)}} \qquad \text{(Equation 1)}$$

Subsequently, plants were washed in 0.1 M HCl solution and in distilled water, and separated into leaves, stems and roots; they were dried in forced-air ventilation oven at 65°C, until reaching constant mass. Leaf, stem and root dry matter (LDM, SDM and RDM, respectively) were determined.

Nutrient concentration in plant tissue and nutrient use efficiency

Leaf, stem and root dry matter was ground and subjected to nitro-perchloric digestion in order to determine total P, K, Ca, Mg, Cu, Zn, Fe and Mn contents, based on the methodology by Embrapa (2000). Readings of total Cu, Zn, Fe, Mn, Ca and Mg concentrations were performed in atomic absorption spectrophotometer (EAA; Varian SpectrAA-600, Australia). Potassium (K) concentration was determined in flame photometer (B262 Micronal). Phosphorus (P) concentration was determined in UV-visible spectrophotometer (1105, Bel Photonics), at wavelength of 882 nm, based on Murphy and Riley (1962).

Nutrient use efficiency (NUE) was calculated for each nutrient, based on Siddiqi and Glass (1981), through equation 2:

$$\mathrm{NUE(g^2mg^{-1})} = \frac{\mathrm{(Total\ plant\ dry\ matter(g))^2}}{\mathrm{Total\ nutrient\ accumulation\ in\ plant\ (mg)}} \qquad \text{(Equation 2)}$$

Gas exchange in leaves

Photosynthesis Analyzer - IRGA (Li-6400, Li-COR Inc., Neb., USA) was used to quantify gas exchange parameters in the last fully expanded leaf of each plant 15 days after plants' exposure to Cu. Net CO_2 assimilation rate (A_{net}), stomatal CO_2 conductivity (Gs), water use efficiency (WUE) and instantaneous carboxylation efficiency (A/Ci) (by ribulose-1,5-

bisphosphate-carboxylase/oxygenase) were the evaluated parameters. These variables were determined in chamber at CO_2 concentration of 400 μmol mol^{-1}, temperature of 20/25°C, relative humidity of 50 ± 5% and photon flow density of 1500 μmol m^{-2} s^{-1}.

Root morphology analysis

One plant from each experimental unit was randomly collected after 15-day exposure to Cu, thus totaling three plants per treatment. Their roots were washed in running and distilled water and scanned in EPSON Expression 11000 scanner equipped with additional light (TPU), at 600 dpi definition for roots. Subsequently, images were analyzed in the WinRhizo Pro 2013 image analysis system software (Régent Instr. Inc.) in order to determine root length, root surface area, mean root diameter and root volume.

Statistical analysis

Results were subjected to Lilliefors and Shapiro-Wilk tests in order to investigate the normality and homogeneity of variance assumptions. Subsequently, analysis of variance ($p \leq 0.05$) was performed; means recorded for NUE were compared through Scott-Knott test ($p \leq 0.05$), whenever H_0 was rejected. The other variables were subjected to polynomial regression analysis ($p \leq 0.05$), when they were significant in the F test ($p \leq 0.05$).

Results

Biomass growth and partition

Copper (Cu) increase in the nutrient solution affected the growth of all species in different ways (Fig. 1). The growth rate of grass species *C. dactylon* and *A. affinis* has linearly decreased as Cu concentrations increased (Fig. 1a). Increased Cu doses have decreased leaf and stem dry matter production in *A. affinis* (Fig. 1b and 1c). The exposure of this species to the highest Cu concentration (45 µM) reduced its stem dry matter production by 85%. On the other hand, *P. plicatulum* presented increased growth rate up to Cu concentration of 30 µM in the solution. This species has also presented quadratic response in leaf, stem and root dry matter production (Fig. 1b, 1c and 1d). *Cynodon dactylon*, *A. affinis* and *P. notatum* did not show significant variations in root dry matter (Fig. 1d).

Copper (Cu) content and distribution in plants

Increased Cu concentrations in the culture medium have affected Cu contents in the leaves, stems and roots of all grass species in different ways (Fig. 2). Copper (Cu) levels in the leaves and roots of invasive species *C. dactylon* were adjusted through quadratic model; this species recorded maximum concentrations of 27.87 mg Cu kg^{-1} in leaves and 386.03 mg Cu kg^{-1} in roots, after the addition of approximately 30 µM of Cu to the nutrient solution (Fig. 2a and 2c). *Paspalum plicatulum* leaves and stem recorded higher Cu increase than that observed for the same organs in the other species (Fig. 2a and 2b). On the other hand, Cu content in *P. plicatulum* roots grown in the 45µM solution has increased by 11 times, whereas Cu content in *A. affinis*, *C. dactylon* and *P. notatum* roots increased by 85, 18 and 22 times, respectively (Fig. 2c).

Based on Cu partition in plant tissues, it was possible to show that the investigated species presented different metal distribution patterns when they were subjected to different Cu concentrations (Fig. 2d). *Axonopus affinis*, *C. dactylon* and *P. notatum* subjected to 0.32 µM of Cu were capable of maintaining more than 80% of the absorbed Cu in shoot organs, on average, whereas *P. plicatulum* concentrated most Cu in the root system (65%, on average). However, these plants presented similar metal distribution patterns in their tissues when they were grown in environments subjected to high Cu concentration; approximately 70% of the absorbed Cu remained in the root system, whereas 30% of it was transferred to the shoot (Fig. 2d).

Gas exchanges

The impact of Cu increase in the nutrient solution on gas exchange parameters strongly depended on plant species (Fig. 3). The net CO_2 assimilation (A_{net}) rate recorded for species *A. affinis* did not adjust to any regression model as Cu concentrations increased (Fig. 3a). A^{net} rates have linearly decreased in the other investigated species. However, the A_{net} value recorded for species *C. dactylon* was higher than the ones observed for species *P. notatum* and *P. plicatulum* at all Cu concentrations. The Anet value recorded for *C. dactylon* was three times higher than the ones recorded for native species *P. notatum* and *P. plicatulum*, on average, when all these species were exposed to 45 µM of Cu (Fig. 3a).

Stomatal conductance (Gs) has also varied between species at all tested Cu concentrations; the highest value was observed for *A. affinis,* after the addition of 15 µM of Cu to the nutrient solution (Fig. 3b). However, *C. dactylon* recorded higher Gs than that of Pampa biome-native species at all other Cu concentrations. The Gs recorded for *P. plicatulum* was mostly affected by Cu concentration increase. The exposure of this species to the highest Cu concentration reduced its Gs by approximately 70% (Fig. 3b).

Increased Cu concentrations have also decreased the water use efficiency (WUE) of species *A. affinis*, *P. plicatulum* and *C. dactylon* (Fig. 3c). Despite this reduction, *A. affinis* presented better WUE than the other investigated species, at all tested Cu concentrations. *Axonopus affinis* subjected to excessive Cu concentration (45 µM) recorded 101% better WUE than that of species *C. dactylon* and *P. plicatulum*, and 374% better than that of *P. notatum*. The instantaneous carboxylation efficiency (A/Ci) of all four investigated species has decreased as Cu concentrations in the nutrient solution increased. However, similar to what was observed for WUE, species *A. affinis* maintained A/Ci higher than that observed for the other species, after Cu concentration increase in the solution (Fig. 3d).

Morphological changes in the root system

Copper (Cu) exposure has limited the root surface area in all species, mainly in *C. dactylon* and *P. plicatulum* (Fig. 4b) grown at the highest Cu concentration (45µM Cu), which presented root surface area reduction by 62% and 72%, respectively. These two species have also shown significantly inhibited root volume and length as Cu concentrations increased (Fig. 4d and 4a). Volume reduction took place in roots belonging to all diameter classes, mainly in those larger than 0.8 mm (Fig.S1c S1l). On the other hand, length decrease prevailed in roots whose diameter was smaller than 0.4 mm (Fig. S1a and S1j). *Axonopus affinis* roots did not change in length or volume as Cu levels in the solution increased.

Native species *A. affinis*, *P. notatum* and *P. plicatulum* presented increased mean root diameter (Fig. 4c) as Cu concentrations in the nutrient solution increased. Although species *C. dactylon* underwent significant decrease in root length and surface area at the highest Cu levels, these two variables recorded higher values than the ones observed for the other investigated species (Fig. 4a and 4b).

Nutrients in plant tissue

Copper (Cu) exposure has changed nutrient levels in plants in different ways (Fig. 5). *Axonopus affinis* leaves recorded the largest changes in nutrient levels as Cu concentrations in the nutrient solution increased (Fig. 5a, 5d, 5g and 5j). Macronutrient levels have decreased, with emphasis on K, which had its leaf content reduced by more than 40%. Similarly, nutritional changes in *P. plicatulum* were more evident in leaves. Increased Cu levels in the culture medium have led to increased P and Mg levels, as well as to decreased K and Ca levels, in *P.*

plicatulum leaves. *Paspalum notatum* stem was the organ presenting the strongest nutritional sensitivity (Fig. 5b, 5h and 5k). Increased Cu levels have favored linear Cu increase in the stem, whereas Mg and P levels reduced in it. *Cynodon dactylon* roots and stem subjected to increased Cu levels presented the greatest nutritional changes; *C. dactylon* roots and stem subjected to the highest Cu concentration (45 µM) presented decreased P, K, Ca and Mg levels. However, roots and stem subjected to intermediate Cu concentrations presented increased K concentrations, which exceeded the control treatment concentration by 69% (roots) and 21% (stem).

Micronutrient concentrations have also changed due to increased Cu levels in the root medium (Fig. 6). Manganese (Mn) concentrations have decreased in all *A. affinis* organs (Fig. 6d, 6e and 6f). In addition, Fe and Zn levels in the stem of this species have decreased. Manganese (Mn) concentrations in *P. notatum* and *P. plicatulum* leaves and stems have also decreased. *Paspalum notatum* leaves and roots have also shown decreased Fe and Zn contents, respectively (Fig. 6g and 6c). Unlike what was observed for the other species, *P. plicatulum* exposed to higher Cu levels presented increased Fe levels in leaves and stems, as well as increased Mn levels in the root system. The main changes recorded for *C. dactylon* were observed in leaves, which presented significantly reduced Zn and Fe levels (Fig. 6a and 6g).

Nutrient use efficiency

Cynodon dactylon and *A. affinis* grass species subjected to the control treatment (0.32 µM of Cu) presented high efficiency in the use of all nutrients (Table 1). However, increased Cu levels in the culture solution have significantly reduced NUE in *A. affinis*. *C. dactylon* subjected to this very same condition presented outstanding behavior in comparison to other species, since it recorded increased NUE for all evaluated nutrients.

Discussion

Copper (Cu) is easily absorbed by plant roots; high Cu absorption leads to changes in root development, as well as in plants' nutritional composition, photosynthetic activity, growth and biomass accumulation (Adrees et al., 2015; Marques et al., 2018). The intensity of these effects depends on plant species. Nowadays, most studies about Cu phytotoxicity focus on agricultural species; thus, the sensitivity of most native, or even invasive, plants to Cu remains poorly understood (Struckhoff et al., 2013).

Invasive species *C. dactylon* and native species *A. affinis* subjected to the control treatment (0.32 µM of Cu) have shown similar growth rates. *P. notatum* and *P. plicatulum* subjected to this very same growth condition presented clear similarity in growth rates (Fig. 1a). These results have shown the similarity between *C. dactylon* and *A. affinis*, and between *P. notatum* and *P. plicatulum*, in their ability to use resources - such as nutrients, light and water - available in the growth medium.

However, it was clear that all species have unique responses to increased Cu concentrations in the solution. Although *C. dactylon* presented reduced growth rate, it was more capable of tolerating high Cu levels than *A. affinis* (Fig. 1a). *A. affinis* growth rate decreased by approximately 75%, whereas that of *C. dactylon* decreased by 48%. Growth responses recorded for *P. plicatulum*, in their turn, confirmed and reinforced differences in nutrient requirements among the evaluated species (Fig. 1a, 1b, 1c and 1d). It happened because *P. plicatulum* cultivation at intermediate Cu levels has favored its growth rate, as well as increased the dry matter of all its organs. This outcome has indicated that *P. plicatulum* likely needs differentiated Cu levels to reach its maximum growth. All four species presented Cu levels in the leaf within the range established as excellent in the literature (5 to 20 mg kg^{-1}) when they reached maximum growth at different Cu concentrations in the solution (Kabata-Pendias, 2011).

The absorbed Cu has mainly accumulated in the root system of all species grown in solution added with high Cu concentrations (Fig. 2d). Results in the current study meet data from other studies, which have reported low Cu translocation rate from the root to the shoot (De Conti et al., 2018; Marques et al., 2018). This low translocation rate likely resulted from Cu retention in the root apoplast (Lequeux et al., 2010). Previous studies have suggested that cell walls have strong ability to bind to heavy metals, mainly to Al and Cu, as well as that they can act as barrier to prevent these metals from entering plant-cell cytoplasm (Krzesłowska, 2011; Torasa et al., 2019). This high binding ability is attributed to Cu sorption by cell wall components such as lignin, pectin, and some polysaccharides and proteins (Colzi et al., 2011; Torasa et al., 2019). Thus, Cu accumulation in the root system has been described as the main process limiting its accumulation in leaves and, consequently, it minimizes the effect of Cu on plant shoot, mainly on the photosynthetic apparatus (Li et al., 2019; Marques et al., 2018).

Although all evaluated species accumulated greater Cu amount in the root system after Cu concentrations in the nutrient solution increased, *C. dactylon* was the species presenting the highest Cu allocation in the roots (Fig. 2 d), mainly specimens grown in solution added with 15 µM and 45 µM of Cu. The greater Cu allocation in the root system was associated with increased Cu concentration, as well as with the maintenance of dry matter production under Cu excess condition (Fig. 2c and 1d). This process reinforces plants' strategy to develop this plant tissue in order to accumulate this metal.

C. dactylon exposed to Cu concentrations higher than 10 µM presented changes in biomass partition, which was likely the feature that gave its greater competitive advantage than that of other species. Unlike native species, invasive grass *C. dactylon* grown under high Cu concentrations invested more resources in stem production (Fig. 1c). This species has stolon-type stems, which are shoot stems that creep along the surface of the soil and have buds in the nodes capable of growing roots and leaves, thus forming new plants. The greater investment in stolon production allows *C. dactylon* to rapidly occupy the space available in the landscape and to easily spread towards new areas, even towards the ones contaminated with Cu. These results corroborate the study by Silva et al. (2020), who showed that despite the increased Cu concentrations in vineyard soils, *C. dactylon* degree of invasiveness has increased in areas whose soil presented the highest Cu levels. According to the aforementioned authors, shoot biomass production by *C. dactylon* accounts for 20% of all biomass produced between rows in these vineyards, on average.

Although the increased Cu content in leaf tissues was relatively low in comparison to that in the root system (Fig. 2a and 2c), it was enough to change several physiological processes in the investigated grass species, as evidenced by gas exchange parameters (Fig. 3). Decreased A_{net}, Gs, WUE and A/Ci are common plant responses to Cu toxicity (Marques et al., 2018; Trentin et al., 2019; Zehra et al., 2020) and may result from structural damage to plants' photosynthetic apparatus (Feigl et al., 2015). Excess of Cu had negative impact on the lipid and pigmentary composition of thylakoids, as well as decreased the photochemical activity of PSII (Sağlam et al., 2015; Trentin et al., 2019; Xu et al., 2013), and chlorophyll and carotenoid contents (Silva et al., 2018; Tiecher et al., 2018). In addition, it is possible ruling out limitations in rubisco carboxylation efficiency, since Cu toxicity appears to be associated with reduced rubisco enzyme concentration and/or decreased activity (Mateos-Naranjo et al., 2015; Sağlam et al., 2015). According to Simova-Stoilova et al. (2002), rubisco content has decreased in barley leaves subjected to toxic copper concentrations. This reduction likely derived from oxidative stress caused by reactive oxygen species (ROS) formation due to Cu toxicity.

Among the herein investigated grass species, *A. affinis* was the one whose gas exchange parameters were the least affected by Cu excess (Fig. 3). This outcome indicates that, despite the stress caused by increased Cu concentrations, *A. affinis* had significant ability to maintain the photosynthetic activity. However, this ability was not enough to maintain its high growth rate, mainly in shoot organs such as leaves and stem (Fig. 1b and 1c). Nevertheless, similar to

C. dactylon, *A. affinis* was efficient in maintaining high dry root biomass production (Fig. 1d), which enabled it to accumulate large Cu amounts in this tissue in order to prevent further damage to the photosynthetic apparatus.

Increased Cu concentrations in the nutrient solution have also changed root morphological parameters such as root length, surface area, mean diameter and volume in all investigated species (Fig. 4a, 4b, 4c and 4d). Overall, plant exposure to Cu excess often induces root morphology remodeling (De Conti et al., 2019; Lequeux et al., 2010; Marques et al., 2018; Trentin et al., 2019). Reduced root growth may result from decreased cell division and lead to increased root cell wall thickness in plants exposed to heavy metals. Similar effects were observed by Song et al. (2017), who investigated the impact of different Cu concentrations in the growth medium on morphogenetic features of *Arabidopsis thaliana* (model plant) roots. The aforementioned authors have found that root growth inhibition resulted from reduced meristematic and elongation zones, as well as that this inhibitory role was mediated, at least in part, by the modulation of biosynthesis, sensitivity, transport or auxin interactions with other phytohormones.

Copper (Cu) phytotoxicity has also induced imbalance in the absorption of essential elements for proper plant growth and development. The effect of Cu on nutrient absorption is determined by the degree of environmental contamination, as well as by species and plant organs, which express different tolerance to heavy metals at toxic levels (Fig. 5 and 6). With respect to cationic macronutrients such as K, Ca and Mg, *C. dactylon* recorded the highest K concentrations in the shoot in comparison to native species (Fig. 5d and 5e). The K plays a key role in energy processes, as well as in metabolite transport and enzyme activation; besides, it is directly involved in the relief of biotic or abiotic stress conditions in plants, such as Cu toxicity (Siddiqui et al., 2012). Thus, the higher K concentration observed in the leaves and stem of the investigated invasive species can enable this plant to deal with Cu-induced stress. Copper (Cu) toxicity in native species *P. plicatulum* appeared to be associated with sudden changes in Ca and Mg concentrations in leaves, stems and roots (Fig. 5). However, the linear Mg content increase in *P. plicatulum* leaves did not appear to be advantageous for this species, as indicated by decreased biomass production in all organs after plant exposure to toxic Cu levels (Fig. 1b, 1c and 1d).

Among all micronutrients assessed in plant organs, Fe concentration in the root system of all species did not significantly change as Cu levels in the nutrient solution increased (Fig. 6i). However, Fe in plant shoot has shown contrasting behavior among species, since its concentration in *P. notatum* and *C. dactylon* leaves, as well as in *A. affinis* stem, decreased, whereas its content in *P. plicatulum* leaves increased. Excess of heavy metals often disturbs Fe homeostasis (Lešková et al., 2017). Several studies have shown Fe and Cu competition for absorption and metabolism sites in plants (Ferreira et al., 2015; Palmer and Guerinot, 2009; Ravet and Pilon, 2013).

Variations in nutrient absorption and accumulation in plants, as well as in plant growth after exposure to Cu excess have changed the NUE of the investigated species (Table 1). Species *A. affinis* stood out among other species for its high efficiency in using all nutrients at low Cu concentration (0.32 µM of Cu). However, invasive species *C. dactylon* stood out under high-exposure-to-Cu conditions; it presented the best NUE in comparison to Pampa biome-native species; this outcome shows the importance of its greater biomass allocation in the stems and roots under the worst Cu contamination condition.

The present study has investigated the impacts of increasing Cu levels on specific features such as photosynthetic activity, metal distribution in biomass, plant growth, root morphology and nutritional status of Pampa biome-native and invasive plants coexisting in vineyards contaminated by Cu. However, it is worth emphasizing the need to carry out complementary studies focused on investigating adsorbent materials and/or immobilizers capable of minimizing the toxic effects of Cu on these species since, based on the present results, native species such as *A. affinis* presented remarkable ability to compete with aggressive invasive species such as *C. dactylon* at low Cu concentrations. Both species were highly efficient in using nutrients, as well as presented high growth rate, under low Cu availability conditions. In addition, *A. affinis* was highly efficient in using water, which is an important feature to help cover plants tolerate additional environmental variations such as drought.

Conclusion

Despite high Cu concetration affects photosynthetic activity, root morphology, nutritional status and growth, *C. dactylon* was capable of maintaining high nutrient use efficiency and root (organ where Cu accumulation prevailed) dry matter production, regardless of the stress condition. In addition, increased Cu concentrations have favored the development of *C. dactylon* stolons. The ability of *Cynodon dactylon* to accumulate Cu in roots and stolons has increased its tolerance to phytotoxicity and competitive ability. All these responses have indicated that species *C. dactylon* was more tolerant to Cu than Pampa biome-native species, a fact that may have contributed to its establishment in vineyards contaminated with Cu in Southernmost Brazil. *Axonopus affinis* stood out among the investigated native grass species for maintaining its high photosynthetic activity at stressful Cu concentrations, whereas *P. plicatulum* stood out for increasing its biomass production at intermediate Cu concentrations.

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Tables

Table 1- Nutrient use efficiency (NUE, g^2 mg^{-1}) of cover grass species grown in nutrient solution added with 0.32 µM and 45 µM of Cu.

Cu dose (µM)	*A. affinis*	*C. dactylon*	*P. notatum*	*P. plicatulum*
		P		
0.32	0.58 aA	0.36 bB	0.15 cA	0.16 cB
45	0.35 bB	0.59 aA	0.19 cA	0.29 bA
		K		
0.32	1.14 aA	1.14 aA	0.50 bA	0.68 bB
45	1.31 aA	1.27 aA	0.45 bA	1.29 aA
		Ca		
0.32	0.67 aA	0.43 bB	0.21 cA	0.13 cA
45	0.41 bB	0.68 aA	0.27 bA	0.28 bA
		Mg		
0.32	0.80 aA	0.68 aB	0.22 bA	0.17 bA
45	0.55 bB	1.08 aA	0.30 cA	0.22 cA
		Cu		
0.32	360.74 aA	233.00 bA	52.25 cA	62.67 cA
45	7.73 bB	53.90 aB	12.11 bB	20.34 bB
		Zn		
0.32	73.26 aA	52.20 bB	14.73 cA	10.69 cB
45	51.60 bB	77.10 aA	19.54 cA	25.81 cA
		Fe		
0.32	43.00 aA	29.47 aB	11.78 bA	9.30 bA
45	17.00 bB	56.31 aA	19.81 bA	15.81 bA

Means followed by the same lowercase letter on the line and by the same uppercase letter in the column did not significantly differ from each other in the Scott-Knott test ($p < 0.05$).

