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Lessandro De Conti

PLANTAS DE COBERTURA DO SOLO E VIDEIRAS: TOXIDEZ, FITORREMEDIAÇÃO E MECANISMOS DE TOLERÂNCIA AO EXCESSO DE COBRE

Santa Maria, RS
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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciência 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 **Doutor em Ciência do Solo.**

Orientador: Prof. Dr. Gustavo Brunetto

Santa Maria, RS
2018

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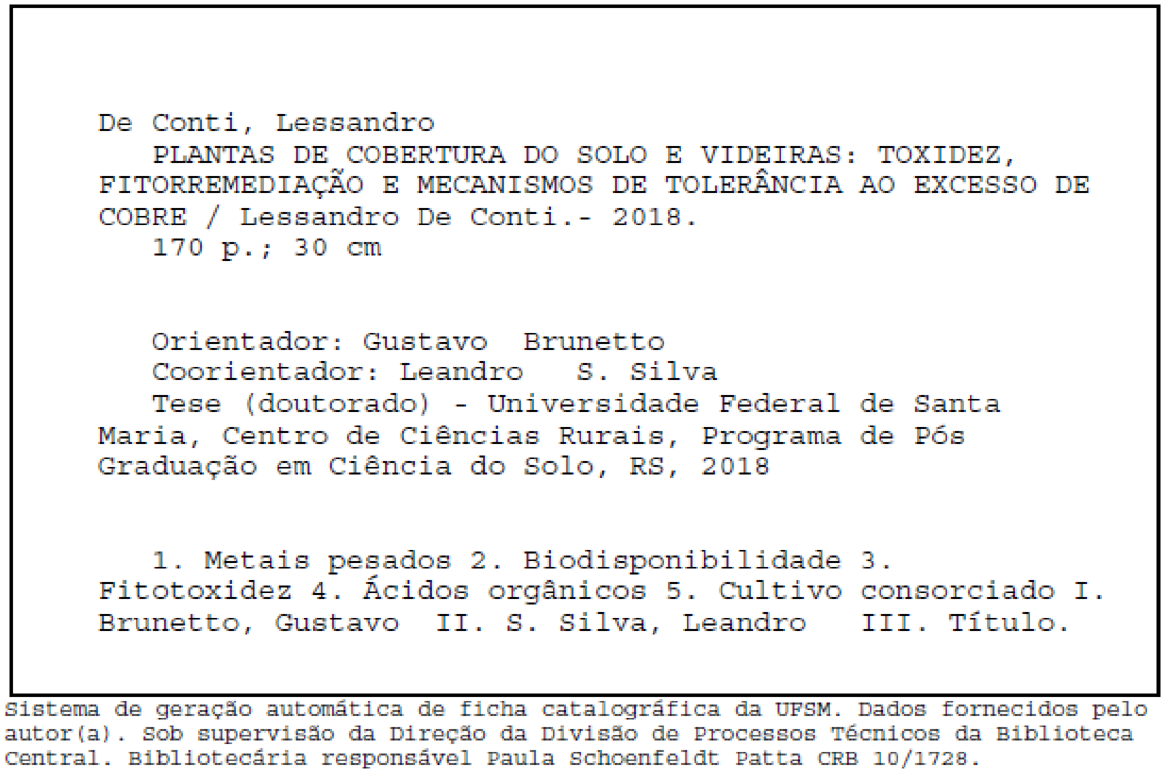

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Aprovado em 28 de junho de 2018:

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Gustavo Brunetto, Dr. (UFSM)
(Orientador - Presidente)

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Tanja Mimmo, PhD. (UNIBZ) – Participou por vídeo conferência

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Rodrigo Josemar Seminoti Jacques, Dr. (UFSM)

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Eduardo Girotto, Dr. (IFRS)

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Tadeu Luis Tiecher, Dr. (IFFar)

Santa Maria, RS

2018

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“A lição número um eu aprendi com meu pai,
quem não sabe aonde vai
não vai a lugar nenhum.”
(Luiz Marenco)

“Você nunca sabe os resultados que virão da sua ação. Mas se você
não fizer nada, não existirão resultados.”
(Mahatma Gandhi)