Figures

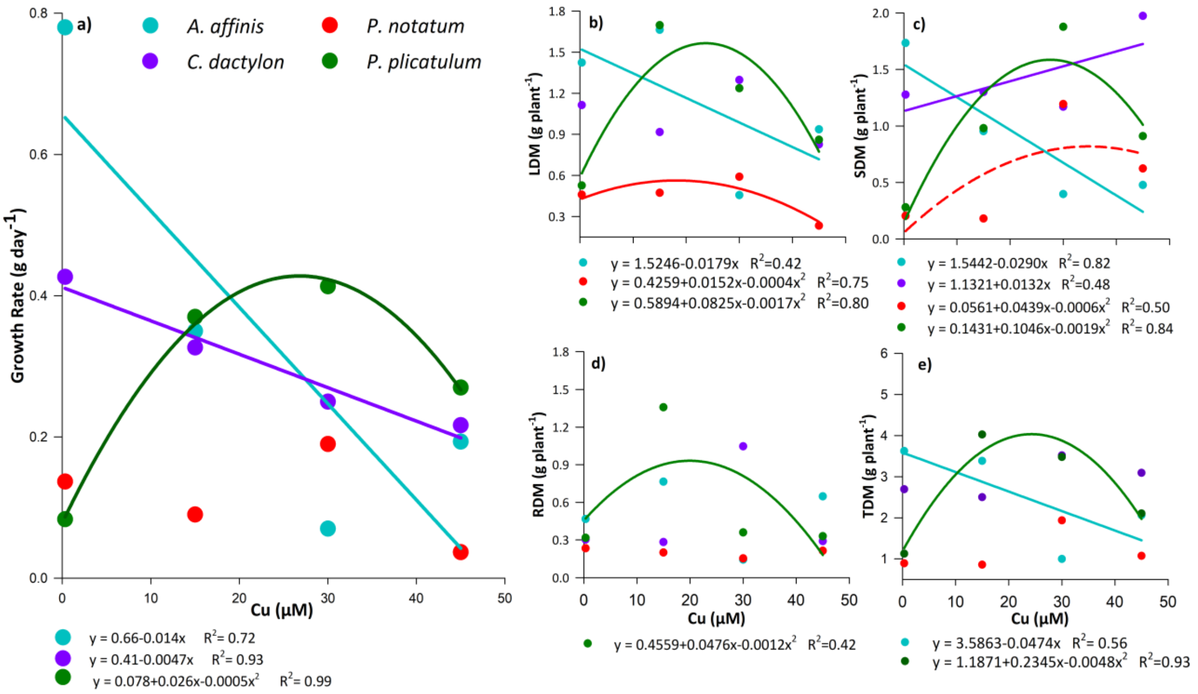

Fig. 1 Growth rate (a), (b) leaf, (c) stem, (d) root and (e) total dry matter production recorded for invasive species *C. dactylon*, and for Pampa biome-native species *A affinis*, *P. notatum* and *P.plicatulum*

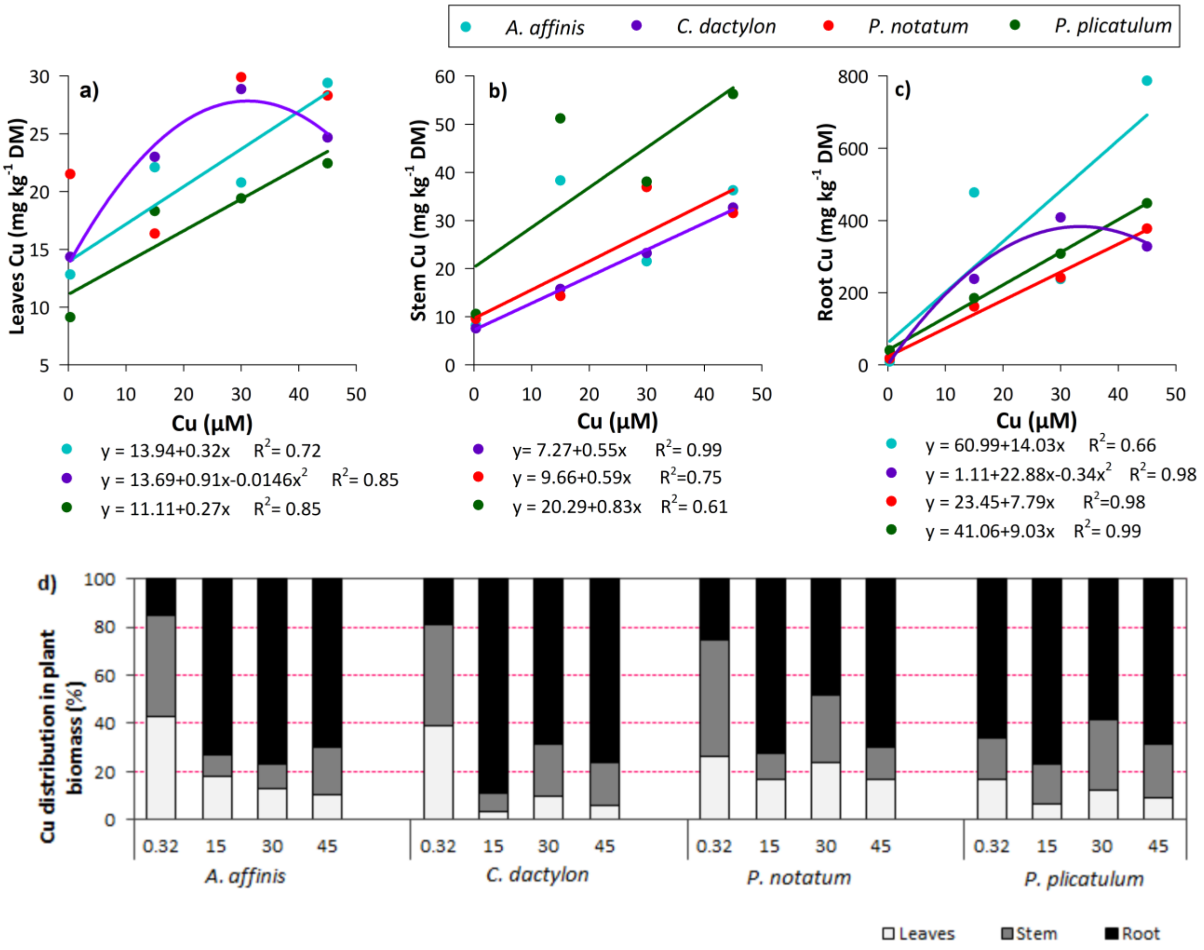

Fig. 2 Cu concentrations in (a) leaves, (b) stems and (c) roots, and (d) Cu distribution in the biomass of invasive species *C. dactylon*, and of Pampa-biome-native species *A. affinis*, *P. notatum* and *P. plicatulum*

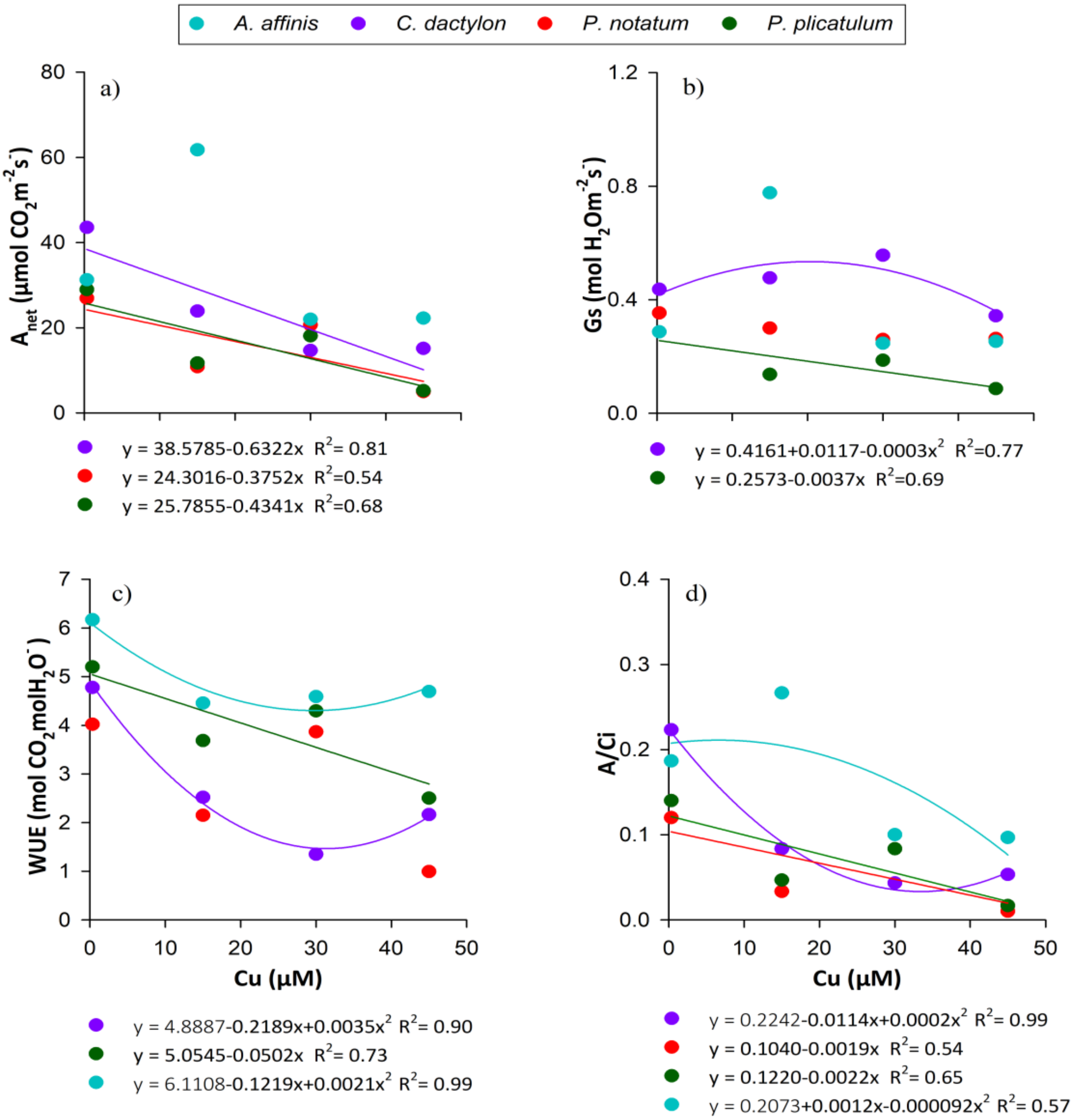

Fig. 3 Net photosynthetic rate (A_{net}), Stomatal conductance (Gs), Water Use Efficiency (WUE) and Instantaneous carboxylation efficiency (A/Ci) of invasive species *C. dactylon*, and of Pampa biome-native species *A. affinis*, *P. notatum* and *P. plicatulum*

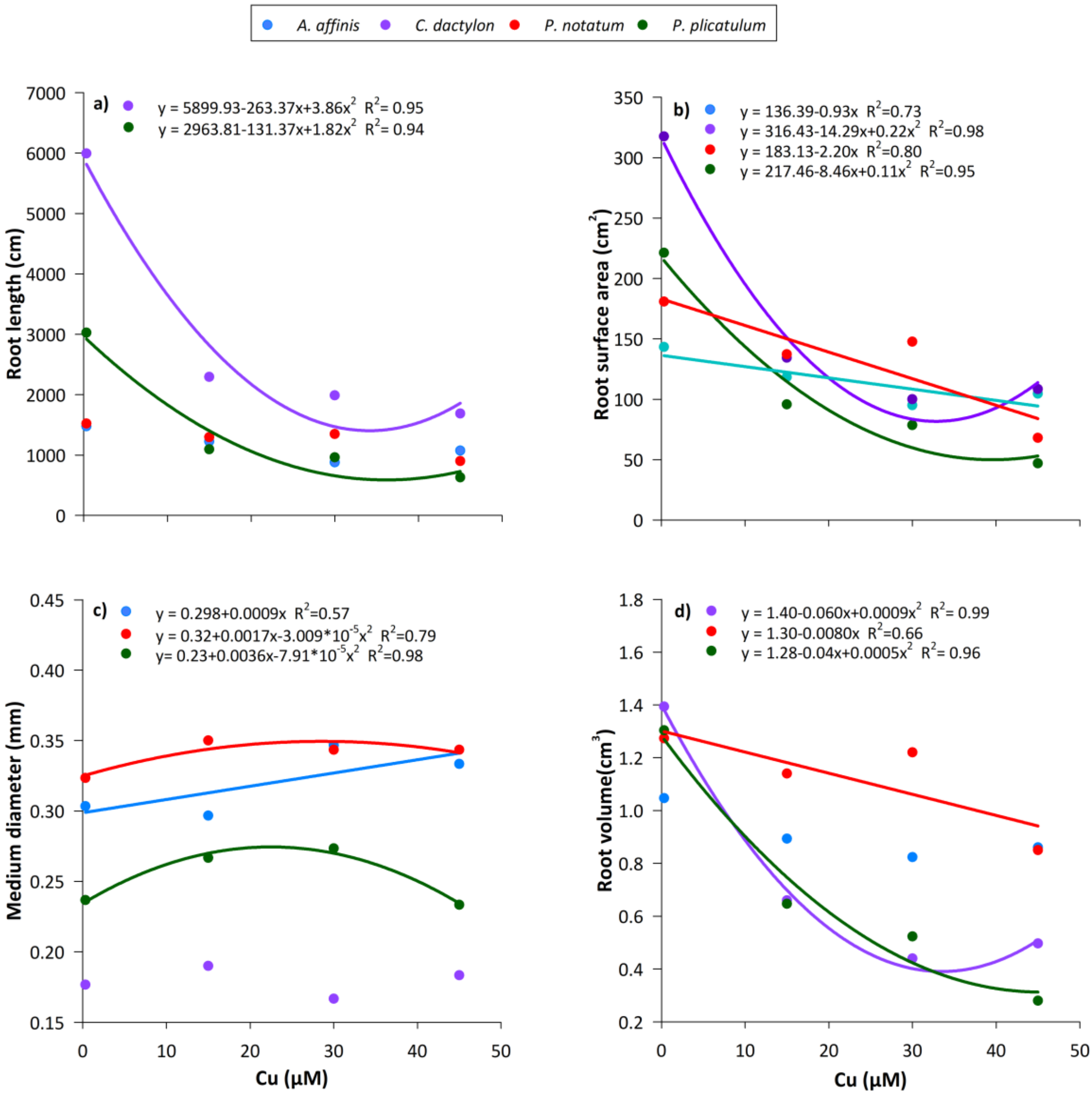

Fig. 4 Root morphological characteristics. (a) root length, (b) surface area, (c) medium diameter and (d) volume recorded for invasive species *C. dactylon*, and for Pampa biome-native species *A. affinis*, *P. notatum* and *P. plicatulum*

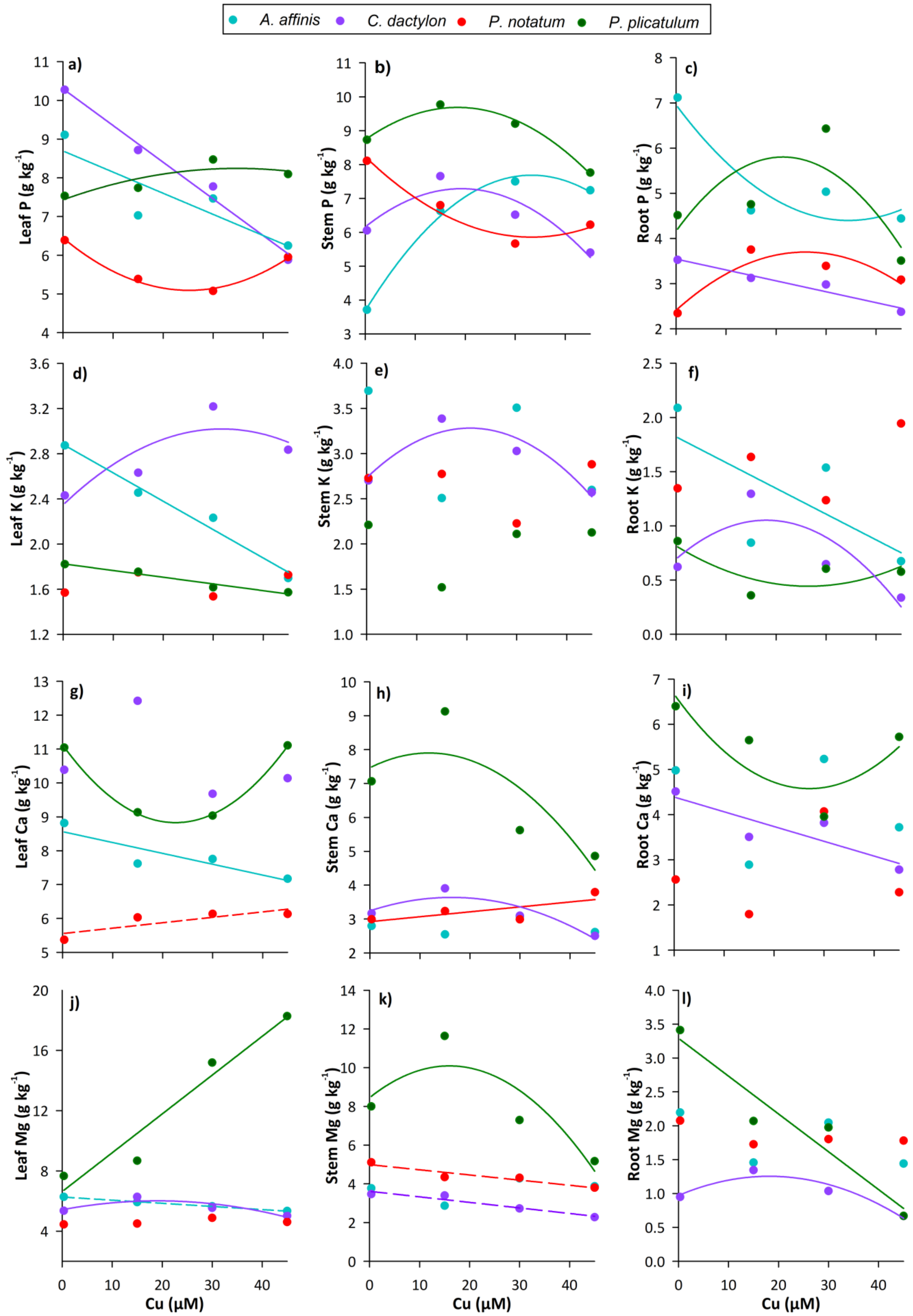

Fig. 5 Phosphorus-P (a, b, c), Potassium-K (d, e, f), Calcium-Ca (g, h, i) and Magnesium-Mg (j, k, l) concentrations in the leaves, stems and roots of invasive species *C. dactylon*, and of Pampa biome-native species *A. affinis*, *P. notatum* and *P. plicatulum*. All regression equations can be seen in supplementary material (Table S1)

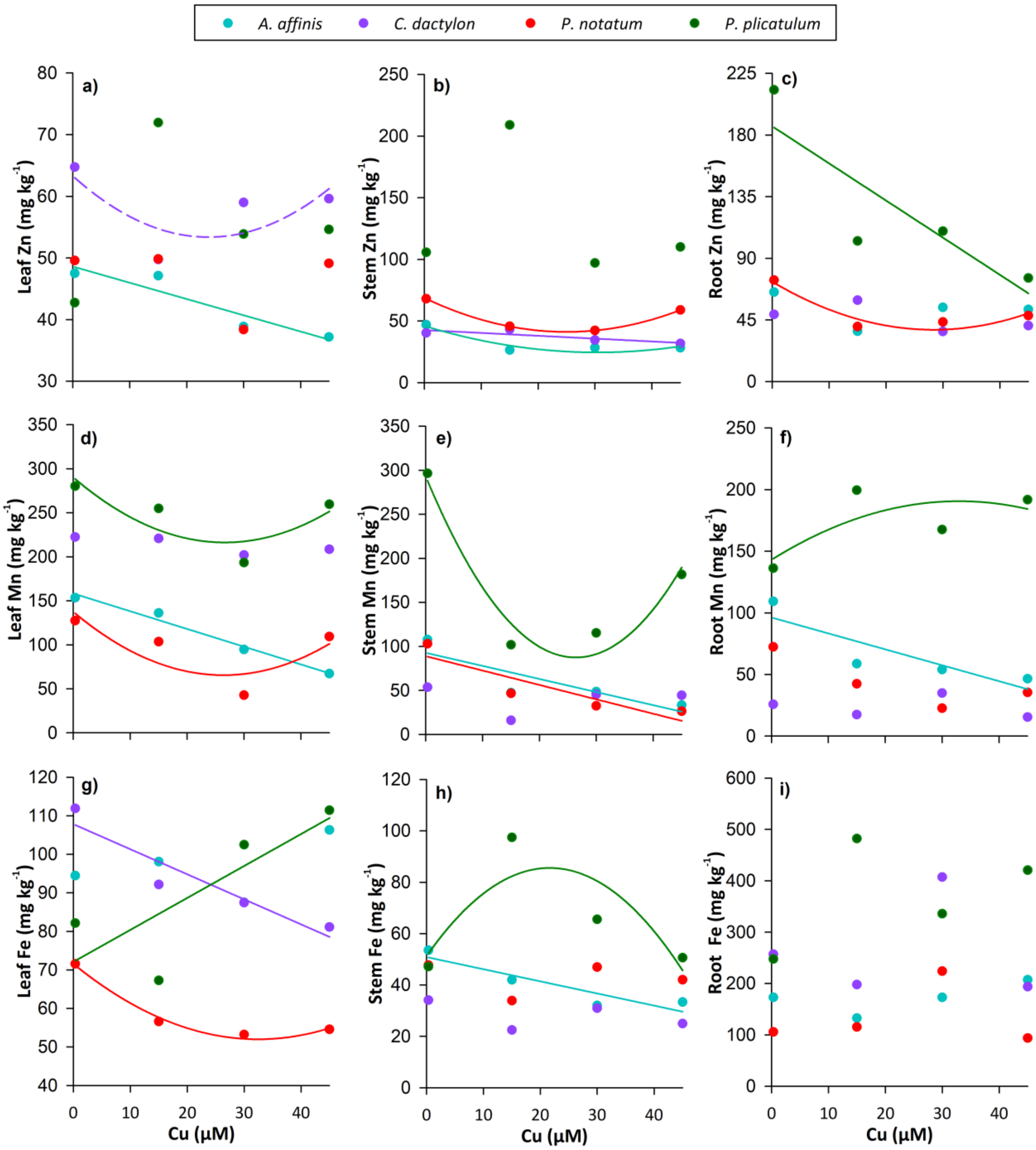

Fig. 6 Zinc-Zn (a, b, c), Manganese-Mn (d, e, f) and Iron-Fe (g, h, i) concentrations in the leaves, stems and roots of invasive species *C. dactylon*, and of Pampa biome-native species *A. affinis*, *P. notatum* and *P. plicatulum*. All regression equations can be seen in supplementary material (Table S2)

Supplementary material

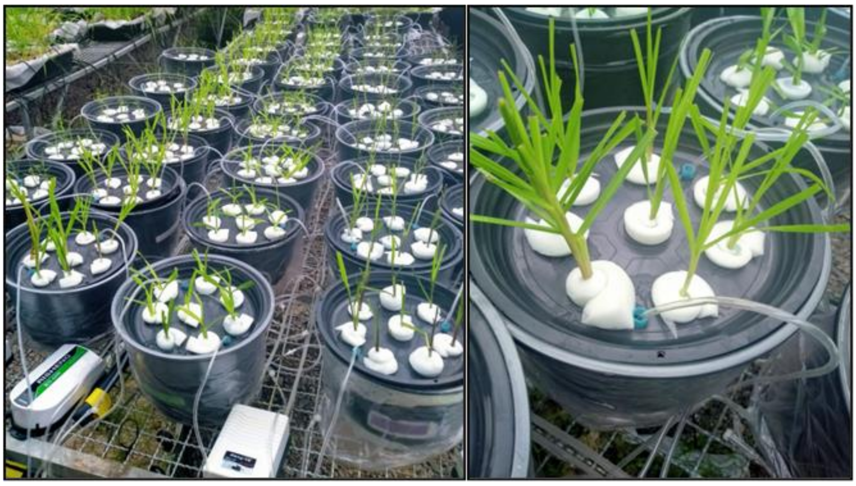

Figure S1 – Overview of the initial layout of experimental units on greenhouse countertops. Emphasis on the continuous aeration of the nutrient solution with the aid of air pumps

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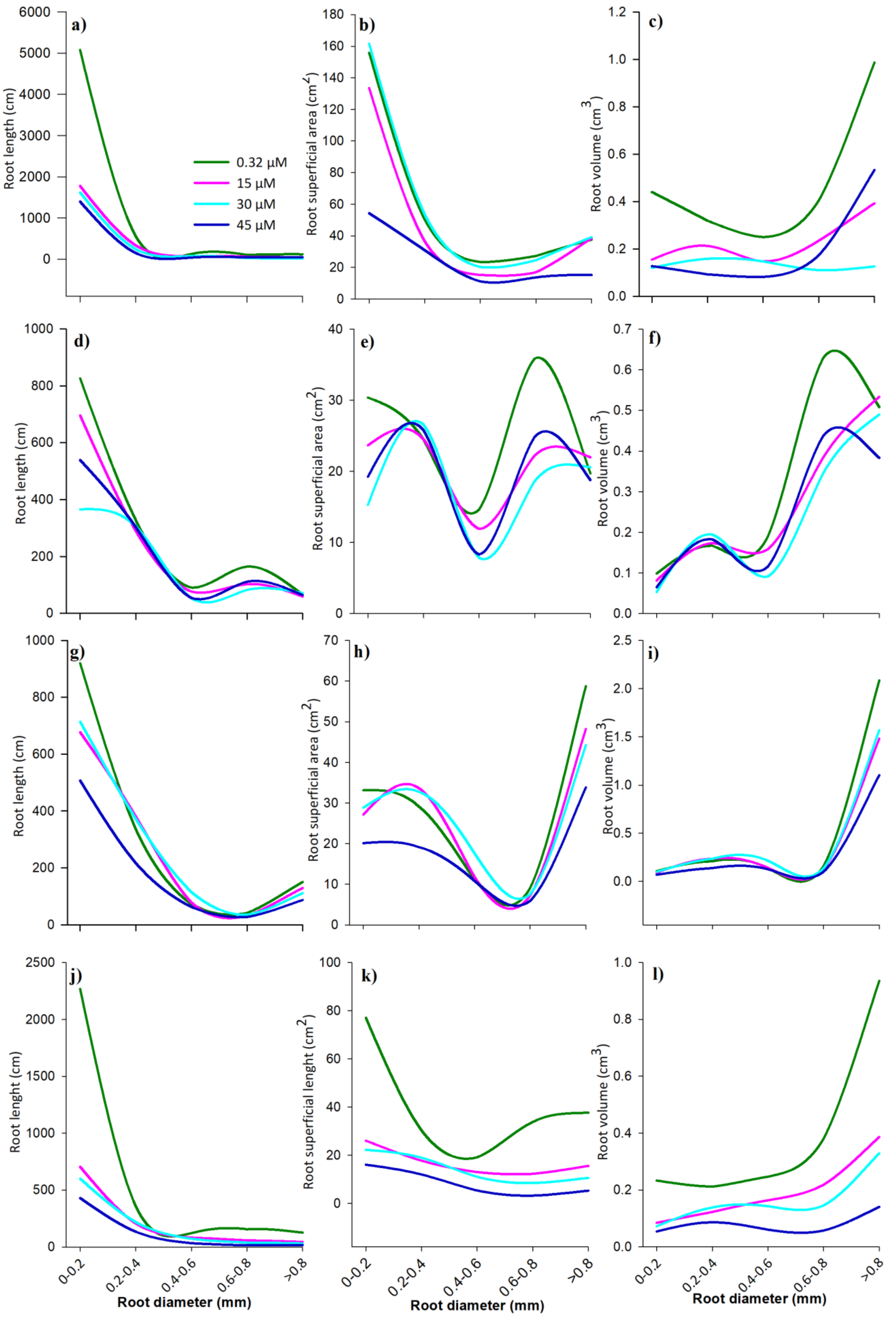

Figure S2 - Root length, surface area and volume distributed across diameter classes of invasive species *C. dactylon* (a, b, c), and of Pampa biome-native species *A. affinis* (d, e, f), *P. notatum* (g, h, i) and *P. plicatulum* (j, k, l).

Table S1- Regression equations adjusted among macronutrient contents (P, K, Ca and Mg) in the leaves, stems and roots of species *C. dactylon*, *A. affinis*, *P. notatum* and *P. plicatulum* in response to the addition of increasing Cu doses to the nutrient solution.

Nutrient (g kg^{-1})	Species	Equation	R^2	Vmáx	Nmax
		Leaves			
P	*A. affinis*	$Y= 8.6937-0.0546x$	0.75	8.6762	0.32
	C. dactylon	$Y= 10.2979-0.0947x$	0.98	10.268	0.32
	P. notatum	$Y= 6.4454-0.1071x+0.0021x^2$	0.99		
	P. plicatulum	$Y= 7.4329+0.0466x-0.0007x^2$	0.74	8.2085	33.29
K	*A. affinis*	$Y= 2.8817-0.0251x$	0.98	2.8737	0.32
	C. dactylon	$Y= 2.3481+0.0424x-0.0007x^2$	0.73	2.9902	30.29
	P. notatum	$Y= 1.6449$	–	–	–
	P. plicatulum	$Y= 1.8254-0.0059x$	0.96	1.8235	0.32
Ca	*A. affinis*	$Y= 8.5615-0.0320x$	0.79	8.5510	0.32
	C. dactylon	$Y= 10.6536$	–	–	–
	P. notatum	$Y= 5.5539+0.0159x$	0.70	6.2694	45
	P. plicatulum	$Y= 11.1217-0.2031x+0.0045x^2$	0.99		
Mg	*A. affinis*	$Y= 5.7933$	–	–	–
	C. dactylon	$Y= 5.4231+0.0619x-0,0016x^2$	0.99	6.0200	19.34
	P. notatum	$Y= 4.6036$	–	–	–
	P. plicatulum	$Y= 6.6274+0.2578x$	0.94	1,8228	45
		Stem			
P	*A. affinis*	$Y= 3.6876+0.2404x-0.0036x^2$	0.99	7.7009	33.39
	C. dactylon	$Y= 6.1611+0.1173x-0.0030x^2$	0.85	7.3077	19.55
	P. notatum	$Y= 8.2306-0.1424x+0.0021x^2$	0.97		
	P. plicatulum	$Y= 8.7359+0.1031x-0.0028x^2$	0.99	9.6849	18.41
K	*A. affinis*	$Y= 3.0766$	–	–	–
	C. dactylon	$Y= 2.7334+0.0531x-0.0013x^2$	0.88	3.2755	20.42
	P. notatum	$Y= 2.6521$	–	–	–
	P. plicatulum	$Y= 1.9907$	–	–	–
Ca	*A. affinis*	$Y= 2.7549$	–	–	–
	C. dactylon	$Y= 3.2402+0.0487x-0.0015x^2$	0.84	3.6353	16.23
	P. notatum	$Y= 2.9224+0.0145x$	0.55	3.5749	45
	P. plicatulum	$Y= 7.4656+0.0737x-0.0031x^2$	0.67	7.9039	11.89
Mg	*A. affinis*	$Y= 3.6941$	–	–	–
	C. dactylon	$Y=3.6105-0.0286x$	0.93	3.6013	0.32
	P. notatum	$Y= 4.9881-0.0266x$	0.89	4.9796	0.32
	P. plicatulum	$Y= 8.4519+0.2055x-0.0064x^2$	0.76	10.1010	16.05
		Root			
P	*A. affinis*	$Y= 6.961-0.1486x+0.002 x^2$	0.83		
	C. dactylon	$Y= 3.5457-0.0241x$	0.95	3.5380	0.32
	P. notatum	$Y= 2.4086+0.0997x-0.0019x^2$	0.84	3.7165	26.24
	P. plicatulum	$Y= 4.1595+0.1536x-0.0036x^2$	0.59	5.7974	21.33
K	*A. affinis*	$Y= 1.8217-0.0237x$	0.49	1.8141	0.32
	C. dactylon	$Y= 0.6936+0.0397x-0.0011x^2$	0.72	1.0516	18.04
	P. notatum	$Y= 1.5399$	–	–	–
	P. plicatulum	$Y= 0.8157-0.0281x+0.0005x^2$	0.58		
Ca	*A. affinis*	$Y= 4.2029$	–		–
	C. dactylon	$Y= 4.3901-0.0327x$	0.77	4.3796	0.32
	P. notatum	$Y= 2.6758$	–	–	–
	P. plicatulum	$Y= 6.6679-0.1549x+0.0029x^2$	0.71		
Mg	*A. affinis*	$Y= 1.7836$	–	–	–
	C. dactylon	$Y= 0.9713+0.0311x-0.0009 x^2$	0.91	1.2410	17.31
	P. notatum	$Y= 1.8445$	–	–	–
	P. plicatulum	$Y= 3.2898-0.0558x$	0.92	3.2719	0.32

Table S2- Regression equations adjusted among micronutrient contents (Zn, Fe and Mn) in the leaves, stems and roots of species *C. dactylon*, *A. affinis*, *P. notatum* and *P. plicatulum* in response to the addition of increasing Cu doses to the nutrient solution.

Nutrient ($g\ kg^{-1}$)	Species	Equation	R^2	Vmáx	Nmax
		---**Leaves**---			
Zn	*A. affinis*	$Y= 46.0990+0.04861x$	0.88	48.2860	45
	C. dactylon	$Y= 63.2862-0.8318x+0.0175x^2$	0.53		
	P. notatum	$Y= 46.7117$	–		
	P. plicatulum	$Y= 55.7867$	–		
Fe	*A. affinis*	$Y= 96.5808$	–		
	C. dactylon	$Y= 107.8190-0.6501x$	0.89	107.61	0.32
	P. notatum	$Y= 71.5682-1.2049x+0.0185x^2$	0.92		
	P. plicatulum	$Y= 72.0895+0.8295x$	0.64	109.42	45
Mn	*A. affinis*	$Y= 158.3124-2.0138x$	0.98	157.67	0.32
	C. dactylon	$Y= 213.3767$	–		
	P. notatum	$Y= 137.4776-5.4418x+0.1030x^2$	0.67		
	P. plicatulum	$Y= 290.1575-5.5502x+0.1043x^2$	0.68		
		---**Stem**---			
Zn	*A. affinis*	$Y= 3.6876-1.4140x+0.0232x^2$	0.89		
	C. dactylon	$Y= 42.6639-0.2306x$	0.71	42.59	0.32
	P. notatum	$Y= 68.7703-2.2051x+0.0441x^2$	0.99		
	P. plicatulum	$Y= 130.4383$	–		
Fe	*A. affinis*	$Y= 50.8449-0.4721x$	0.84	50.6940	0.32
	C. dactylon	$Y= 28.0792$	–		
	P. notatum	$Y= 42.6092$	–		
	P. plicatulum	$Y= 51.2289+3.1710x-0.0732x^2$	0.68	85.5710	21.66
Mn	*A. affinis*	$Y= 104.8397-3.8078x+0.0512x^2$	0.90		
	C. dactylon	$Y= 39.7350$	–		
	P. notatum	$Y= 88.9176-1.6336x$	0.81	88.3950	0.32
	P. plicatulum	$Y= 293.2558-15.5866x+0.2951x^2$	0.94		
		--**Root**--			
Zn	*A. affinis*	$Y= 52.2117$			
	C. dactylon	$Y= 46.4367$	–		
	P. notatum	$Y= 72.9989-2.4736x+0.0436x^2$	0.91		
	P. plicatulum	$Y= 186.4091-2.7098x$	0.75	185.54	0.32
Fe	*A. affinis*	$Y= 171.3417$	–		
	C. dactylon	$Y= 263.8383$	–		
	P. notatum	$Y=134.5750$	–		
	P. plicatulum	$Y= 371.4658$	–		
Mn	*A. affinis*	$Y= 107.8607-3.5152x+0.0490x^2$	0.95		
	C. dactylon	$Y= 23.3567$	–		
	P. notatum	$Y= 74.4076-3.0814x+0.0487x^2$	0.98		
	P. plicatulum	$Y= 143.0010+2.8886x-0.04383x^2$	0.52	190.59	32.95

4.3 ESTUDO III

Cu toxicity in native and invasive grass species in the Pampa biome: Subcellular distribution, chemical forms and physiological effects of it[3]

Isley C. B. Silva ; Vanessa Marques Soares; Luana Maria L. Alberti; Camila Peligrinotti Tarouco; André Somavilla; Tadeu Luis Tiecher, Edicarla Trentin; Raíssa Schwalbert; Fernando Teixeira Nicoloso; Paulo Ademar Avelar Ferreira; Gustavo Brunetto

Abstract

Despite the high level of soil pollution by Cu in vineyards grown in far Southern Brazil, it is common observing grass species native to the Pampa biome, such as *Axonopus affinis*, *Paspalum notatum* and *Paspalum plicatulum,* as well as the invasive grass species *Cynodon dactylon,* between crop rows. However, information about the tolerance mechanisms to Cu of these species remains scarce. The present study assessed the subcellular distribution pattern and the chemical forms of Cu in species *C. dactylon*, *A. affinis*, *P. notatum* and *P. plicatulum,* as well as the response of their antioxidant system when they are grown under increasing Cu concentrations (0.32, 15, 30 and 45 µM). The study also analyzed the influence of these defense mechanisms on species' tolerance to Cu. Species *P. plicatulum* and *C. Dactylon* recorded the highest indices of tolerance to Cu (1.87 and 1.15, respectively). The high tolerance index shown by *P. plicatulum* seems to be associated with Cu deposition on cell wall and with the formation of phosphate precipitate in root cells. Furthermore, the highest increase in the activity of antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD) in the leaves and roots of *P. plicatulum* was essential to mitigate the ultra-structural damages caused by the greater generation of reactive oxygen species. The high Cu deposition in cell wall of *C. dactylon* plants and the combination between metal and protein, phosphates and oxalates likely play critical role in reducing the toxicity of absorbed Cu. *A. affinis* was the species presenting the lowest index of tolerance to Cu (0.57), most of the absorbed Cu stayed in the cell wall. These strategies, as well as the increased activity of the antioxidant enzymes SOD and POD have contributed to mitigate the impact of Cu excess on the photosynthetic apparatus of this species.

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[3] Artigo elaborado de acordo com as normas de formatação da revista Chemosphere**.**

Introduction

Copper (Cu) is an essential element for plant growth and development. It acts as electron acceptor in several enzymatic systems, some of which are involved in antioxidant processes (Yruela 2009; Adrees et al. 2015). Cu is a plastocyanin component that acts in transporting electrons, either at mitochondrial or chloroplast level (Taiz et al. 2017). However, excessive Cu concentrations in plant tissues can cause toxicity and lead to growth inhibition and, in the most severe cases, to plant death (D. M. Marques, Veroneze Júnior, et al. 2018; Lessandro De Conti et al. 2019; Trentin, Facco, Hammerschmitt, et al. 2019; Cambrollé et al. 2015). The toxic effects of Cu affect different biochemical and physiological processes accountable for impairing water- and nutrient-use efficiency, for decreasing the concentration of photosynthetic pigments and for reducing leaf expansion (Adrees et al. 2015; Petit et al. 2012; Saleem et al. 2020). Given the nature of Cu's active redox, its excessive concentration in plants causes the oxidation of cell biomolecules because it increases the production of reactive oxygen species (ROS), such as superoxide anions ($O2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$), in different plant organs (C. H. R. De Vos and Schat 1991). Girotto et al. (2016) reported that Cu excess has increased H_2O_2 in the leaves of *Avena strigosa*. Similarly, Karimi et al. (2012) reported that Cu excess increased lipid peroxidation in cell membranes in the roots of *Astragalus neo-mobayenii*, and it resulted in considerable accumulation of the by-product malondialdehyde (MDA).

Despite the phytotoxic effects caused by Cu excess, its accumulation in the soil is often reported in regions where viticulture is a traditional activity (Lagomarsino et al. 2010; Mackie et al. 2014; Soja et al. 2018; Coppa et al. 2020). This process derives from the successive application of cupric fungicides on grapevines. Soil enrichment with vineyards grown in far Southern Brazil is also widely reported in the literature (Brunetto et al. 2016; Alcione Miotto et al. 2017; P. A. A. Ferreira et al. 2018). Soil pollution rates due to Cu in the oldest vineyards is already classified as high (I. C. B. da Silva et al. 2020). However, different from what happened in other wine producer regions worldwide, most of soil Cu in these sites is found in the bioavailability fractions (Alcione Miotto et al. 2014), assumingly due to the sand soil texture and to the low contents of organic matter in it (I. C. B. da Silva et al. 2020; Tadeu Luis Tiecher et al. 2013). Therefore, Cu contamination in these sites can lead to grapevines' phytotoxicity and to soil cover plants throughout the years, because they present a more superficial root system. Besides the phytotoxic issues, increased soil Cu level represents a severe environmental problem, since it contaminates the soil, groundwater and surface water through different mechanisms (Santos et al., 2018), as well as compromises the stability of microbial communities (Vázquez-Blanco et al. 2020), affects the botanical composition of the local ecosystem and may lead to increased degree of invasiveness by exotic plant species in these sites (Morgado, Loureiro, and González-alcaraz 2018; I. C. B. da Silva et al. 2020).

Even with the Cu concentrations available reaching values much higher than those observed in natural soils in the oldest vineyards in far Southern Brazil (Alcione Miotto et al. 2017; Girotto et al. 2016), it is possible finding different coverage vegetation comprising species native to the Pampa biome, such as *Paspalum notatum, Paspalum plicatulum,* and also invasive species like *Cynodon dactylon* (I. C. B. da Silva et al. 2020), between rows in these sites. The growth of these species, despite the high concentrations of bioavailable Cu in the soil, is indicative that these plants assumingly have efficient mechanisms to tolerate Cu excess in their tissue. Overall, vegetal species growing in environments presenting high metal concentrations in the soil develop their basic strategies to tolerate the metal, namely: (1) exclusion - plants avoid excessive metal absorption and (2) accumulation and sequester – absorbed metallic ions are complexed with organic binders and partitioned into subcellular structures, such as the vacuole (Hall 2002). Therefore, plants express the extracellular and intracellular detoxification mechanism to reduce the phytotoxicity of free Cu.

Among the intracellular tolerance mechanisms, metal partition into subcellular structures and changes in the chemical forms of Cu play an important role in decreasing Cu phytotoxicity. When the heavy metal is absorbed by the plant, it is selectively distributed among the subcellular partitions and/or immobilized by several substances found in vegetal cells (Xin et al. 2017; Ye et al. 2018; Mwamba et al. 2016b). Consequently, one part of the metal observed by plants is phytotoxic (Van Assche and Clijsters 1987; Ganesan and Konganapuram Chellappan 2018); therefore, somehow, Cu mobility and toxicity are determined by its chemical forms and subcellular distribution. Previous studies have shown that metal deposition in cell wall, vacuole partition or Cu/protein combination, and phosphate and oxalates play critical role in reducing Cu toxicity (Q. Y. Wang, Liu, and Hu 2016; L. Li et al. 2019; Huang et al. 2017). Mwamba et al. (2016) compared Cu subcellular distribution and chemical forms to Cu sensitive and tolerant genotypes of *Brassica napus.* They found that the sensitive genotype presented higher Cu concentrations in its inorganic and complexed forms (with soluble organic acids in water) than the tolerant genotype. On the other hand, as for the tolerant genotype, Cu concentrations integrated to pectates and proteins, sequestered with phosphates and bond to the oxalate, exceeded the values observed for the sensitive genotype. The activation of the antioxidant defense mechanism is another tolerance strategy developed by plants, at intracellular level, to limit Cu toxicity. This system mostly works to stop excessive ROS production by antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), Guaiacol peroxidase (POD), catalase (CAT), and of non-enzymatic binders, such as ascorbic acid, carotenoids, glutathione (GSH) and phenolic compounds (Taiz et al. 2017).

The presence of more robust intracellular tolerance mechanisms in soil-coverage plant species allows them to tolerate larger amounts of Cu in their tissue, without presenting severe toxicity symptoms. Thus, these species are capable of immobilizing larger amounts of Cu in their tissue, and it contributes to decrease Cu concentration in the soil and, consequently, its phytotoxicity potential in other plants, including grapevines. However, so far, information available about the physiological responses involved in Cu toxicity variation and about the detoxification mechanisms used by these species remains scarce. Therefore, the aims of the current study were to (1) identify Cu subcellular distribution patterns and chemical forms in species *C. dactylon, A. affinis, P. notatum* and *P. plicatulum,* as well as the responses of their antioxidant system to Cu excess; and (2) to analyze the influence of these defense mechanisms on these species' tolerance to Cu.

Materials and Methods

Study site

The study was performed in greenhouse at Federal University of Santa Maria, in Santa Maria County, Rio Grande do Sul State, Southern Brazil. Mean temperature throughout the experiment in greenhouse was of 26°C, at 50% relative air humidity.

The tested plant species were *Cynodon dactylon* (bermudagrass), *Paspalum notatum* (Bahiagrass), *Paspalum plicatulum* (Brownseed) and *Axonopus affinis* (Narrowleaf carpet grass). Plants were collected in a 35-year-old vineyard located in Santana do Livramento County (30°46'36" S, 55°22'03" W), Southern Brazil. Copper (Cu) bioavailability in the soil in the collection site, in layer 0-20 cm, was 40.38 mg kg^{-1} (extracted by EDTA). The index of soil pollution by Cu was classified as high (I. C. B. da Silva et al. 2020). The collected species were grown in hydroponic culture in sand, based on protocol by Marques et al. (2020). Every two months, for one year, the vegetative propagation of the species was carried out to broaden the bank of plants and to increase homogeneity among seedlings belonging to each species. The seedlings were irrigated twice a day with complete nutrition solution added with (mg L^{-1}): 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.

Experimental treatments and procedures

Seedlings used in the study were selected, washed, standardized by species (three roots and three fully-expanded leaves were left in each plant) and taken to 5-L vessels filled with nutrition solution by Hoagland and Arnon (1950), with 50% ionic strength – they were allowed to acclimate for 15 days. Eight seedlings were planted in each vessel; they were fixed with the aid of a plastic support (Figure S10).

After acclimation, the nutrition solution was supplemented with different Cu concentrations by using $CuSO_4.5H_2O$ as metal source. Sixteen (16) treatments were assessed in four grass species (*Cynodon dactylon*, *Paspalum notatum*, *Paspalum plicatulum* and *Axonopus affinis*). Each species was grown under four different Cu concentrations: 0.32, 15, 30 and 45 µM. Treatments were delivered based on completely randomized design (CRD), with three repetitions. The nutrition solution was systematically aerated by air pump and completely removed every 3 days throughout the whole experimental period. The pH was adjusted to 5.5±0.2 at all solution replacements, by using NaOH 1 M or HCl 1 M. Four (4) plants from each experimental unit were randomly collected after 15 days. Root and leaf samples were collected and immediately stored in liquid N_2. The collected samples were stored in ultra-freezer at -80°C until laboratory analysis time (Cu cellular distribution and chemical forms; concentration of photosynthetic pigments and biochemical analysis).