RESUMO

PLANTAS DE COBERTURA DO SOLO E VIDEIRAS: TOXIDEZ, FITORREMEDIAÇÃO E MECANISMOS DE TOLERÂNCIA AO EXCESSO DE COBRE

AUTOR: Lessandro De Conti
ORIENTADOR: Gustavo Brunetto

A aplicação de fungicidas cúpricos para o manejo das doenças foliares nas videiras (*Vitis vinífera*) aumenta os teores de cobre (Cu) nos solos de vinhedos, fato já diagnosticado em diversas partes do Mundo com tradição na produção de uvas. No entanto, o problema de contaminação dos solos com Cu é mais expressivo nas regiões que possuem solos ácidos e apresentam elevados índices pluviométricos, combinado com temperaturas elevadas durante o ciclo de produção, a exemplo da região Sul do Brasil, o que aumenta a severidade das doenças e consequentemente a utilização do controle químico. O acúmulo de Cu no solo pode atingir níveis tóxicos às videiras e plantas de cobertura do solo que coabitam os vinhedos, com maior intensidade em regiões que possuem solos arenosos e com baixo teor de matéria orgânica, como os encontrados na região da Campanha Gaúcha no Rio Grande do Sul (RS). Estas características dos solos conferem baixa capacidade de sorção ao Cu, promovendo incrementos nos teores de Cu em formas disponíveis, o que potencializa o risco de fitotoxidez. A expansão da viticultura na região da Campanha Gaúcha ocorreu sobre áreas de pastagem nativa do Bioma Pampa. Com isso, as espécies nativas deste Bioma coabitam espontaneamente os vinhedos desta região e são manejadas como plantas de cobertura de solo, através de roçadas. No período de inverno são comumente introduzidas espécies hibernais, como aveia preta (*Avena strigosa*) e azevém perene (*Lolium perene*) nas entrelinhas das videiras. Estas plantas podem exsudar substâncias quelantes que reduzem a bidisponibilidade do Cu e consequentemente, a toxidez às videiras. O trabalho objetivou avaliar os efeitos do excesso de Cu sobre o crescimento, estado nutricional e morfologia radicular em videiras jovens e plantas de cobertura de solo, os mecanismos de tolerância por elas desencadeados em resposta ao excesso do metal e o potencial de utilização das plantas de cobertura na fitorremediação dos solos de vinhedos contaminados com Cu. Cinco estudos em condições controladas foram realizados, utilizando amostras de Argissolo Vermelho, coletado em uma pastagem nativa da região da Campanha Gaúcha, para a realização dos estudos II, III, IV e V. O estudo I foi realizado em solução nutritiva. Após a correção do pH e adição de macronutrientes no solo, foram criados três níveis de contaminação de Cu, através da adição de 0, 40 e 80 mg Cu kg^{-1}. No estudo I os níveis de Cu foram 0,2; 5; 25 e 50 μM Cu L^{-1}. Em todos os estudos foram avaliados parâmetros de crescimento das plantas. Nos estudos I, II, III, e IV foram avaliados também parâmetros nutricionais e morfologia do sistema radicular, sendo também determinados os ácidos orgânicos de baixo peso molecular no estudo I. No estudo V foi determinadas as trocas gasosas das videiras e a taxa de crescimento. Níveis elevados de Cu reduziram o crescimento das videiras jovens e plantas de cobertura do solo, tanto das nativas do Bioma Pampa como as introduzidas. A absorção excessiva de Cu reduziu a eficiência da fotossíntese e desencadeou alterações morfológicas no sistema radicular, reduzindo o volume de solo explorado, contribuindo para os desequilíbrios nutricionais observados. As plantas cultivadas em condições de toxidez pelo Cu expressaram mecanismos de tolerância ao excesso do metal, que consistiram no aumento do pH e da exsudação de ligantes orgânicos, elevando a complexação do Cu^{+2} na solução do solo. O cultivo consorciado de espécies nativas favoreceu o crescimento das videiras jovens, em baixos e moderados níveis de contaminação por Cu. As plantas nativas do Bioma Pampa apresentam grande potencial de utilização na fitorremediação dos solos de vinhedos contaminado com Cu, devido a sua ocorrência espontânia e adaptação as condições locais.

Palavras-chave: Complexação. Biodisponibilidade. Fitotoxidez. Metais pesados. Balanço nutricional. Fungicida cúprico. Rizosfera. Cultivo consorciado. Ácidos orgânicos. Fenóis.