Biomass partition and tolerance index

After 15-day exposure to Cu, three plants from each experimental unit were randomly collected, washed in HCl 0.1 M solution and in distilled water, separated into roots and shoot, and dried in forced air circulation oven at 65°C, until reaching constant mass. Shoot and root dry matter (SDM and RDM) were determined and biomass partition was calculated based on equation 1.

$$\text{Biomass partition} = (\text{SDM or RDM}/\text{Total dry matter of the plant})\ \text{x}100 \quad \text{(Equation 1)}$$

Tolerance index (TI) of plants at Cu excess was calculated as the ratio between total dry matter of the plant in the treatment based on Cu excess and the total dry matter of the control (0.32 µM Cu) (Zhou et al. 2017).

Biochemical analysis applied to leaf tissue and roots

Sample preparation for photosynthetic pigment concentration determination in leaves was carried out based on the methodology suggested by Hiscox and Israelstam (1979). Tissue samples (0.05 g) were macerated in liquid N_2 and incubated in 5 mL of dimethylsulfoxide (DMSO), at 65°C, until full pigment removal. Absorbance was determined in the supernatant extract, at wavelengths of 663, 645 and 470 nm, in spectrophotometer (Bel Photonics, 1105, Brasil). Chlorophyll *a*, *b* and total (a+b) and carotenoids' concentrations were estimated based on the formula by Lichtenthaler (1987), expressed in mg g^{-1} FM (fresh matter).

Leaf and root lipid peroxidation was assessed through malondialdehyde concentration determination (MCD), as final product from lipid peroxidation through the reaction to the thiobarbituric acid (TBA) (El-moshaty et al. 1993). The samples (0.5g) were homogenized in 4 mL of 0.2 M citrate-phosphate buffer (pH 6.5) added with 0.5% Triton X-100, at ratio of 1:10 (w/v). Subsequently, they were centrifuged at 20000xg, for 15 minutes, at 4°C. Aliquots of 1.5 mL at supernatant fraction were added to equal TBA volume, equal to 0.5% (w / v) and 20% trichloroacetic acid (TCA) (w / v), and incubated in water bath at 95°C, for 40 minutes, to find the thiobarbituric acid reactive substances (TBARS). The reaction was stopped with ice water bath for 15 minutes and, next, the samples were centrifuged at 10000xg, for 5 min. Then, absorbance was determined at 532 nm, non-specific absorbance was subtracted at 600nm, in spectrophotometer (Bel Photonics, 1105, Brazil). MCD concentration was calculated and results were expressed in nM MDA mg^{-1} protein.

The concentration of hydrogen peroxide (H_2O_2) in the leaves and roots was determined based on Loreto and Velikova (2001). The samples (0.1g) were homogenized in .0 mL of 0.1% of TCA (w/v) and centrifuged at 12000xg, for 15 minutes, at 4°C. Subsequently, 0.5 mL of supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer solution (pH 7.0) and 1 mL of KI 1M. Absorbance was determined in spectrophotometer at wavelength of 390 nm. The concentration of H_2O_2 was expressed in μmol g^{-1} MF.

The activity of enzymes dismutase (SOD) and Guaiacol peroxidase (POD) was determined in previously frozen leaf and root samples. The samples (0.5g) were macerated in liquid N_2 and homogenized in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8), 1 mM EDTA and 1% Triton X-100. The homogenate was centrifuged at 13000xg, for 20 min, at 4 ºC. Supernatant was used in enzyme activity and protein content assays (Zhu et al. 2004). The activity of superoxide dismutase (SOD) was assessed based on the spectrophotometric method described by Giannopolitis and Ries (1977). One SOD unit was defined as the amount of enzyme inhibiting nitroblue tetrazolium (NBT) through 50% photoreduction expressed in U

mg^{-1} protein. The activity of enzyme Guaiacol peroxidase (POD) in the leaves and roots was determined based on the methodology suggested 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). This solution was added with 50 µL of plant extract, after the homogenization. Guaicol oxidation to tetraguaiacol was measured through absorbance increase, at 470 nm.

Subcellular Cu distribution

Subcellular Cu distribution was assessed in leaf and root samples of plants grown under the lowest and highest Cu concentrations in nutrition solution (0.32 and 45 µM of Cu, respectively). Root and leaf samples, in these cases, were separated into four different subcellular fractions (Cu in cell wall, nuclei and plastids, mitochondria and soluble fraction) by using the differential centrifugation techniques suggested by Huang et al. (2017), with some adaptations. Tissue samples (1.5 g of leaves and 1.0 g of roots) were homogenized in buffer solution – previously cooled to 4°C – at ratio of 1:10 (w: v). The used buffer solution comprised 250 mM sucrose, 50 mM Tris-HCl (pH 7.5) and 1.0 mM dithioerythritol (DTE). The homogenate was centrifuged at 300×g, for 5 min, at 4°C. The *pellets* deposited on the bottom of the tube were labeled as Fraction 1 (Fi) - they represented the cell wall. The supernatant was transferred to a new tube and centrifuged at 15000×g, for 30 minutes. The resulting deposit was labeled as mitochondrial fraction (Fiii), whereas the supernatant was labeled as soluble fraction and named fraction 4 (Fiv). The whole fractionation process was carried out at 4°C. Subsequently, the fractioned material was dried in oven at 70°C until reaching constant mass and, then, it was subjected to nitroperchloric digestion (3:1, v/v). The Cu concentration in different fractions were quantified in atomic absorption spectrophotometer (EAA; Varian SpectrAA-600, Australia).

Chemical forms of Cu

The chemical forms of Cu were also determined in the leaves and roots of plants grown under the lowest and highest Cu concentrations in the nutrition solution (0.32 and 45 µM of Cu, respectively). The chemical forms of Cu in leaf tissue and roots were determined based on the methodology suggested by Zhao et al. (2015), with adaptations proposed by Xin et al. (2017). Five extracting solutions were used at the following order: (i) 80% ethanol, to extract inorganic Cu, including nitrate, chloride and aminophenol (Fi); (ii) deionized water, to extract Cu bond to complexes of water-soluble organic acids (Fii); (iii) NaCl 1 M, to extract Cu integrated to pectates and proteins (Fiii); (iv) 2% acetic acid, to extract Cu bond to phosphate (Fiv) and (v) HCl 0.6 M, to extract Cu bond to oxalate (Fv).

Samples (1.0 g of leaves and 0.5 g of roots) were homogenized in 80% ethanol at ratio of 1: 10 (w: v) – the resulting homogenate was stirred for 22 hours, at 25°C. Next, the homogenate material was centrifuged at 5000×g, for 10 min. The resulting supernatant was reserved in a tube and the sediment was re-suspended two more times, by using the same extracting solution – it was stirred for 2h, at 25°C, at every new suspension, and centrifuged at 5000xg, for 10 min. The supernatants of the three centrifugations – with the same extracting solution – were gathered in a common tube. The pellet material was subjected to the same extraction procedure by using the other extracting solutions, according to the indicated order. The supernatants extracted with each solution and residual fraction (Fvi) were evaporated at 70°C until reaching constant mass. They were subsequently subjected to nitroperchloric digestion (3:1, v/v). The Cu concentration in each fraction was determined in the obtained acidic extract in atomic absorption spectrophotometer (EAA; Varian SpectrAA-600, Australia).

Statistical analysis

Results were subjected to analysis of variance and when the effect of the treatments was significant in the F test ($p \leq 0.05$) the samples were compared through Scott-Knott test ($p \leq 0.05$). Variance analysis and the test of means recorded for fata concerning subcellular distribution and the chemical forms of Cu were carried out by taking into account the two-factor scheme. The principal factor, in these cases, was “plant species grown under concentrations of 0.32 and 45 µM of Cu”, which totaled 8 combinations; the secondary factor was “Cu chemical forms (6 forms)” or “subcellular Cu fractions (4 fractions)”. Analysis of variance and the test of the means were carried out by taking into account the 16 treatments resulting from each species under the four Cu concentrations in nutrition solution (0.32, 15, 30 and 45 µM of Cu), in order to assess the other variables. The SISVAR software (D. F. Ferreira 2011) was used to the conduction of all statistical analyses.

Results

Biomass partition and tolerance index

The increased Cu concentration in the growth medium has had different effects on the development of the tested species. The highest total dry biomass productions were observed in s *P. plicatulum* (doses of 15 and 30 µM Cu), *A. affinis* (0.32 and 15 µM Cu) and *C. dactylon* (30 µM Cu) (Figure 1a). *C. dactylon* and *P. notatum* presented maximum dry matter production when they were cultivated in nutrition solution added with 30 µM Cu (Figure 1a). Biomass production in the root system of *C. dactylon* increased by approximately 18% under this Cu concentration in comparison to plants grown in control solution (0.32 µM) (Figure 1c). The total dry mass production observed in *P. notatum* mainly resulted in shoot biomass production increase (Figure 1b). Similarly, *P. plicatulum* presented increased total dry matter production when it was grown under Cu concentration higher than 0.32 µM. The greatest *P. plicatulum* development was observed when it was grown in 15 µM Cu, since it increased total dry matter production by more than 3.5 times in comparison to plants grown in control solution. Different from the other species, *A. affinis* growth was much reduced after its cultivation under increasing Cu doses. Its dry matter production was minimal when it was grown at 30 µM of Cu; its root biomass contribution was also much lower.

The greatest tolerance recorded for species *C. dactylon* and *P. plicatulum* in environments contaminated with Cu was evidenced by their high tolerance indices (TI), mainly when these plants were grown under the highest Cu concentrations in nutrition solution, 45 µM of Cu (Table S1). *C. dactylon* and *P. Plicatulum* recorded IT 2 and 3 times higher than that recorded for *A. Affinis* (the species recording the lowest IT under all tested concentrations) (Table S1) under this very same treatment, 45 µM of Cu.

Photosynthetic pigments

The exposure of grass species to the evaluated Cu levels has affected the concentration of photosynthetic pigments, as well as the associations observed between chlorophyll *a* and *b*, and between carotenoids and chlorophylls. Overall, *C. dactylon* presented the highest concentrations of photosynthetic pigments, even when it was grown under the highest Cu concentrations in nutrition solution (45 µM of Cu) (Figure 2).

C. dactylon and *P. plicatulum* grown under the highest Cu concentrations, 45 µM, presented the maximal reduction in the content of chlorophyll *a* and *b*, and of carotenoids. The concentrations of chlorophyll *a* and *b*, and carotenoids in the leaves of *C. dactylon* decreased to 31%, 43% and 28%, respectively, in comparison to the control treatment, 0.32 µM. The concentration of chlorophyll *a* dropped to 74%, that of chlorophyll *b* decreased to 59% and the content of carotenoids reached 67% in *P. plicatulum.* Different from what was observed for the above cited grasses, the concentration of photosynthetic pigments in the leaves of *A. affinis* increased when it was grown under concentrations of 15, 30 and 45 µM of Cu, respectively. The maximal chlorophyll *a* and *b*, and carotenoid concentrations in *P. notatum* have undergone small changes when it was grown under concentrations of 15, 30 45 µM Cu.

Oxidative stress

Increase in Cu concentration in the growth medium accounted for oxidative stress in the leaves and roots of all species, as well as affected the H_2O_2 and MCD concentrations in plant leaves and roots (Figure 3). H_2O_2 increase in *C. dactylon* and *A. Affinis* was mainly observed in the leaves (Figure 3a), whereas *P. notatum* and *P. plicatulum* presented the highest rise in H_2O_2 concentrations in root tissue (Figure 3b). Exposure to Cu had impact on lipid peroxidation, which was measured based on the MCD content (Figures 3c and 3d). This effect was mainly highlighted in plants' root system. The highest increase in lipid peroxidation was observed in *C. dactylon* and the lowest one in *A. Affinis:* 40.74 and 75.43 nM MCD mg^{-1} protein in *C. dactylon,* and 40.46 and 44.20 nM MCD mg^{-1} protein in *A. affinis,* when they were grown in nutrition solution added with 0.32 and 45 µM of Cu, respectively.

Activity of antioxidant enzymes

SOD activity is naturally higher in species *P. notatum* and *P. plicatulum* (Figures 4a and 4b). SOD activity in the leaves and roots of *C. dactylon* and *P. plicatulum* increased when these species were grown in nutrition solution with Cu concentration higher than 0.32 µM (Figures 4a and 4b). SOD activity reached its maximum rate in both organs of these species in the treatment with 30 µM of Cu. The increased SOD activity in *A. affinis* and *P. notatum* was mostly observed in the leaves, which reached its peak when they were grown under the highest Cu concentration, 45 µM.

The activity of enzyme POD in the leaves of *P. notatum* and *P. plicatulum* is also naturally higher than that in the other species. It was significantly changed due to Cu concentration in the nutrition solution (Figure 4c) and reached its peak when these species were grown in 30 µM of Cu. The POD activity in the roots of *C. dactylon* and *P. plicatulum* reached its maximum rate when these plants were grown in solution added with 32µM of Cu (Figure 4d). Different from the other species, the POD activity in the roots of *P. notatum* decreased when it was grown at concentrations higher than 0.32 µM of Cu in the nutrition solution (Figure 4d).

Subcellular Cu distribution

Increase in Cu concentration in the nutrition solution from 0.32 to 45 µM of Cu also increased Cu concentration in different subcellular fractions of leaf and root tissue in the tested grass species (Table 1). Cu concentration in cell wall (Fi), plastids and nucleus (Fii), mitochondria (Fiii) and soluble fraction (Fiv) of the roots of *C. dactylon* increased 28.5, 2.2, 1.3, 25.8 times, respectively, in comparison to the concentrations of 0.32 and 45 µM of Cu. These values corresponded to 20.9, 2.9, 2.1 and 39.1 times increase in Fi, Fii, Fiii and Fiv, respectively, in species *A. Affinis.* The Cu concentrations in these fractions increased 22.6, 7.1, 2.7, 35.2 times in the roots of *P. notatum* and 28.9, 4.1, 1.8 and 25.7 times in the roots of *P. plicatulum*.

Copper (Cu) increase in the subcellular fractions in the leaves of all grass species was mostly observed in fractions Fi, Fii and Fiv (Table 1). Cu concentration in these three fractions increased 1.8, 1.9 and 1.8 times in the leaves of *C. dactylon,* and 2.7, 2.3 and 2.1 times in the leaves of *A. affinis,* respectively. Cu concentration in Fi, Fii and Fiv in the leaves of *P. Notatum* increased 1.4, 2.1 and 2.7 times, respectively. Cu concentration in the leaves of *P. plicatulum* increased 2.3 times in Fi, 2.3 times in Fii and 4.4 times in Fiv. Such a trend of change in Cu concentrations in the different subcellular fractions has shown that the species *C. dactylon* was the one recording the lowest Cu increase in the most sensitive organelles (plastids, nuclei and mitochondria). On the other hand, the highest increase in Cu in these organelles was observed in the roots of *P. notatum* and in the leaves of *A. affinis*. The maximum concentrations of Cu in cell walls were observed in the root tissue of *C. dactylon* and *P. plicatulum*, and in the leaves of *A. affinis*.

The mean rate of Cu (between the two plant tissues – leaf and root) was observed in plastids, nucleus and mitochondria (6.72%) in *C. dactylon,* 11.76% in *A. affinis*, 12.96% in *P. notatum* and 10.63% in *P. plicatulum,* after the exposure to 45 µM of Cu in nutrition solution. With respect to Cu ratio in cell wall after plant cultivation under high Cu concentration, it was possible observing that the 50.74% Cu absorbed by *C. dactylon* was removed from the fractions. The ratio of Cu withheld in cell wall in *A. affinis*, *P. notatum* and *P. plicatulum* reached 39.48%, 38.59% and 42.25%, respectively. As for the amount of Cu in the soluble fractions, it was possible seeing that species *C. dactylon, A. affinis* and *P. plicatulum* concentrated similar amounts of Cu in this fraction of the root system after they were cultivated in solution added with 45 µM of Cu - 61.15 mg kg^{-1} of FM, on average (Table 1). On the other hand, the highest Cu concentration in the leaves in the soluble fraction was observed in *P. plicatulum.* It was observed that, among all organelles, Cu accumulated in the leaves and roots of all plants, mainly in platinum and nuclei (Fii), in comparison to mitochondria (Fiii) (Figure 5).

Thus, the cultivation of plants under the highest Cu concentration made it possible observing that the general metal distribution pattern in subcellular fractions has changed due to the tested plant species and also to the assessed organ. Cu distribution pattern in the roots, in the subcellular fractions, followed the order Fi>Fiv>Fii=Fiii in *C. dactylon*; Fiv>Fi>Fii=Fiii in

A. affiins; Fiv>Fi>Fii>Fiii in *P. notatum*; and Fi>Fiv>Fii>Fiii in *P. plicatulum.* Cu distribution pattern in the leaves followed the order Fi>Fiv>Fii=Fiii in *C.dactylon*; Fi>Fiv>Fii>Fiii in *A. affinis*; Fi=Fiv>Fii>Fiii in *P. notatum*; and Fiv>Fi>Fii>Fiii in *P. plicatulum*.

Chemical forms of Cu

Concentrations of different chemical forms of Cu in plant leaves and roots were affected by Cu level in the cultivation medium, by plant species and plant organ (Table 2). In most cases, Cu concentration in each one of its chemical forms has increased as Cu concentration in the medium also increased, except for the residual Cu, which did not face any significant change in leaves and roots. Regardless of plant species, the increase observed in Cu concentration, in all its chemical forms, was always higher in the root system than in the leaves.

The most toxic Cu form observed in the roots was found in the inorganic fraction (Fi) and in the water-soluble fraction (Fii) - species *A. affinis* presented the highest increase. Cu concentration in the roots of this species increased from 0.64 to 31.50 mg kg^{-1}FM in fraction Fi; and from 0.0 to 42.07 mg kg^{-1}FM in fraction Fii (Table 2). Despite the remarkable amounts of Cu, in its active forms (Fi and Fii), in all grass species, mainly when they were exposed to the highest level of this metal (45 µM), most Cu in the root system was concentrated in the least active forms of it: Fiii (bond to pectates and proteins), Fiv (bond to phosphate) and Fv (bond to oxalate). The largest amount of Cu, in its least active forms, in the root system was mainly evident in roots of species *C. dactylon* and *P.plicatulum*, which maintained approximately 65% and 70% of Cu in these fractions, respectively (Figure 6). Cu accumulation, in its least toxic forms and in its forms presenting the lowest migration ability, mainly happened in the fraction bond to phosphate (Fiv), which was followed by the fraction integrated to the pectates and proteins (Fiii). These outcomes seem to indicate the active involvement of phosphate (Fiv) in Cu toxicity relief. Pectates and proteins (found in Fiii) and the oxalates (Fiv) can also contribute to neutralize the intracellular activity of Cu.

Different from the distribution observed in the root system, after the exposure to the highest Cu concentration in the solution, the leaves of *A. affinis*, *P. notatum* and *P. plicatulum* kept most of the absorbed Cu - in its toxic forms – in Fi and Fii. The maximum Cu increase in these fractions was observed in the leaves of *P. Plicatulum* in comparison to the control treatment (0.32 µM). It increased from 0.937 to 4.677 mg kg^{-1}FM in fraction Fi; and from 0.0 to 0.487 mg kg^{-1}FM in fraction Fii (Table 2). Cu concentration, in its most active forms, in the leaves of *C. dactylon* also significantly increased; it increased 2.47 times in Fi and 9.1 times

Fii. However, most absorbed Cu by this species remained in its least toxic form in Fiii, Fiv and Fv (Figure 6).

Discussion

Cu is an essential element for plant growth and development, however its excessive concentration in the root growth medium can have severe toxic effect on plants (D. M. Marques, Veroneze Júnior, et al. 2018; Lessandro De Conti et al. 2019). Biomass production inhibition and reduction are visible and common responses of several species when they are grown under stressing Cu level (Q. Li et al. 2019; Trentin, Facco, Hammerschmitt, et al. 2019; Lessandro De Conti et al. 2019). However, the intensity plants respond to such a feature is highly specific, since it depends on their tolerance to this metal (Adrees et al. 2015; D. M. Marques, Veroneze Júnior, et al. 2018; Lessandro De Conti et al. 2018). The production of total dry matter by *C. dactylon*, *P. notatum* and *P. plicatulum* in the present study reached its maximum rate when they were grown in nutrition solution at intermediate Cu concentrations, 15 or 30 µM of Cu (Figure 1). Although growth in these species only recorded negative effect when they were grown under the highest Cu concentration, 45 µM, ultra-structural damages in the cellular system was triggered by its excess – it started being observed at intermediate concentrations, 15 and 30 µM of Cu (Figure 3). Besides the growth inhibition in *C. dactylon, P. notatum* and *P. plicatulum,* which was induced by Cu concentration increase in the nutrition solution, it was possible observing increase in H_2O_2 concentration in the leaves of *dactylon* and *P. notatum* and in the roots of *P. notatum* and *P. plicatulum* (Figures 3a and 3b). The increase in lipid peroxidation in these three species was mainly observed in the roots; this is another strong indicative of oxidative damage in the structure of the root system (Figures 3c and 3d). The highest H_2O_2 concentration, as well as the concentration of other reactive oxygen species (ROS), such as superoxide anions ($O_2 \bullet^-$) and hydroxyl radicals (HO ●), cause oxidation in cells' biomolecules. Lipid peroxidation of the membranes is one of the main observed damaging effects, since it results in lower membrane selectivity and subsequent cell content leak and cell death (P. Sharma et al. 2012). Lipid peroxidation occurrence was confirmed by increase in MCD content in cells in the root system of these species (Figure 3d). MCD is the product of both lipid peroxidation and extended damage in the membrane system (Z. Xiao et al. 2020; Ayala, Muñoz, and Argüelles 2014; P. Sharma et al. 2012). Therefore, assumingly, the intensification of these damages in the root system was one of the factors accountable for the significant reduction in the biomass production by these grass species when they were grown in solution added with 45 µM of Cu.

The reduced biomass production in *C. dactylon* and *P. plicatulum* may have also resulted from the decreased concentrations of photosynthetic pigments: chlorophyll *a* and *b*, and carotenoids (Figures 2a, 2b and 2d) and from photosynthesis inhibition (Silva et al, data not published). Cu excess inhibits pigment accumulation and delays chlorophyll integration to the photosystems (Caspi et al. 1999; Petit et al. 2012). Furthermore, it inhibits the synthesis of the chlorophyll precursor, as well as δ-aminolaevulinic acid, and the activity of protochlorophyllide reductase (enzyme that catalyzes the reductive formation of chlorophyllide from protochlorophyllide during chlorophyll biosynthesis) (Stiborová, Ditrichová, and BŘEzinová 1987; Petit et al. 2012). Reduction in the photosynthetic pigment contents in treatments based on the addition of heavy metals may have been caused by other factors, such as oxidative break of the pigment, destruction of thylakoid membranes and abnormal absorption of nutrients (Pan et al. 2019b).

The toxic effect of Cu on biomass production in *A. Affinis* seem to be more intense than on the other grass species (Figure 1, Table S1). Similar to what was observed in other grass species, the decreased total dry matter in *A. affinis* may have resulted from oxidative stress, as indicated by increase in H_2O_2 concentration in the leaves and by the NCD content mainly observed in the root system (Figures 3a and 3d). Besides, as highlighted in our previous study, the lowest biomass production recorded for *A. affinis* can be associated with morphological changes in the root system, as well as with severe nutritional disturbances and, consequently, with reduced efficiency to use nutrients when this species is grown under stressing Cu concentration in the growth medium (Silva et al, data not published).

Different from species *C. dactylon* and *P. plicatulum*, increase in Cu concentration in the growth medium has led to increased concentrations of photosynthetic pigments in the leaves of *A. affinis* (Figures 2a, 2b and 2d). Increase in the concentrations of chlorophyll *a* and *b*, and carotenoids (Figure 2) has contributed to maintain the high photosynthetic ability of *A. affinis* (Silva et al, data not published), even when it was grown under high Cu concentrations. However, it was not enough to keep its high growth rate (Silva et al, data not published) and biomass production, due to the lowest growth of this organ. Furthermore, part of the necessary energy to keep biomass production in species *A. affinis* may have been used for Cu partition processes in less sensitive subcellular structures, such as cell wall and vacuoles; and/or for ROS elimination processes and for the consequent protection of important systems in cells, such as the photosynthetic apparatus (Bazihizina et al. 2015; Calow 1991; Hall 2002).

Despite the oxidative damages caused by Cu excess and its effects on important cell structures and processes, such as photosynthesis and membrane permeability, plants have several biochemical defense mechanisms at cell level, including the antioxidant defense system (enzymatic, and non-enzymatic) to protect them from oxidative damage (C. H. R. De Vos and Schat 1991; Taiz et al. 2017). According to the present study, the activity of protective antioxidant enzymes, such as SOD and POD, which help keeping balance between ROS production and removal (P. A. A. Ferreira et al. 2018; Z. Xiao et al. 2020; L. Li et al. 2019), has increased (Figure 4). This significant increase in SOD activity in the roots of *C. dactylon*, *A. affinis* and *P. plicatulum*, and in the leaves of *P. notatum* and *P. plicatulum* gradually increased in order to remove H_2O_2, which was generated by SOD and later on converted into H_2O and O_2 by POD, and mitigated cell damages. This finding indicates that SOD and POD play important roles in grass species tolerance to stress caused by Cu excess. Although these antioxidant enzymes are fundamental for the cell detoxification process, the increase in MCD, mainly observed in the roots of grass species grown under the highest Cu concentration (Figure 3d), indicates that the cleaning ability of antioxidant enzymes is limited. ROS excess has attacked the unsaturated fat acids in cell membrane, mainly in the roots of *C. dactylon* and *P. plicatulum*, and weakened the cell membrane structure, a fact that can increase membrane permeability and favor cell leakage (P. Sharma et al. 2012; Z. Xiao et al. 2020).

Similar to the antioxidant system, Cu subcellular partition represents an important tolerance mechanism of plants against this metal. This mechanism involves the removal of toxic metals from the metabolic active cell areas and their storage in less active partitions, since it allows greater plant adaptation to stress caused by Cu excess (Xin et al. 2017; Zhao et al. 2015). The analysis of Cu concentration in different cell components indicated that cell wall was the prevalent location for metal accumulation in the roots and leaves of *C. dactylon*. This finding suggests that the cell wall played key role in this species’ tolerance to Cu. On the other hand, there were large Cu ratios in the soluble fraction, in the leaves of *P. plicatulum* and in the roots of *A. affinis* and *P. notatum* (Figura 5). It suggests that the vacuole in the soluble fraction, mainly in *A. affinis* and *P. notatum*, is the site for the accumulation of excessively absorbed Cu. The present results are in compliance with some previous studies that have shown cell wall and vacuole as the prevailing sites for Cu storage (Wang et al. 2016; Mwamba et al. 2016; Huang et al. 2017; Li et al. 2019).

Cell wall is rich in polysaccharides, such as cellulose, hemicellulose and pectin, and proteins that provide anionic structures due to the presence of ionizable groups such as carboxyls, hydroxyls, phosphates, amino and aldehydes, which are capable of participating in the adsorption process, as well as in ion exchange, and metal complexation and precipitation (Fu et al. 2011; J. Wang et al. 2015). Thus, cell wall acts as first protection barrier for cell protoplast, which limits Cu entrance in the cell, and its move to the shoot (X. Wang et al. 2008; Mwamba et al. 2016b; Huang et al. 2017). The vacuole, as well as cell wall, represent an important location for the accumulation of excessive metals. The vacuole is a huge dynamic organelle found in most plant cells; it acts as central storage partition (Wink 1993). The presence of sulfur-rich peptides and organic acids in the vacuoles ensures Cu complexation and, therefore, reduces the activity of the free ion and metal toxicity. Assumingly, after Cu concentration reaches a certain level in nutrition solution, there may be saturation in the exchange locations in cell wall, and it likely increases Cu concentration in other organelles and damage to cellular homeostasis.

Besides Cu subcellular distribution, Cu degree of mobility and toxicity in plants also depend on its chemical forms inside the cell (Dučić and Polle 2005). According to Mwamba et al. (2016) and Wang et al. (2008), overall, this heavy metal, in its inorganic and organic forms (Fractions i and ii), is more capable of migrating and toxic to plant cells in comparison to the metal integrated to pectates and proteins (fraction iii), to that bond to phosphates (fraction iv) and to oxalates (fraction v). With respect to the present study, most Cu in the roots of *C. dactylon* and *P. plicatulum* was integrated to pectates and proteins, phosphates and oxalates; this finding is indicative that these species are lesser capable of transporting Cu, which is an important mechanism to tolerate this metal (Huang et al. 2017; L. Li et al. 2019). Mwamba et al. (2016) also found that keeping Cu in its non-dissolved phosphate form and in its forms bond to pectates and proteins, can be the main strategy to reduce Cu mobility and toxicity in *Brassica napus*. Toxicity reduction in the leaves of *C. dactylon* mainly happened through ligations to peptide and phosphate binders (Table 2). Previous studies have shown that insoluble phosphates of Cu are mainly found in cell wall and vacuoles, whereas Cu integrated to pectates and proteins are often found in vacuoles (Q. Y. Wang, Liu, and Hu 2016; Zeng et al. 2011). Therefore, the chemical forms of Cu observed in the herein assessed grass species quite correspond to subcellular Cu distribution results.

In comparison to the other grass species, *C. dactylon* presented the lowest Cu concentration in its mobile forms (Fractions i and ii) either in the root or leaves, and relatively higher rate of Cu integrated to pectates and proteins, and of Cu bond to phosphates, in both tissues. This finding suggests that *C. dactylon* can adopt a more efficient way to reduce Cu mobility and toxicity, as well as that it can be more tolerant to Cu than the other species.

Finally, the analysis of grass species regarding their intracellular tolerance mechanisms has indicated that Cu deposition in cell wall and the formation of precipitate with phosphate in root cells were essential to reduce Cu toxicity and mobility in *P. plicatulum.* Thus, it avoided excessive Cu accumulation in plant shoot, where the harming effects of it are much more significant. Despite the reduction in Cu entering to the inside of root cells, and the consequent reduction in mobility to the shoot, the highest increase in the activity of antioxidant enzymes SOD and POD in the leaves and roots of *P. plicatulum* was essential to mitigate the ultra-structural damages caused by the greatest ROS generation. All these strategies have contributed to the high TI rates in *P. plicatulum* (Table S1). On the other hand, the high TI value recorded for *C. dactylon* seems to be mainly associated with lower Cu absorption by plants (Silva et al, data not published), and it indicates that this species is more capable of avoiding Cu absorption. This process can possibly explain extracellular tolerance mechanisms, such as the release of root exudates. This process leads to changes in the pH of the rhizosphere environment and to increase in dissolved organic carbon, which changes the solubility, activity and distribution of Cu chemical species in the solution (Meier et al., 2012; Li et al., 2013; De Conti et al., 2015) and reduces its absorption. Besides the high Cu concentration conditions in the growth medium (45 µM), the highest production of Cu absorbed by *C. dactylon* was removed from the cell wall, either in the roots or leaves, as well as its least toxic chemical forms, such as Cu integrated to pectates and proteins, and bond to phosphate and oxalate (Figure 6). *A. affinis,* which was the species recording the lowest TI, showed high Cu concentration, either in the root system and in the shoot (Silva et al, data not published). Most of the absorbed Cu inside this plant was removed from the soluble fraction of root cells, which is mainly composed of vacuoles, (Table 1, Figure 5), and it mitigated this metal's translocation to the shoot. On the other hand, most of the translocated Cu in the leaves of *A. affinis* was removed from cell wall. These strategies helped mitigating the impact of Cu excess on the photosynthetic apparatus of this species. Although changes in the activity of antioxidant enzymes SOD and POD in the leaves and roots of *A. affinis* was lower than that in *P. plicatulum,* this strategy was also important to keep balance in the association between ROS production and elimination, mainly in roots, in order to mitigate oxidative damage. The combined use of all these detoxification strategies have contributed to protect the photosynthetic apparatus and the consequent maintenance of high photosynthetic activity in plants.

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Tables

Table 1. Concentrations of copper (Cu) in subcellular fractions in the leaves and roots of species *C. dactylon, A. affinis, P. notatum and P. plicatulum*.

Tissue	**Species**	**Cu concentration (µM)**	**Cu concentration (mg kg^{-1} FW)**							
			Cell wall		**Plastids and nucleus**		**Mitochondria**		**Soluble fraction**	
Leaf	*C.dactylon*	0.32	1.060	dA[1]	0.090	bC	0.090	aC	0.823	dB
		45	1.923	aA	0.170	bC	0.100	aC	1.487	bB
	A.affinis	0.32	0.557	fA	0.153	bB	0.093	aB	0.557	eA
		45	1.493	cA	0.353	aC	0.153	aD	1.193	cB
	P.notatum	0.32	0.977	dA	0.127	bC	0.093	aC	0.527	eB
		45	1.403	cA	0.270	aB	0.100	aC	1.437	bA
	P.plicatulum	0.32	0.710	eA	0.147	bC	0.093	aC	0.513	eB
		45	1.610	bB	0.333	aC	0.170	aD	2.263	aA
Root	*C.dactylon*	0.32	2.330	dA	0.523	dA	0.173	bA	1.817	cA
		45	66.400	aA	5.177	cC	3.067	aC	60.227	bB
	A.affinis	0.32	1.573	dA	0.520	dA	0.233	bA	1.537	bA
		45	32.990	cB	4.533	cC	3.307	aC	61.603	bA
	P.notatum	0.32	2.273	dA	0.413	dA	0.240	bA	1.753	cA
		45	51.390	bB	16.040	aC	6.077	aD	80.037	aA
	P.plicatulum	0.32	2.393	dA	0.733	dA	0.323	bA	2.770	cA
		45	69.163	aA	9.917	bC	4.243	aD	61.630	bB

[1] Means followed by the same lowercase letter in the column and by the same capital letter on the line did not significantly differed from each other in the Scott-Knot test ($p \leq 0.05$)

Table 2. Concentration of different chemical forms of copper (Cu) in the leaves and roots of species *C. dactylon*, *A. affinis*, *P. notatum and P. plicatulum.*

Tissue	**Species**	**Cu concentration (µM)**	**Cu concentration (mg kg^{-1} FW)**					
			Fi[2]	**Fii**	**Fiii**	**Fiv**	**Fv**	**Fvi**
Leaf	*C.dactylon*	0.32	0.693 gC[1]	0.030 cE	1.707 cA	1.103 aB	0.380 dD	0.023 aE
		45	1.717 dA	0.273 bC	1.720 cA	1.007 aB	0.370 dC	0.000 aD
	A.affinis	0.32	0.800 gB	0.017 cD	1.403 dA	0.237 dC	0.280 dC	0.000 aD
		45	3.443 bA	0.193 bE	1.907 bB	0.400 cD	0.647 bC	0.077 aF
	P.notatum	0.32	1.127 eB	0.000 cE	1.777 cA	0.267 dD	0.380 dC	0.030 aE
		45	2.603 cA	0.190 bD	1.403 cB	0.197 dD	0.523 cC	0.007 aE
	P.plicatulum	0.32	0.937 fB	0.000 cD	1.847 bA	0.270 dC	0.350 dC	0.000 aD
		45	4.677 aA	0.487 aE	2.627 aB	0.607 bD	0.770 aC	0.053 aF
Root	*C.dactylon*	0.32	1.983 eA	0.003 dA	2.100 cA	0.060 eA	0.140 cA	0.000 aA
		45	14.133 dB	9.317 cB	10.527 bB	30.907 dA	2.137 bD	0.033 aD
	A.affinis	0.32	0.640 eA	0.000 dA	2.113 cA	0.800 eA	0.060 cA	0.000 aA
		45	31.500 cC	42.073 aB	17.160 aD	54.220 cA	2.863 bE	0.163 aE
	P.notatum	0.32	3.417 eA	0.000 dA	1.890 cA	1.523 eA	0.190 cA	0.000 aA
		45	88.620 aB	36.947 aC	17.690 aD	100.407 bA	9.453 aE	0.317 aF
	P.plicatulum	0.32	4.320 eA	0.270 dA	1.877 cA	1.140 eA	0.453 cA	0.050 aA
		45	42.400 bB	18.817 bC	15.707 aC	119.083 aA	6.357 aD	0.640 aE

[1] Means followed by the same lowercase letter in the column and the same capital letter on the line did not significantly differ from each other in the Scott-Knot test ($p \le 0.05$). [2] Fi, Fii, Fiii, Fiv, Fv and Fvi represented inorganic Cu, Cu bond to complexes of water-soluble organic acids, Cu integrated pectates and proteins, Cu sequestered with phosphate, Cu bond to oxalate and Fvi represents the residual fraction.

Figures

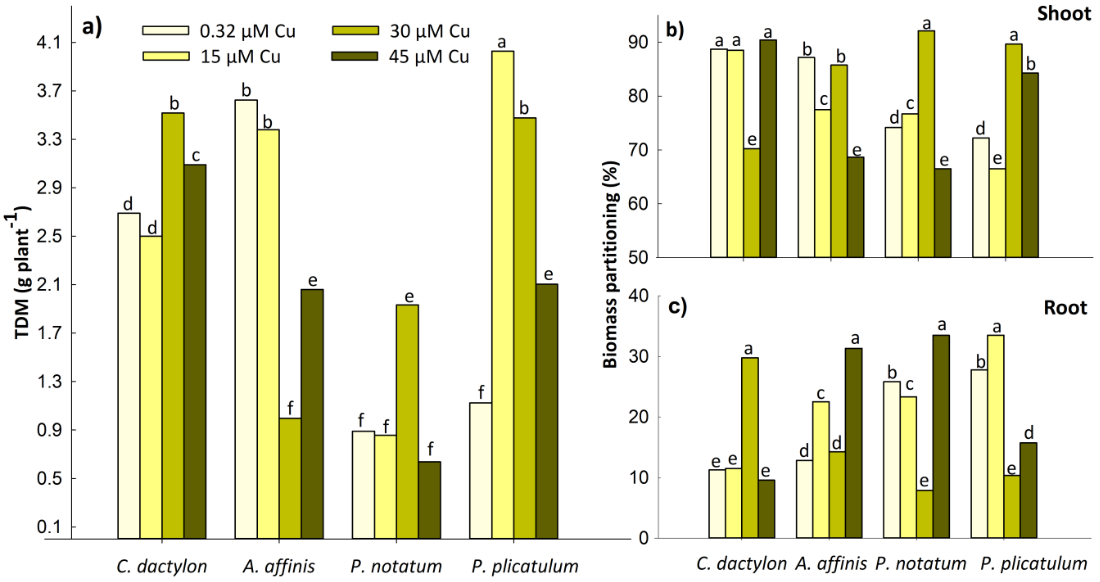

Figure 1. Total dry matter (TDM) (a) and biomass partitioning between shoot (b) and roots (c) of species *C. dactylon*, *A. affinis*, *P. notatum* and P. *plicatulum*. The same letters represent no difference in the Scott-Knott test ($p<0.05$).

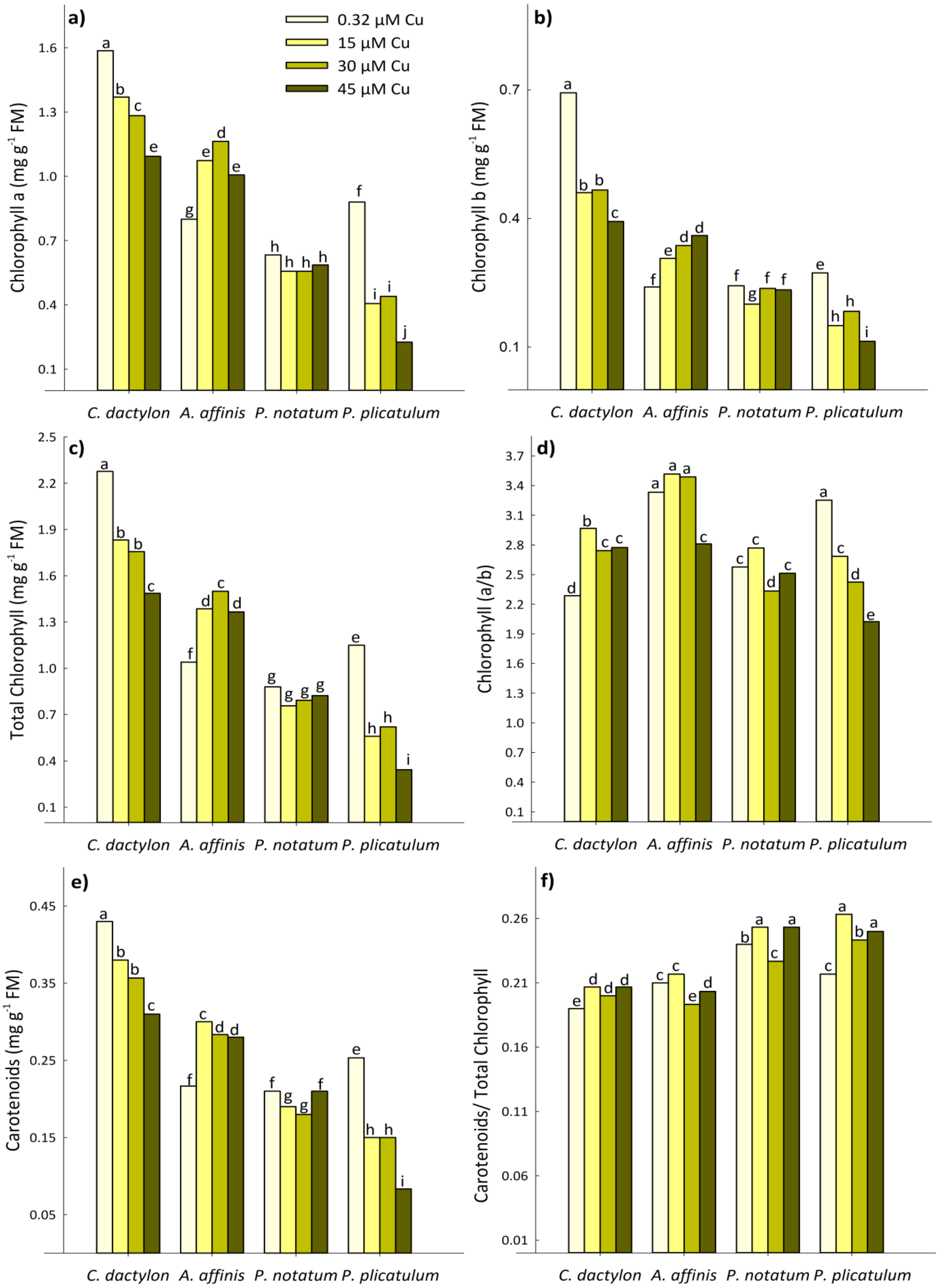

Figure 2. Concentrations of Chlorophyll a (a), Chlorophyll b (b), Total chlorophyll (c), Chlorophyll a / b (d) ratio, Carotenoids (e) and Carotenoids / Total chlorophyll (f) ratio in the leaves of species *C. dactylon*, *A. affinis*, *P. notatum* and P. *plicatulum*. The same letters represent no difference in the Scott-Knott test ($p<0.05$).

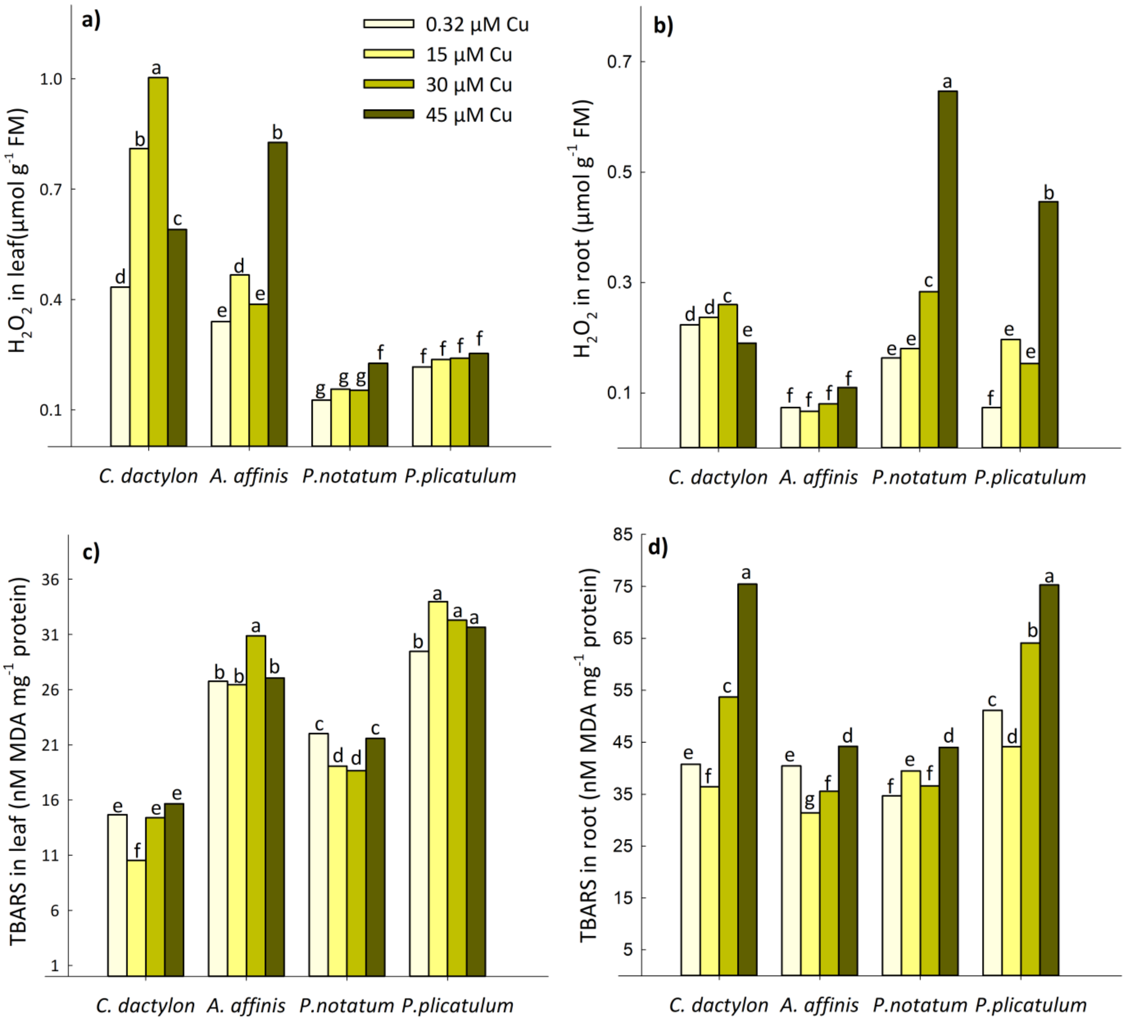

Figure 3. H_2O_2 concentration in the leaves (a) and roots (b); and MCD in the leaves (c) and roots (d) of species *C. dactylon, A. affinis, P. notatum* and P. *plicatulum.*

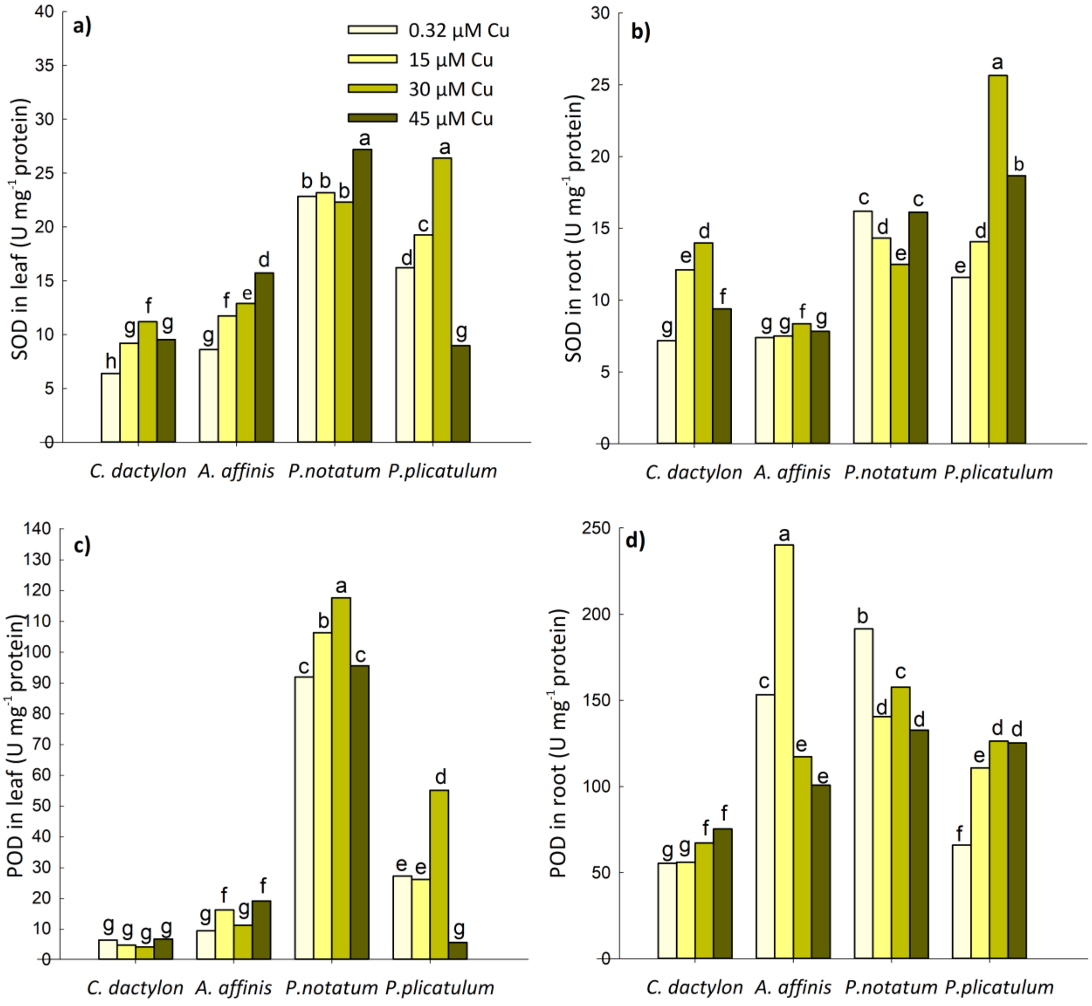

Figure 4. Activity of enzymes superoxide dismutase (SOD) in the leaves (a) and roots (b) and peroxidases (POD) in the leaves (c) and roots (d) of species *C. dactylon, A. affinis, P. notatum* and *P. plicatulum*.

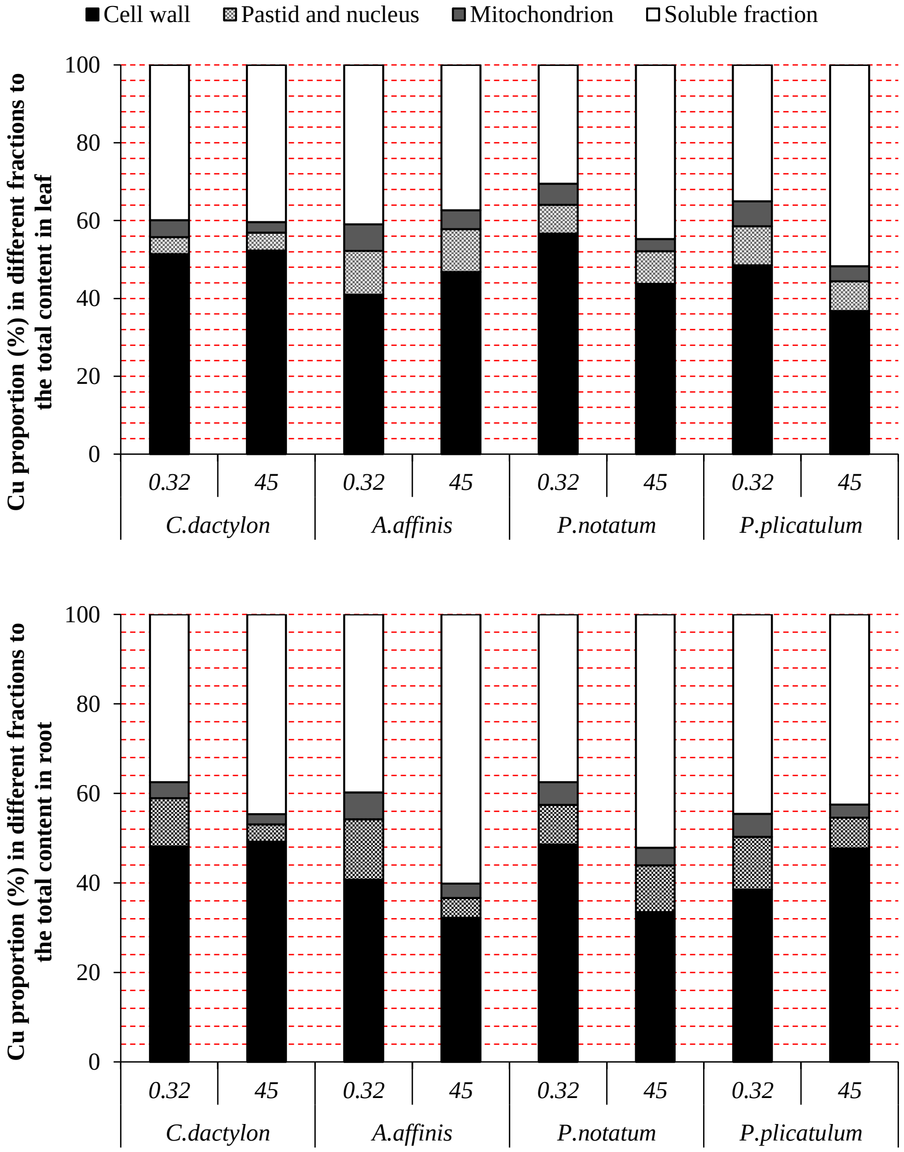

Figure 5. Mean ratios of copper (Cu) in each subcellular fraction in the leaves and roots of species *C. dactylon*, *A. affinis*, *P. notatum* and P. *plicatulum.*

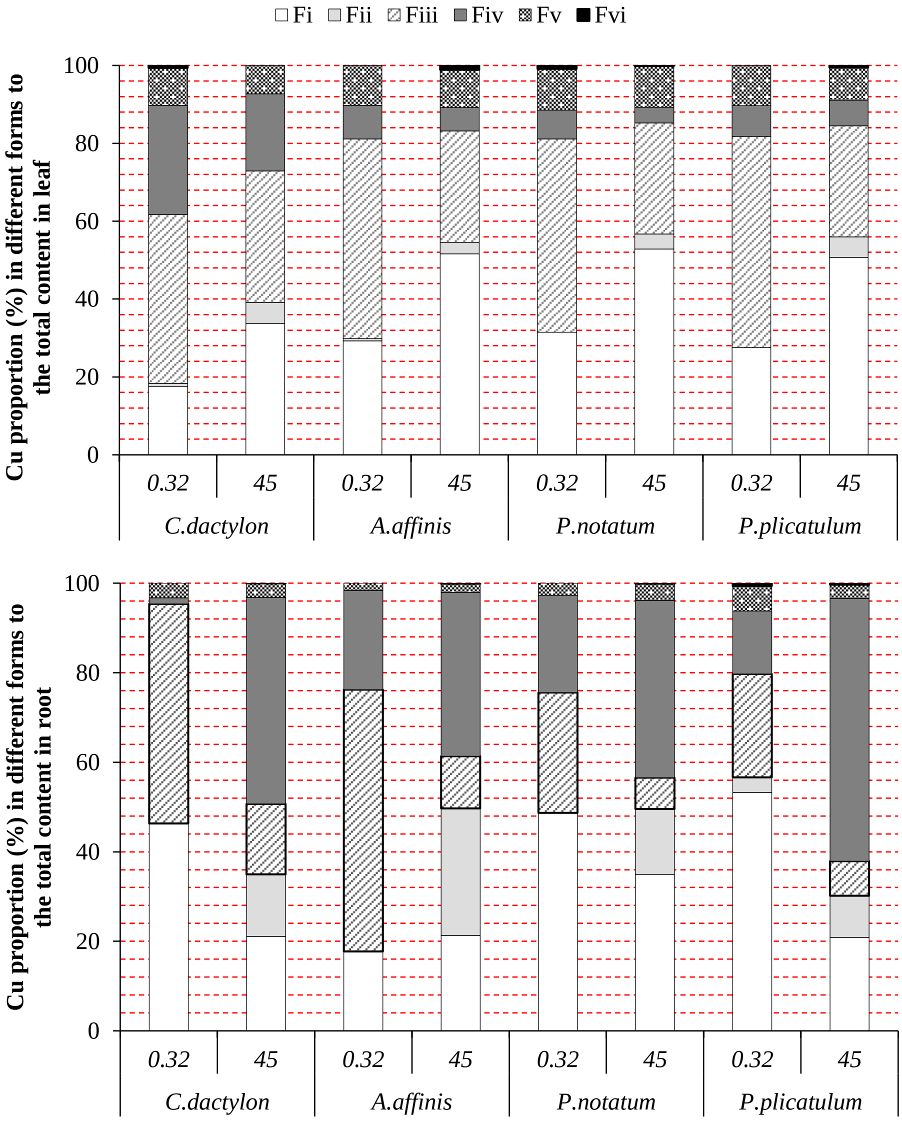

Figure 6. Distribution copper (Cu) chemical forms in the leaves and roots of species *C. dactylon*, *A. affinis*, *P. notatum* and *P. plicatulum*. Fi, Fii, Fiii, Fiv, Fv represent inorganic Cu, Cu bond to the complexes of water-soluble organic acids, Cu integrated to pectates and proteins, Cu sequestered with phosphate, Cu bond to oxalate and Fvi represents the residual fraction.

Supplementary material

Figure S1 – Overview of the initial layout of experimental units on greenhouse countertops. Emphasis on the continuous aeration of the nutrient solution with the aid of air pumps

Table S1 Cu tolerance index of *C. dactylon*, *A. affinis*, *P. notatum* and *P. plicatulum*, grown under increasing concentrations of Cu in nutrient solution.

	C. dactylon	*A. affinis*	*P. notatum*	*P.plicatulum*
15 µM Cu	0.93	0.93	0.96	3.58
30 µM Cu	1.31	0.28	2.17	3.09
45 µM Cu	1.15	0.57	0.72	1.87

4.4 ESTUDO IV

Potential phytoremediation of Pampa biome native and invasive grass species cohabiting vineyards contaminated with Cu in Southern Brazil [4]

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Abstract

The phytoremediation of soils contaminated with heavy metals, such as copper (Cu), depends on the proper selection of plants capable of surviving in such an environment. Thus, 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. *Cynodon dactylon* presented the best performance in soil contaminated with 35 mg of Cu kg^{-1}. Increased Cu concentration in *C. dactylon* tissues has led to increased chlorophyll b and carotenoid concentrations, as well as to increased photosynthetic and plant growth rates. However, the activity of antioxidant enzymes such as SOD and POD remained unchanged. 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. The low Cu translocation-to-shoot factor observed in all species has indicated that these plants have low potential to be used in Cu phytoextraction processes. High values recorded for Cu bioaccumulation-in-grass-root factor (BCF), mainly in *P. plicatulum*, have indicated that the investigated plants are potential phytostabilizers. The greater *C. dactylon* ability to produce

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[4] Artigo elaborado de acordo com as normas de formatação da revista Environmental Science and Pollution Research

biomass makes it more efficient in phytoremediation processes than other species. 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.

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. 2018a; 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; 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).

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).

$$\text{GR}\left(\text{g.month}^{-1}\right)=\frac{\text{FM}_f\ (\text{g}) - \text{FM}_i\ (\text{g})}{\text{T (months)}} \qquad \text{(Equation 1)}$$

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).

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 (Oliva et al. 2010; Houri et al. 2020). 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.

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Tables

Table 1- Chemical characteristics 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 carbono (%)	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

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

Figures

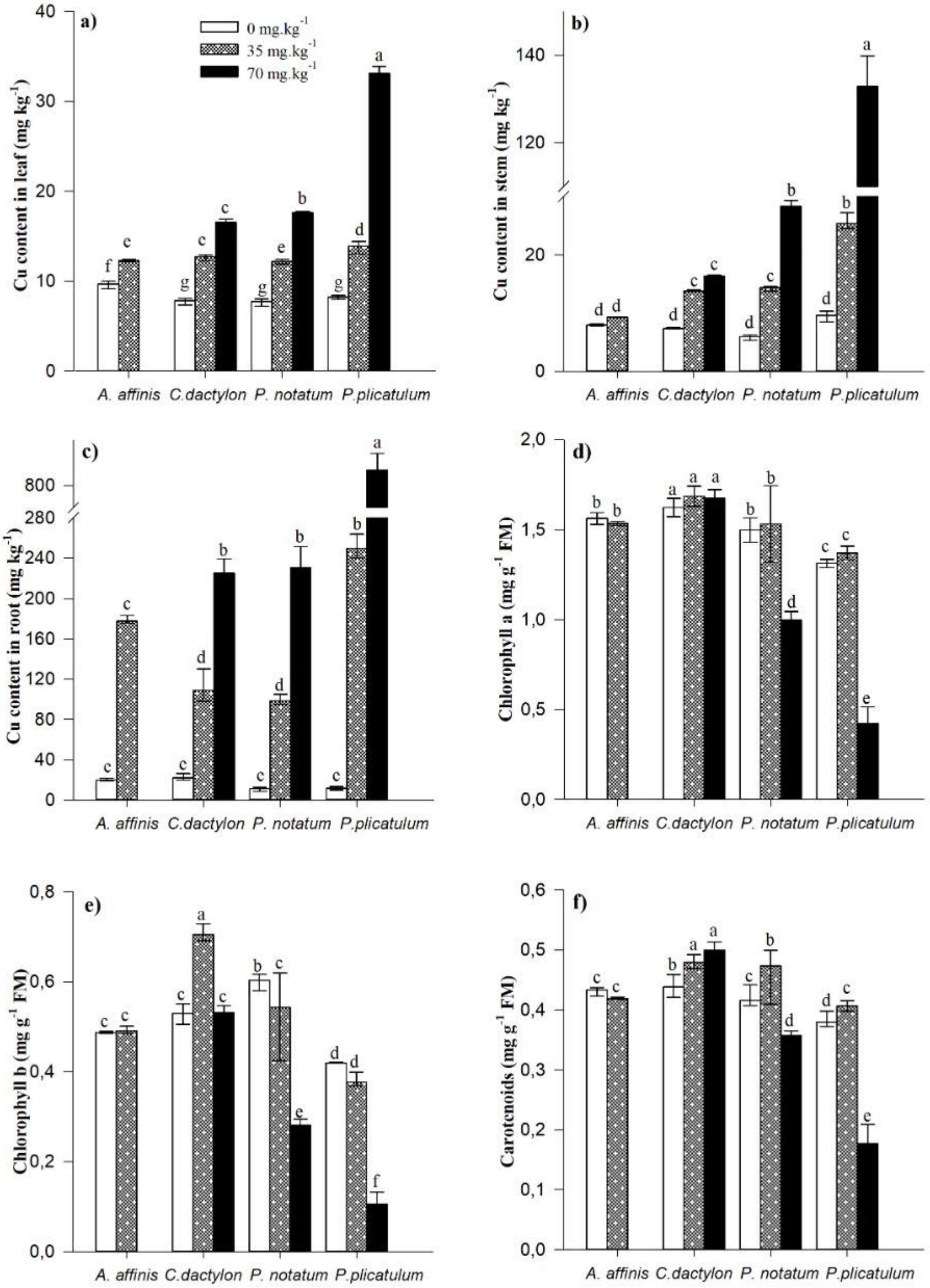

Figura 1 - Copper concentration in leaves (a), stem (b) and roots (c); Chlorophyll a (d), Chlorophyll b (e) and carotenoid (f) concentration in A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with 0, 35 and 70 mg kg-1 of Cu. Equal letters mean that values did not differ from each other in the Scott-Knott test ($p<0.05$).

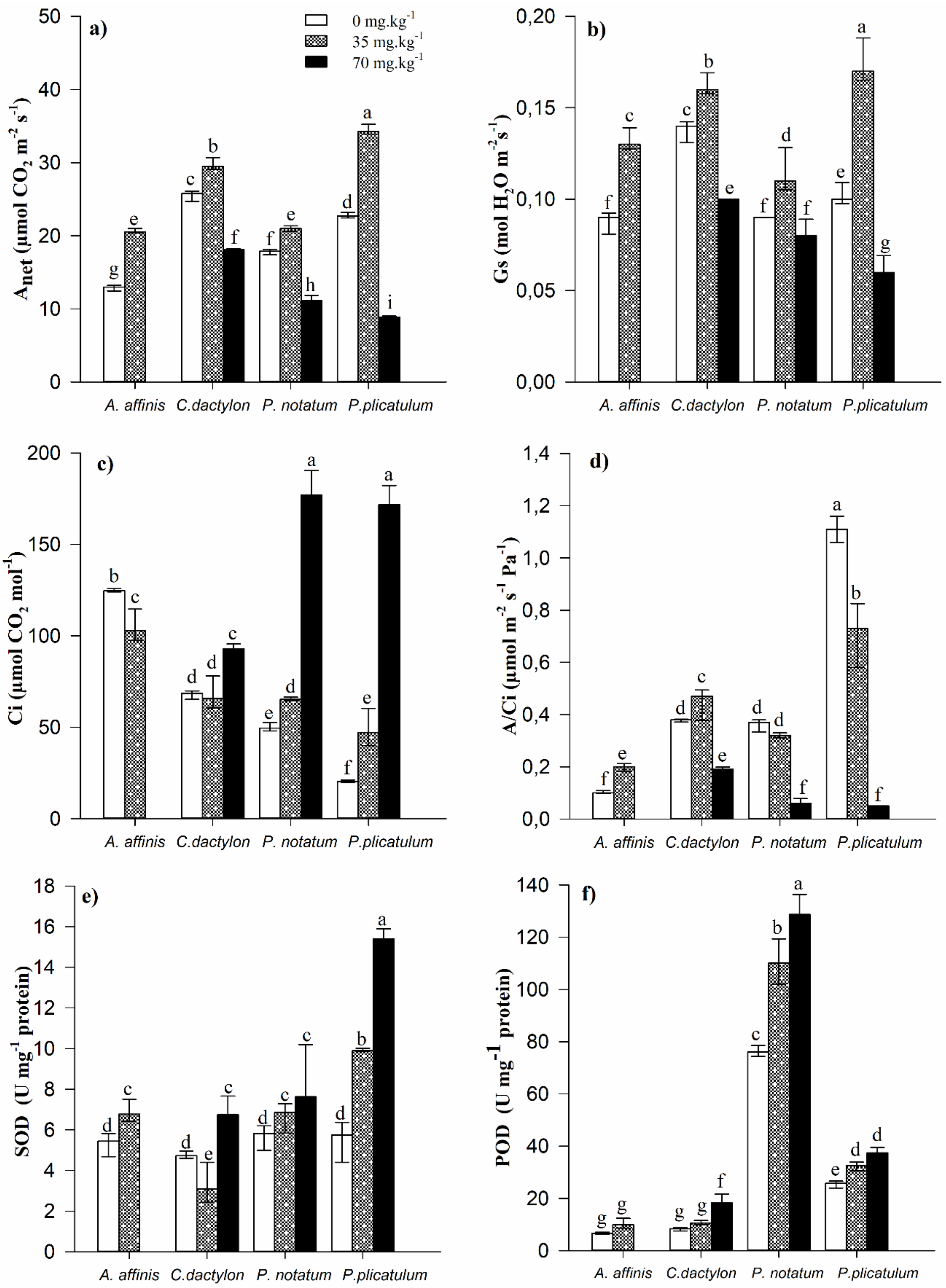

Figura 2 - Net photosynthetic rate (a), Stomatal conductance (b), Intercellular CO2 concentration (c), Instant carboxylation efficiency (d); and SOD (e) and POD (f) enzyme activity in the leaves of A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with 0, 35 and 70 mg kg-1 of Cu. Equal letters mean that values did not differ from each other in the Scott-Knott test ($p < 0.05$).

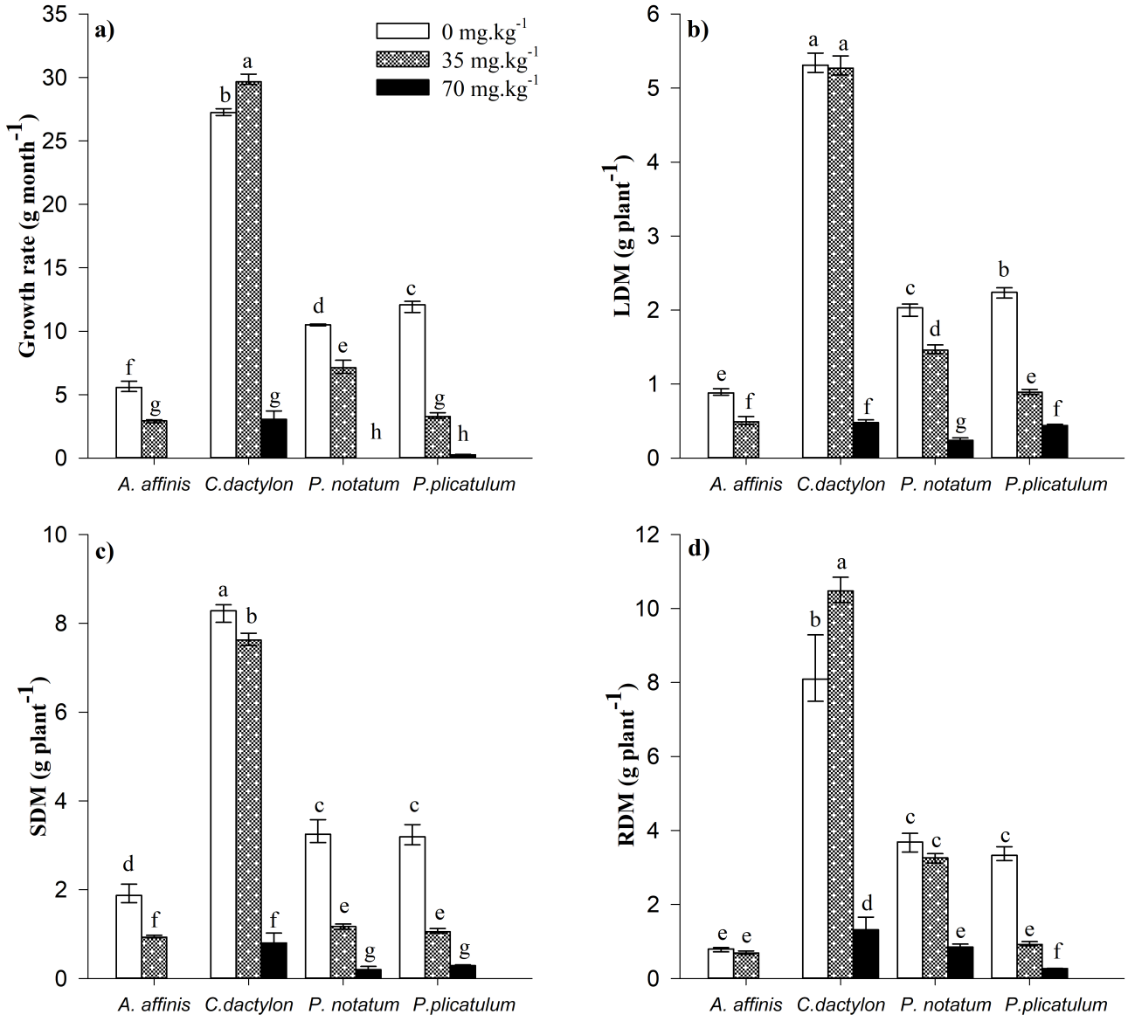

Figura 3 - Growth rate (a), and leaf (b), stem (c) and root (d) dry matter production of A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with 0, 35 and 70 mg kg-1 of Cu. Equal letters mean that values did not differ from each other in the Scott-Knott test (p <0.05).

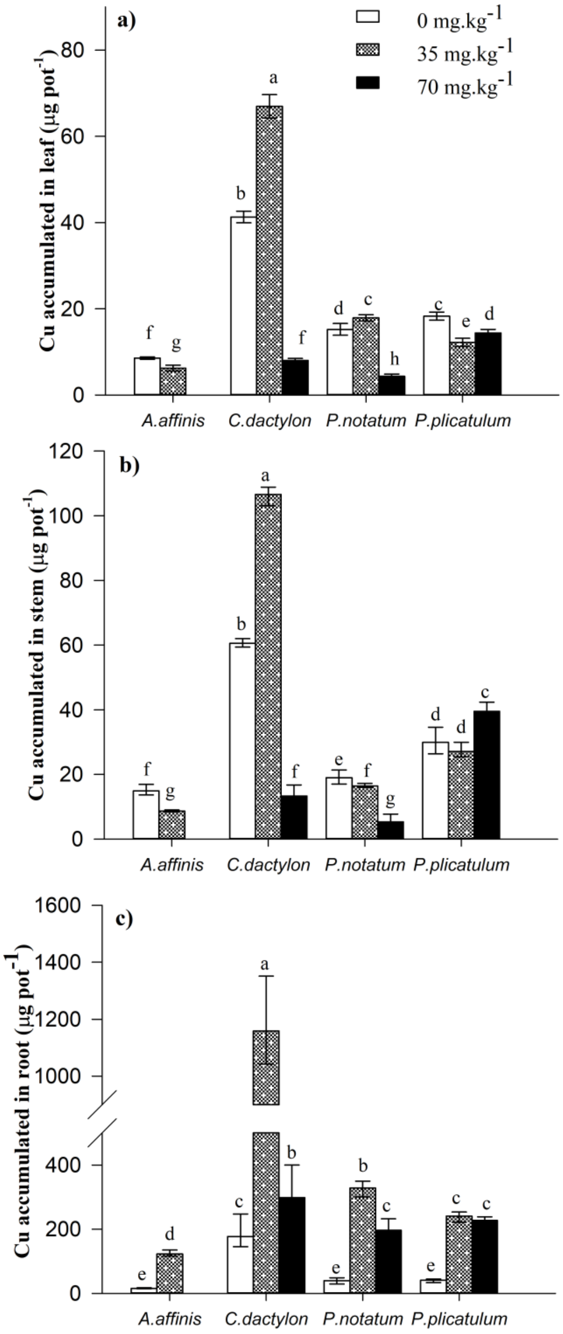

Figura 4 – Cu accumulation in the leaves (a), stem (b) and roots (c) of A. affinis, C. dactylon, P. notatum and P. plicatulum plants grown in soil contaminated with 0, 35 and 70 mg kg- 1 of Cu. Equal letters mean that values did not differ from each other in the Scott-Knott test (p <0.05).

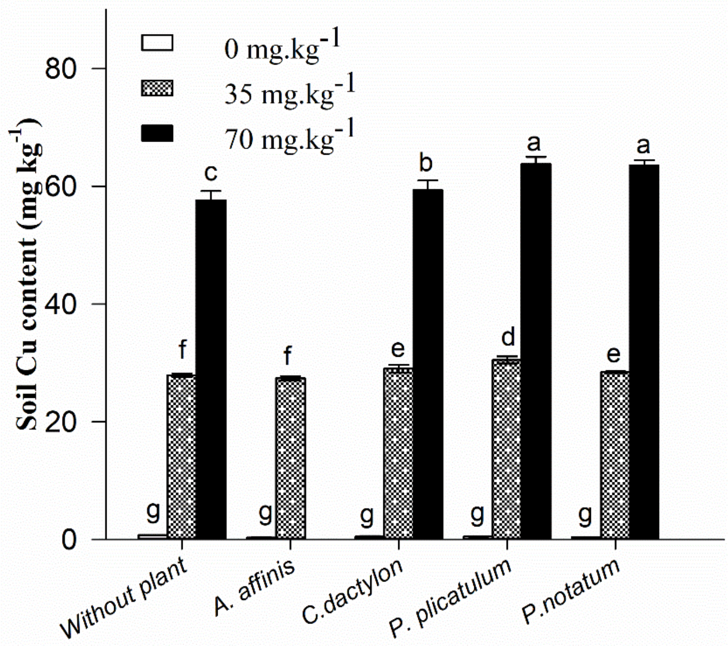

Figura 5 – Mean Cu content in the soil after the cultivation of species A. affinis, C. dactylon, P. notatum and P. plicatulum, and in non-cultivated soil (without plant), based on the applied Cu concentrations. Equal letters mean that values did not differ from each other in the Scott-Knott test ($p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

5. DISCUSSÃO GERAL

Neste trabalho buscou-se compreender a relação entre o aumento do acúmulo de Cu em solos de vinhedos, a dinâmica de espécies nativas do bioma Pampa, a invasibilidade das áreas por espécies exóticas, bem como o potencial das espécies que coabitam os vinhedos em atuar como fitorremediadoras de solos contaminados com Cu. Para isso, primeiramente realizou-se um levantamento botânico nas entrelinhas de três vinhedos com histórico de uso de fungicidas a bases de Cu, e também em uma área de campo natural adjacente aos vinhedos, que foi utilizada como referência. Em seguida, identificamos alguns dos mecanismos de tolerância ao Cu utilizados pelas principais espécies que coabitam vinhedos contaminado com Cu; e realizamos um estudo detalhado dos efeitos do excesso de Cu sobre as respostas morfofisiológicas e nutricionais dessas espécies.

A aplicação de fungicidas a base de Cu em vinhedos, ao longo dos anos, conduz ao enriquecimento do solo com o metal, podendo posteriormente resultar na contaminação do solo. Esse fenômeno é observado em vinhedos de todo o Mundo e amplamente relado na literatura (IÑIGO *et al.*, 2020; LAGOMARSINO *et al.*, 2010; MIOTTO *et al.*, 2017; SOJA *et al.*, 2018). Nos vinhedos da Campanha Gaúcha, localizadas no extremo sul do Brasil, essa realidade não é diferente. Porém, os solos de vinhedos da Campanha possuem a particularidade de serem arenosos e possuirem baixos teores de matéria orgânica. Além disso, estão localizados em um dos mais importantes biomas do Mundo, o bioma Pampa, onde uma grande diversidade de espécies são observadas. O acúmulo de Cu no solo é observado na linha de cultivo, mas também nas entrelinhas da cultura, onde predominam espécies herbáceas nativas do bioma Pampa, como *Paspalum notatum, Paspalum plicatulum, Ageratum conyzoides, Desmodium incanum*; e também exóticas, como *Cynodon dactylon* (Estudo I). Em vinhedos mais antigos, como VN3 (implantado em 1981), o índice de poluição do solo por Cu já é classificado como elevado. Mais de 70% do Cu total do solo encontra-se na fração considerada potencialmente disponível para as plantas. A elevada disponibilidade de Cu nas entrelinhas dos vinhedos provavelmente se deve a combinação de propriedades física e química observadas nos solos das áreas experimentais, como baixos teores de matéria orgânica, textura arenosa e baixa capacidade de troca catiônica. Embora a concentração de Cu seja a propriedade que sofre maior alteração no solo com o passar dos anos de implantação do vinhedo, observa-se também o incremento de outros elementos químicos no solo, como P, K, Ca e Zn, o que ocorre possivelmente em função das práticas de manejo adotadas nos vinhedos, como adubação ou aplicação de outros agroquímicos, como fungicidas a base de Zn.

Assim como as características químicas do solo, a composição botânica das áreas experimentais também foi alterada. Em área de campo natural, onde a concentração de Cu no solo é de 1,43 $mg.kg^{-1}$, gramíneas nativas do bioma Pampa, como *Paspalum notatum* e *Axonopus argentinus* dominam a paisagem. Enquanto que espécies das famílias Fabaceae, Rubiaceae e Asteraceae estão mais associadas aos vinhedos, especialmente aos mais antigos. Além disso, a presença de espécies invasoras, como *C. dactylon*, foi restrita as áreas de vinhedos. Em vinhedos mais antigos, como VN2 e VN3, essa espécie contribui com mais de 15% de toda biomassa produzida nos locais. Provavelmente, a concentração de Cu do solo não é o único fator que explica a variabilidade espacial da comunidade de plantas nas áreas avaliadas. Fatores como a melhoria da fertilidade do solo podem ter sido determinantes para a variação da composição botânica observada nas áreas avaliadas. A maior disponibilidade de nutrientes, que ocorre nos vinhedos, pode beneficiar espécies com maior capacidade de responder à fertilidade do solo, como é o caso de espécies da família Fabaceae, que em geral tem o seu estabelecimento favorecido em áreas com maior suprimento de P, K e Ca (CARLEN; DARBELLAY; GEX, 1998; GAUJOUR *et al.*, 2012).

Nos vinhedos, especialmente naqueles onde a aplicação de fungicidas a base de Cu ocorre por períodos mais longos, observou-se também o incremento das concentrações de Cu na biomassa produzida nas entrelinhas da cultura. A concentração de Cu nessa vegetação depende não apenas da concentração de Cu no solo, mas também das espécies que compõem a comunidade vegetal. Entre as espécies que mais contribuem para a produção de biomassa nas áreas experimentais, *A. conyzoides* e *P. plicatulum* destacaram-se por apresentarem as maiores concentrações de Cu na parte aérea, especialmente quando coletadas em VN2. *P. notatum* e *C. dactylon*, embora apresentem concentrações de Cu inferiores as verificadas na parte aérea de *A. conyzoides* e *P. plicatulum*, destacaram-se pela ampla distribuição nas áreas avaliadas apesar das altas concentrações de Cu no solo. Assim, as espécies nativas do bioma Pampa *P. plicatulum*, *P. notatum* e *A. affinis*; e a espécie exótica *C. dactylon* foram selecionadas para os estudos subsequentes, onde avaliou-se os efeitos do excesso de Cu sobre as respostas morfofisiológicas e nutricionais dessas espécies e identificou-se alguns dos mecanismos de tolerância ao Cu utilizados por elas. Por fim avaliou-se o potencial dessas plantas em atuarem como fitorremediadoras de solos contaminados com Cu.

Elevadas concentrações de Cu no meio de crescimento são tóxicas para a maiorias das plantas. Neste trabalho, o incremento nas concentrações de H_2O_2 nas folhas e raízes das gramíneas e a peroxidação lipídica, ocorrida principalmente nas raízes das plantas, indicam estresse oxidativo em função das altas concentrações de Cu nos tecidos (Estudo III). A maior concentração de H_2O_2, assim como de outras espécies reativas de oxigênio (ERO), como ânions superóxido ($O_2^{\bullet -}$) e radicais hidroxil ($HO^{\bullet}$), causa a oxidação de biomoléculas das células; e a peroxidação lipídica das membranas é um dos principais efeitos danosos observados, resultando em menor seletividade da membrana, com posterior extravasamento do conteúdo celular e morte da célula (SHARMA *et al.*, 2012).

Em geral, a exposição ao excesso de Cu também induz a remodelação da morfologia radicular (DE CONTI *et al.*, 2019; LEQUEUX *et al.*, 2010; MARQUES *et al.*, 2018; TRENTIN *et al.*, 2019). No estudo II, alterações no comprimento radicular, área superficial, diâmetro médio e volume da raiz foram comuns em todas as espécies quando cultivadas nos maiores níveis de Cu. A absorção excessiva de Cu afeta a capacidade de diferenciação celular no sistema radicular das plantas, ocasionando o engrossamento e encurtamento do ápice radicular, bem como o escurecimento das raízes, e aparecimento de raízes secundárias (AMBROSINI et al., 2015; 2018; GUIMARÃES et al., 2016). Segundo Song *et al.* (2017) a inibição do crescimento das raízes, em plantas expostas a altas concentrações de metais pesados, se deve a redução tanto da zona de alongamento como da zona meristemática, e esse papel inibitório é mediado, pelo menos em parte, pela modulação da biossíntese, sensibilidade, transporte ou interações da auxina com outros fito-hormônios.

As alterações no sistema radicular, ocasionadas pela absorção excessiva de Cu, afetam profundamente a capacidade das plantas em absorver água e nutrientes. Além disso, a concentração excessiva de Cu no ambiente de crescimento radicular compete com outros nutrientes no processo de absorção e transporte nas plantas, indicando a presença de transportadores comuns (SAĞLAM *et al.*, 2015; VATANSEVER; OZYIGIT; FILIZ, 2017), isso ajuda a explicar a alteração do equilíbrio nutricional das gramíneas, observada no estudo II.

O incremento de Cu ocorrido nos órgãos aéreos das plantas, ainda que muito inferior ao incremento verificado no sistema radicular, foi suficiente para provocar danos no aparato fotossintético (Estudos II, III e IV). O cultivo das gramíneas sob os maiores níveis de Cu inibiu a taxa fotossintética e prejudicou a assimilação de carbono (Estudos II e IV). A diminuição da taxa fotossintética pode estar associada as alterações nas concentrações de pigmentos fotossintéticos, clorofila *a*, clorofila *b* e carotenóides, nas folhas das gramíneas. As maiores reduções nas concentrações de pigmentos fotossintéticos foram verificadas em *P. plicatulum* (Estudos III e IV). A diminuição da taxa fotossintética pode ser resultado ainda das alterações da composição lipídica e pigmentária dos tilacoides, o que causa diminuição da atividade fotoquímica do PSII (SAĞLAM *et al.*, 2015; TRENTIN *et al.*, 2019; XU *et al.*, 2013). Além disso, uma limitação da eficiência de carboxilação da rubisco não pode ser descartada, já que a toxicidade do Cu parece estar associada à redução da concentração da enzima rubisco e/ou a diminuição da sua atividade (MATEOS-NARANJO *et al.*, 2015a; SAĞLAM *et al.*, 2015).

Vários mecanismos contribuem para a aumentar a tolerância das plantas ao metal pesado. Para combater a produção excessiva de ERO e minimizar os seus efeitos deletérios, as plantas possuem mecanismos de defesa antioxidante, que incluem sistemas enzimáticos e não enzimáticos. O sistema enzimático é composto por enzimas, como superóxido dismutase (SOD), catalase (CAT), ascorbato peroxidase (APX), guaiacol peroxidase (POD), glutationa redutase (GR), monodehidroascorbato redutase (MDHAR) e desidroascorbato redutase (DHAR). Enquanto que o sistema não enzimático inclui compostos como ácido ascórbico (AA), glutationa reduzida, α-tocoferol, carotenóides, flavonóides e osmólito prolina (SINGH; PRASAD; SINGH, 2016). Nos estudos III e IV, a atividade das enzimas antioxidantes SOD e POD foram aumentadas. O aumento na atividade da enzima SOD nas raízes de *C. dactylon*, *A. affinis* e *P. plicatulum* (Estudo III) e nas folhas das quatro gramíneas (Estudos III e IV) resultaram na remoção de $O_2^{\bullet -}$. A atividade de POD nas raízes de *C. dactylon*, *A. affinis* e *P. plicatulum* (Estudo III) e nas folhas de *P. notatum* e *P. plicatulum* (Estudos III e IV) aumentaram gradualmente para remover o H_2O_2, o qual foi gerado pela SOD e posteriormente convertido em H_2O e O_2 pela POD, mitigando os danos celulares (VENKATESWARLU *et al.*, 2012).

Outros mecanismos de defesa, como exclusão do metal pesado, imobilização na parede celular, complexação e compartimentalização vacuolar, estão envolvidos na tolerância das plantas ao metal. Nas gramíneas avaliadas, verificou-se que a parede celular e o vacúolo são os locais predominantes no armazenamento de Cu (Estudo III). As paredes celulares são compostas principalmente de polissacarídeos, como celulose, hemicelulose e pectina e proteínas que fornecem estruturas aniônicas devido à presença de grupos ionizáveis como carboxilas, hidroxilas, fosfatos, amino e aldeídos capazes de participar de processos de adsorção, troca iônica, complexação e precipatação do metal (FU *et al.*, 2011; WANG *et al.*, 2015). Assim, a parede celular atua como uma primeira barreira de proteção para o protoplasto celular, que restringe a entrada do Cu na célula bem como a sua movimentação para as partes superiores das plantas (HUANG *et al.*, 2017; MWAMBA *et al.*, 2016; WANG *et al.*, 2008). Já os vacúolos respondem por 90% do volume celular e são ricos em ácidos orgânicos, proteínas e alcaloides, fitatos, oxalatos, que podem quelar íons metálicos, convertendo os íons em um complexo menos tóxico. Consequentemente, os vacúolos são considerados como a segunda barreira de acúmulo e desintoxicação de metal pesado (CLEMENS, 2006).

As diferentes formas químicas de Cu também são fatores-chave para a tolerância das plantas e influenciam a migração e o acúmulo de Cu em toda a planta. Em geral, o metal pesado em formas inorgânicas e orgânicas tem maior capacidade de migração e é considerado mais tóxico para as células vegetais, em comparação com o metal integrado a pectatos e proteínas e ligado a fosfatos e oxalatos. No estudo III, observou-se que nas raízes de *C. dactylon* e *P. plicatulum* a maior parte do Cu absorvido foi integrado a pectatos e proteínas, fosfatos e oxalatos, indicando essas espécies possuem menor capacidade de transportar o Cu, o que é considerado um importante mecanismo de tolerância ao metal (HUANG *et al.*, 2017; LI *et al.*, 2019). Nas folhas, a diminuição da toxicidade em *C. dactylon* ocorreu provavelmente pela ligação com ligantes peptídicos e ligantes fosfato.

Portanto, as plantas possuem vários mecanismos potenciais a nível celular que estão envolvidos na desintoxicação de metais pesados. Essa capacidade da planta de conviver com o excesso de metal em seu ambiente de crescimento e acumulá-lo em seus tecidos é de fundamental importância para fitorremediação. No estudo IV, o potencial de *C. dactylon*, *A. affinis*, *P. notatum* e *P. plicatulum* em fitorremediar solo contaminado com 35 e 70 mg de Cu kg^{-1} foi avaliado. Em ambos níveis de contaminação do solo, as quatro gramíneas possuem baixa translocação de Cu; não tendo, portanto, utilidade potencial na fitoextração de Cu. Por outro lado, os altos valores do fator de bioacumulação de Cu nas raízes das gramíneas, especialmente de *P.plicatulum*, indicam que, em ambos níveis de contaminação do solo, as plantas são consideradas fitoestabilazadoras potenciais.

Contudo, o sucesso da fitorremediação de solo contaminado é altamente correlacionado com a produção da biomassa vegetal. Em solo contaminado com 35 mg de Cu kg^{-1}, a taxa de crescimento da espécie exótica *C. dactylon* aumentou, enquanto o crescimento das espécies nativas foi acentuadamente reduzido. Quando cultivadas em solo contaminado com 70 mg de Cu kg^{-1}, a espécie *A. affinis* sofreu grandes danos fitotóxicos, o que resultou na morte das plantas. As demais gramíneas, embora tenham resistido a essa condição de contaminação, tiveram o crescimento drasticamente reduzido. Apesar da redução acentuada do crescimento de *C. dactylon* em solo contaminado com 70 mg Cu kg^{-1}, a taxa de crescimento e a produção de biomassa manteve-se superior aos valores observados para as demais gramíneas quando cultivadas nessa mesma condição. Portanto, embora *C. dactylon* apresente baixo valor de Fator de translocação e Fator de bioacumulação de Cu nas raízes inferior ao observado em outras gramíneas, como *P. plicatulum* e *A. affinis*, a sua alta capacidade de produção de biomassa compensa a concentração relativamente baixa do metal nos tecidos; e a extensão da remoção do Cu do solo pode ser ainda maior.

6. CONCLUSÕES GERAIS

O aumento das concentrações de Cu disponível no solo de vinhedos localizados no extremo Sul do Brasil não alterou a biodiversidade da comunidade vegetal que coabita essas áreas. Porém, a composição botânica foi modificada, e isso pode ser resultado do aumento dos teores de Cu no solo e da melhoria da sua fertilidade.

As espécies *P. plicatulum* e *A. conyzoides*, que estão entre as mais representativas do bioma Pampa, apresentaram os maiores valores de fator de bioacumulação na parte aérea e são candidatas potenciais a técnicas de fitorremediação. Outras herbáceas que coabitam os vinhedos, como a espécie nativa *P. notatum* e a exótica *C. dactylon*, destacaram-se pela sua ampla distribuição nas áreas estudadas, apesar das altas concentrações de Cu no solo.

A exposição das gramíneas nativas do bioma Pampa, *A. affinis*, *P. notatum* e *P. plicatulum*, e da gramínea invasora, *C. dactylon*, a altas concentrações de Cu no ambiente de cultivo aumentou a concentração de espécies reativas de oxigênio, como H_2O_2, e a peroxidação lipídica das membranas celulares das plantas. Além disso, a absorção excessiva de Cu diminuiu a concentração de pigmentos fotossintéticos, comprometeu a atividade fotossintética, e alterou a morfologia radicular e o estado nutricional das plantas. Tudo isso contribuiu para o menor crescimento das espécies avaliadas.

Quando cultivadas em ambientes contaminados com Cu, as plantas expressaram mecanismos de tolerância ao excesso do metal. O Cu absorvido pelas plantas foi acumulado predominantemente no sistema radicular, o que minimizou a quantidade de Cu translocada para a parte aérea e evitou sintomas de toxidez em órgãos mais sensíveis das plantas. Para combater a produção excessiva de espécies reativas de oxigênio e minimizar os seus efeitos deletérios, a atividade das enzimas antioxidantes SOD e POD foram aumentadas. Nas células radiculares e das folhas, a maior parte do Cu absorvido foi retido na parede celular e nos vacúolos. Outra estratégia utilizada pelas plantas para minimizar a toxidez do Cu foi a complexação do metal. No sistema radicular das plantas, a maior parte do Cu ficou concentrada nas três formas menos tóxicas, Fiii (ligado com pectatos e proteínas), Fiv (ligado com fosfato) e Fv (ligado com oxalato). Nas folhas de *C. dactylon*, diferentemente do observado nas demais espécies, a maior parte do Cu absorvido foi mantido em formas menos ativas, Fiii, Fiv e Fv.

O cultivo das gramíneas nativas do bioma Pampa *A. affinis*, *P. notatum* e *P. plicatulum* e da gramínea exótica *C. dactylon* em solo contaminado com 35 e 70 mg de Cu kg^{-1}, indica que essas plantas são fitoestabilizadoras potenciais. No entanto, o uso das espécies nativas como fitoestabilizadoras de solo com contaminação moderada ou elevada requer a adoção de estratégias capazes de diminuir a disponibilidade de Cu no solo, de modo a minimizar os efeitos fitotóxicos do metal e favorecer o desenvolvimento da vegetação nativa. O aumento na produção de biomassa das espécies nativas, como *P. plicatulum*, associada ao seu alto fator de bioacumulação de Cu no sistema radicular, possibilitam que elas contribuam mais efetivamente para a fitoestabilização do solo contaminado.

Em solo moderadamente contaminado, a espécie invasora *C. dactylon* apresenta alta tolerância ao Cu e elevada taxa de crescimento. Essas características de *C. dactylon* associadas ao seu alto fator de bioacumulação do Cu no sistema radicular indica que o uso dessa espécie como planta de cobertura é uma estratégia viável para fitoestabilizar a área contaminada. Essa espécie é capaz de imobilizar maiores quantidades de Cu em sua biomassa; e o seu cultivo em áreas contaminadas pode minimizar os processos erosivos e consequente dispersão de poluentes. Contudo, por ser uma espécie exótica, o seu uso como fitoestabilizadora não é recomendado em áreas onde a manutenção da biodiversidade vegetal nativa do bioma Pampa é priorizada.

7. PERSPECTIVAS DE ESTUDOS FUTUROS

I – Acompanhar a dinâmica da vegetação presente nas entrelinhas dos vinhedos ao longo dos anos e avaliar se o aumento das concentrações de Cu no solo contribui para a alteração da composição botânica da vegetação nativa.

II – Avaliar se a reforma dos vinhedos antigos resulta em maior disponibilidade de Cu no solo e, em caso positivo, se a maior disponibilidade de Cu no solo afetou a composição botânica da vegetação presente nas entrelinhas e se favorece o desenvolvimento de espécies mais tolerantes ao metal.

III – Analisar a variabilidade genética de populações das espécies vegetais nativas do bioma Pampa, como *Paspalum notatum, Paspalum plicatulum e Ageratum conyzoides,* em áreas de campo natural e em áreas com longo histórico de aplicação de fungicidas de base cúprica. Este estudo possibilitará relacionar a variação interpopulacional à contaminação por Cu e produzir uma descrição genética de populações dessas espécies que sejam adequadas para uso em práticas de fitorremediação.

IV – Comparar o efeito de diferentes fontes de Cu (sais puros e produtos comerciais) no desenvolvimento vegetal (videiras e plantas de cobertura) e no estabelecimento de comunidades microbianas.

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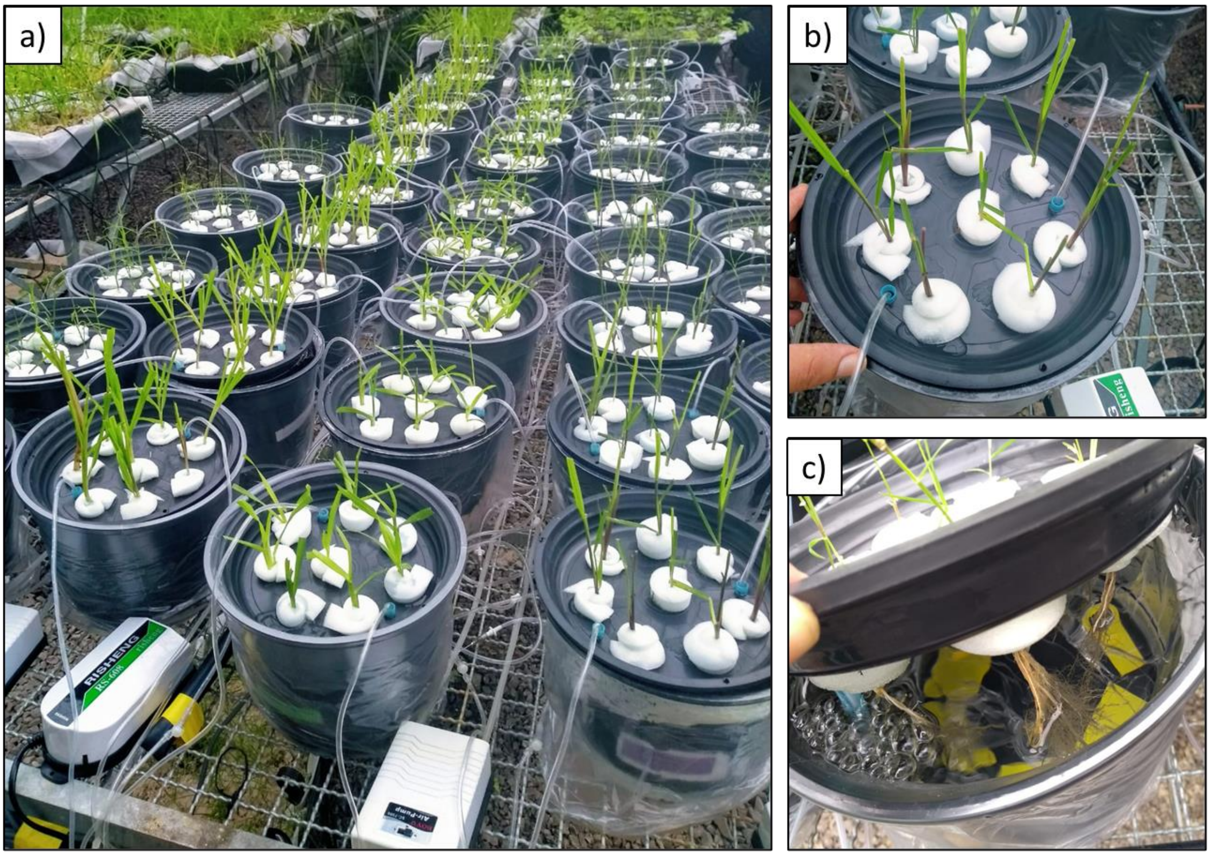
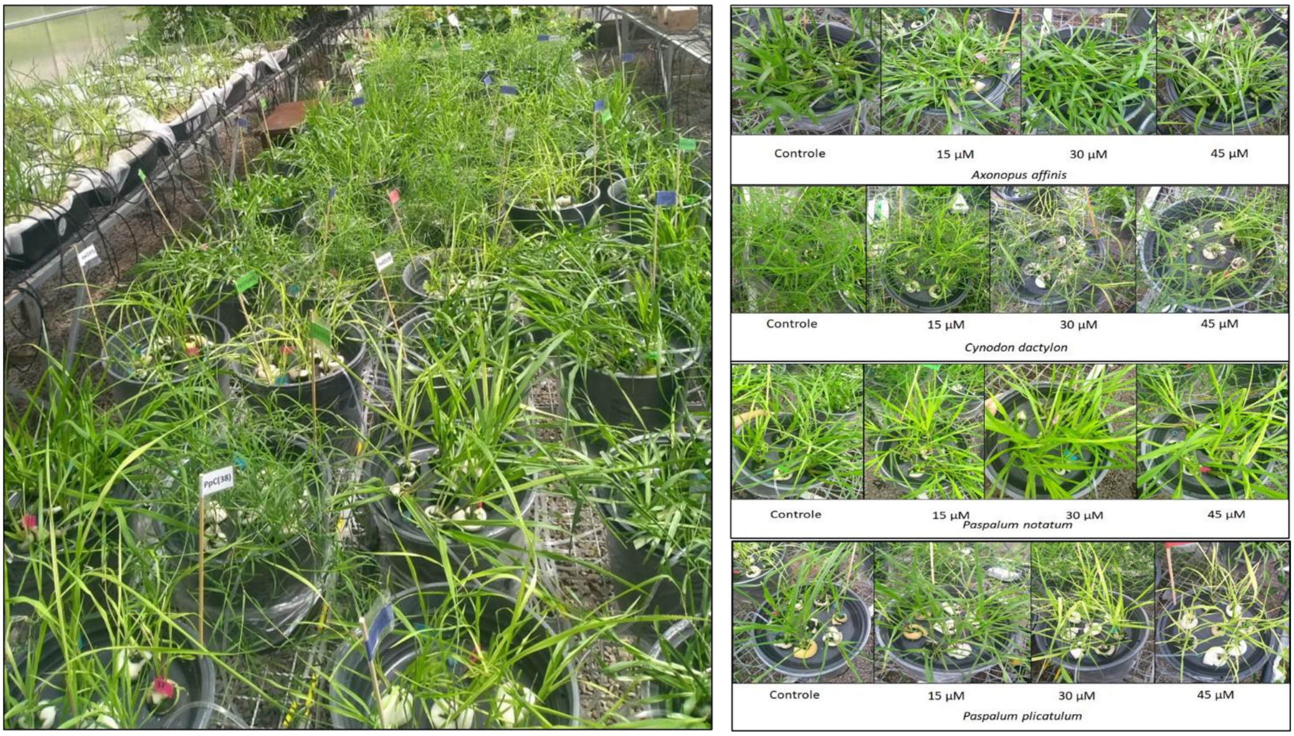
APÊNDICES

APÊNDICE 1: Identificação das espécies herbáceas que compõe a vegetação presente nas entrelinhas dos vinhedos. A imagem acima corresponde a avaliação realizada no estudo 1.

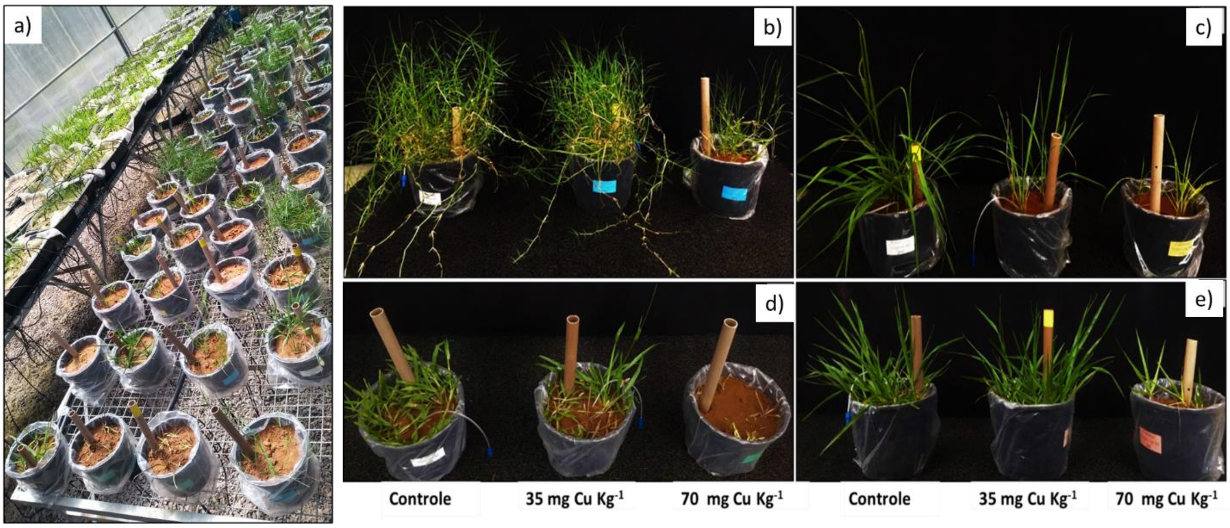
APÊNDICE 2: Multiplicação das gramíneas encontradas com maior frequência em vinhedos contaminados com Cu. As gramíneas coletadas foram multiplicadas em casa de vegetação e utilizadas nos estudos 2, 3 e 4.



APÊNDICE 3: Experimento referente aos estudos 2 e 3. Vista geral do experimento após o plantio das mudas de *Axonopus affinis*, *Cynodon dactylon*, *Paspalum notatum e Paspalum plicatulum* (a); Detalhes estruturais das unidades experimentais (b e c).



APÊNDICE 4: Experimento referente aos estudos 2 e 3. Vista geral do experimento uma semana após a aplicação dos tratamentos (0.32, 15, 30 e 45 µM de Cu); e efeito do incremento de Cu na solução nutritiva sobre a parte aérea das gramíneas (uma semana após a aplicação do tratamentos).



APÊNDICE 5: Experimento referente ao estudo 4. Vista geral do experimento 45 dias após o transplantio das mudas (a); efeito do incremento de Cu no solo sobre o desenvolvimento das gramíneas *Cynodon dactylon* (b), *Paspalum plicatulum* (c), *Axonopus affinis* (d), e *Paspalum notatum* (e) (90 dias após o transplantio das mudas).

Apêndice 6: **Protocololo do Fracionamento subcelular- (Técnica: Centrifugação diferencial)**

Fracionamento subcelular

O fracionamento subcelular é uma estratégia que permite isolar/ purificar diferentes partes das células como, por exemplo, as organelas celulares com o intuito de se poder analisá-las por métodos físicos e químicos. A centrifugação diferencial é a técnica clássica empregada para o fracionamento; e têm como princípio a realização da centrifugação sequencial do homogenato de células ou tecidos para obter estruturas subcelulares como parede celular, núcleos, mitocôndrias e cloroplastos.

Nesta técnica, o primeiro passo para fracionar a célula é a lise, ou seja, o rompimento das membranas e/ou das paredes das células mas de forma tão delicada quanto possível. Para tal, as células são suspendidas numa solução de pH e concentração de sais adequadas, que devem ser semelhantes àquelas encontradas no interior das células. Posteriormente, a suspensão de células é submetida à centrifugação para provocar a lise das células. A mistura obtida com as células lisadas é chamada de extrato celular e é normalmente sujeita à força centrífuga por rotação.

A força centrífuga separa o extrato em duas frações:

a) o *pellet*;

b) e o sobrenadante.

O *pellet* que se forma no fundo do tubo contém materiais mais pesados como os núcleos da célula comprimidos juntos num sedimento. O sobrenadante, o líquido que envolve o pellet, contém partículas mais leves, moléculas dissolvidas e íons. O sobrenadante pode ser recolhido e ser centrifugado novamente a uma velocidade superior de forma a obter um *pellet* que contém os seguintes componentes celulares mais pesados como, por exemplo, as mitocôndrias e os cloroplastos.

Numa centrifugação diferencial, o sobrenadante é girado a velocidades sucessivamente superiores, permitindo a separação dos vários componentes celulares de acordo com as suas diferenças de densidade e de tamanho. Os componentes celulares nos pellets ressuspendidos podem ainda ser mais purificados pela centrifugação em gradiente de densidade.

A sacarose usada para fazer a separação das organelas, em cada centrifugação a densidade da organela se iguala a da sacarose e fica no fundo do tubo. Tris hcl e ddt são inibidores das proteases sendo utilizados para evitar a quebra das proteínas, inibem as proteases que degradam as proteínas, mantendo a conformação das proteínas, é importante lembrar que células são repletas de proteases.

Tampão de extração:

TRIS HCl 50mM (pH 7,5) - $C_4H_{11}NO_3$.Hcl, PM= 157,60

1mM..............157,6mg/l

50mM.............. x

X= 7.880 mg/l ou 7,88g/L pesar isso de tris e diluir em 1 litro de agua

Ajustar o pH com HCL ou NaOH

DTT 1mM - DITHIOTHREITOL - $C_4H_{10}O_2S_2$, PM= 154,25

1mM..............154,25 mg/l **ou 0,154 g/l**

SACAROSE 250mM - $C_{12}H_{22}O_{11}$, PM= 342,3

1mM..............342,3 mg/l

250nM.............85.575 mg/l ou **85,57g/l**

Preparar o tampão sempre no dia, não guardar na geladeira para uso posterior.

Metodologia:

A distribuição subcelular do Cu dentro da planta pode ser avaliada na parte aérea e no sistema radicular, seguindo o protocolo de **Huang et al (2016)** com algumas adaptações. As amostras de tecido fresco ou congelado (1 grama) foram homogeneizadas em 10 mL de solução tampão contendo Tris – HCl 50 mM (pH7.5), sacarose 250 mM e dithioeritritol 1,0 mM), utilizando almofariz e pistilo. O homogenato foi então centrifugado a **300 × g por 5 min a 4 ° C**. Os *pellets* da parede celular (F1) ficaram no fundo do tubo e o sobrenadante foi transferido para um novo tubo e centrifugado a **5000×g por 20 minutos**, obtendo-se assim a fração 2 (F2), a qual continha núcleos e plastídios. A solução sobrenadante da segunda etapa foi finalmente centrifugada a **15.000 ×g por 30 minutos**; o depósito resultante foi referida como fração mitocondrial (F3), enquanto que o sobrenadante foi considerado como a fração solúvel (F4).

Todos os passos foram realizados a 4°C. As diferentes frações subcelulares obtidas foram secas em estufa a 70°C até peso constante e posteriormente submetidas digestão nitroperclórica (3:1, v/v). As concentrações de Cu nas diferentes frações foram analisadas usando o espectrofotômetro de absorção atômica.

Procedimento geral:

- Pesar 1 g de amostra e homogeneizar com 10 ml de tampão em um almofariz, sempre no gelo e após transferir para uma falcon de 50ml

- centrifugar a **300xg durante 5** minutos em centrífuga refrigerada a 4°C. Após transferir o sobrenadante para outro tubo falcon e reservar o precipitado (fração 1 - corresponde a fração da parede celular).

- Centrifugar a **5000x g durante 20 min** (fração 2 - núcleo e plastídios)

- Centrifugar novamente o sobrenadante da segunda centrifugação a **15.000xg durante 30 minutos.** O precipitado corresponde a fração mitocondrial (fração 3) e o sobrenadante é a fração solúvel (Fração 4 - citoplasma, vacúolos...).

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Apêndice 7: **Receita de pão de queijo**

Caracterização:

O pão de queijo é uma receita tipicamente mineira. Macio por dentro, dourado e crocante por fora, com aroma e gosto de queijo de verdade, o pão de queijo foi criado pelos escravos durante o período colonial. Atualmente, essa delícia ultrapassou todas as montanhas de Minas e é consumida em todo o Brasil. Na receita, é possível utilizar queijos mais suaves ou com sabores mais fortes. ~~Para os mais ousados~~, pode até mesmo fazer as versões recheadas, que podem ser de frango, bacon, calabresa e até com algum doce, como a goiabada. O pão de queijo é tão bom (**mais tão bão**) que pode ser consumido em qualquer horário do dia, porém é mais comum no café da manhã e no lanche da tarde. A melhor forma de saborear é quando ele ainda está quentinho, acompanhado de café. Porém, se não gostar de café, pode combinar o pão de queijo com chá, suco, iogurte ou refrigerante (~~para os mais ousados~~).

Materiais e soluções:

- 1 xícara de leite;
- 1 xícara de óleo;
- 1 xícara de água;
- 1 colher de sal;
- 2 xícaras de polvilho doce;
- 2 xícaras de polvilho azedo;
- 4 ovos;
- 2 xícaras de queijo.

Procedimento de análise:

a) Adicionar o polvilho doce e o polvilho azedo em uma vasilha.

b) Misturar leite, água, óleo e sal, em recipiente que possa ir ao fogo; e aquecer até levantar fervura.

c) Adicionar a solução fervente ao polvilho e misturar. Deixar a massa esfriar.

d) Adicionar os ovos e sovar a massa até que se torne homogênea.

e) Adicionar o queijo e sovar a massa novamente.

f) Modelar as bolinhas (com as mão untadas com óleo) e colocar em assadeira grande.

e) Assar em forno preaquecido (200°C) até que os pães estejam dourados.

Determinação:

Para acompanhar esse pão de queijo quentim que acabou de sair do forno, nada melhor do que um cafezim coado na hora. Ê.. trem bão demais da conta!

VITAE

Isley Cristiellem Bicalho da Silva, filha de Jerônimo Emiliano da Silva e Mires Dalva Bicalho da Silva, nasceu em 1º de novembro de 1988, em Montes Claros, Minas Gerais (MG).

Cursou as séries iniciais (1ª à 4ª série) na Escola Estadual Nereide Carvalho. Os anos finais do ensino fundamental (5ª a 8ª série) foram cursados na Escola Estadual Felício Pereira de Araújo (MG). Em 2004, ingressou na Fundação Educacional Montes Claros (FEMC) para cursar o ensino médio concomitantemente ao curso técnico.

Em 2009 ingressou no curso de Engenharia Agrícola e Ambiental da Universidade Federal de Minas Gerais (UFMG), no Instituto de Ciências Agrárias (ICA), em Montes Claros, MG. Durante a graduação foi bolsista de ensino das disciplinas de química analítica, bioquímica geral e poluição ambiental; foi bolsista do Laboratório de Solos sob orientação do Professor Luiz Arnaldo Fernandes; e foi voluntária em projetos de iniciação científica, sob orientação do Professor Fernando Colen.

Entre os anos de 2014 e 2016, realizou o Curso de Mestrado em Produção Vegetal do Programa de Pós-Graduação em Produção Vegetal da UFMG, sob orientação dos Professores Luiz Arnaldo Fernandes e Fernando Colen. A dissertação foi intitulada "Atributos do solo e características agronômicas do feijoeiro adubado com biochar".

Em agosto de 2016 iniciou seu Curso de Doutorado em Ciência do Solo no Programa de Pós-Graduação em Ciência do Solo da Universidade Federal de Santa Maria (UFSM), sob orientação do Professor Gustavo Brunetto. Durante o curso de doutorado, seus estudos foram concentrados na área de Processos Químicos e Ciclagem de Elementos, com ênfase na dinâmica de nutrientes e de elementos tóxicos. Além disso, foi membro do GEPACES - Grupo de Estudo de Predição de Adubação e Potencial de Contaminação de Elementos no Solo (www.gepaces.com.br), onde atuou em inúmeras atividades de pesquisa em laboratório e a campo e atividades de extensão.

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