ABSTRACT

COVER CROPS AND GRAPEVINES: TOXICITY, PHYTOREMEDIATION AND TOLERANCE MECHANISMS TO EXCESS COPPER

AUTHOR: Lessandro De Conti
ADVISOR: Gustavo Brunetto

The application of copper fungicides for the management of foliar diseases in grapevines (*Vitis vinifera*) increases copper (Cu) content in vineyard soils. This has been diagnosed in several traditional grape growing regions of the world. However, soil contamination with Cu is more significant in regions with acidic soils and high rainfall rates, which combined with elevated temperatures during the production cycle, increase the severity of diseases and consequently the use of chemical control. Such is the case in the southern region of Brazil. Cu accumulation in soil can reach toxic levels to grapevines and cover crops cohabiting the vineyards. It is more intense in regions that have sandy soils with low organic matter content, such as those found in the Campanha Gaúcha region of the state of Rio Grande do Sul (RS). These characteristics of the soils attribute low sorption capacity of Cu, promoting increased Cu levels in available forms, which increases the risk of phytotoxicity. The expansion of viticulture in the Campanha Gaúcha region occurred on native grasslands of the Pampa Biome. Therefore, the native species of the Biome spontaneously cohabit the vineyards of this region and are managed as cover crops, by mowing. In the winter period, hibernal species such as black oat (*Avena strigosa*) and perennial ryegrass (*Lolium perene*) are commonly introduced between the rows of grapevines. These plants may exude chelating substances that reduce Cu bioavailability and thus toxicity to grapevines. The study aimed to evaluate the effects of excess Cu on growth, nutritional status and root morphology in young grapevines and cover crops, the tolerance mechanisms triggered by plants in response to excess Cu, and the potential use of cover crops in the phytoremediation of Cu-contaminated vineyard soils. Five studies were carried out under controlled conditions using samples of an Typic Hapludalf Soil of a native grassland of the Campanha Gaúcha region for studies II, III, IV and V. Study I was done in nutrient solution. After the correction of pH and addition of macronutrients to the soil, three levels of Cu contamination were created by adding 0, 40 and 80 mg Cu kg^{-1}. In study I, Cu levels were 0.2; 5; 25 and 50 µM Cu L^{-1}. Plant growth parameters were evaluated in all the studies. Nutrient contents and root morphology were also evaluated in studies I, II, III and IV. Low molecular weight organic acids were determined in study I. Grapevine gas exchange and growth rate were determined in study V. High levels of Cu reduced the growth of young grapevines and cover crops, both those native to the Pampa Biome as well as introduced species. Excessive uptake of Cu reduced the efficiency of photosynthesis and triggered morphological changes in the root system, which reduced the volume of soil explored by roots and contributed to nutritional imbalances. Plants grown under Cu toxicity expressed tolerance mechanisms, which consisted in increased pH and exuding organic ligands, increasing Cu^{+2} complexation in the soil solution. The intercropping of native species promoted the growth of young grapevines at low and moderate levels of Cu contamination. The native plants of the Pampa Biome exhibit great potential for the phytoremediation of Cu-contaminated vineyard soils, because of their spontaneous occurrence and adaptability to local conditions.

Keywords: Complexation. Bioavailability. Phytotoxicity. Heavy metals. Nutritional balance. Copper-based fungicides. Rhizosphere. Intercropping. Organic acids. Phenols.

SUMÁRIO

1. INTRODUÇÃO GERAL

O avanço tecnológico e a intensificação dos sistemas de produção agrícola, permitiu elevar os índices de produção e expandir as áreas de cultivo no Mundo. No entanto, elevou a necessidade de adição de insumos, para o fornecimento de nutrientes e controle de patógenos, promovendo o aumento nos teores de metais pesados em solos agrícolas de diversas partes do Mundo, como diagnosticado nos solos de vinhedos (KOMÁREK et al., 2010; ALI et al., 2013; SARWAR et al., 2017).

A viticultura é uma atividade de grande importância econômica e social no Brasil, onde é desenvolvida em uma área de aproximadamente 63.816 hectares (PROTAS et al., 2014). A importância da produção de uvas é maior em estados do Sul do país, como o Rio Grande do Sul (RS), que nacionalmente é o maior estado produtor de uvas e vinhos. Neste estado a produção de uvas ocupa uma área de, aproximadamente, 50.500 hectares, distribuídos em cerca de 13.000 propriedades, que produzem anualmente entre 500 e 600 milhões de toneladas de uva (MELLO, 2011; FLORES e MEDEIROS, 2013). No estado, a principal região e mais tradicional no cultivo de videiras é a Serra Gaúcha, que também é a principal região produtora. Entretanto, nas últimas décadas a atividade se expandiu para outras regiões dentro do estado, principalmente para a região da Campanha Gaúcha, que atualmente concentra grande parte da produção de uvas destinadas a elaboração de vinhos e espumantes. Na região da Campanha Gaúcha o cultivo ocorreu em áreas anteriormente ocupadas com campo natural do Bioma Pampa, anteriormente utilizadas para pecuária extensiva, que a partir da década de 70 foram convertidas à vinhedos.

As condições edafoclimáticas, principalmente os elevados índices pluviométricos das regiões produtoras, entre elas a Campanha Gaúcha, favorecem a incidência de doenças fúngicas foliares, como o míldio (*Plasmopora viticola*). Por isso, torna-se necessário a aplicação de produtos fitossanitários para o controle das doenças fúngicas foliares. Entre os principais fungicidas utilizados no controle das doenças está à calda bordalesa ($Ca(OH)_2$ + $CuSO_4$), por aliar alta eficiência no controle e baixo custo. Mas, em sua composição este fungicida apresenta alta quantidade de cobre (Cu), que somado as frequentes aplicações durante o ciclo produtivo, adiciona anualmente expressivas quantidades deste metal pesado, incrementando o teor no solo com o passar dos anos de cultivo. O acúmulo de Cu no solo potencializa o risco de contaminação de águas superficiais, através da transferência pela água escoada na superfície de solos de vinhedos e também percolada no perfil, quando o Cu está presente em altas concentrações na solução do solo (WENG et al., 2002). Somado ao impacto ambiental negativo, altos teores de Cu em solos podem causar toxidez as videiras e demais espécies que coabitam os vinhedos.

A contaminação por Cu em solos cultivados com vinhedos é observada em vários países do Mundo com tradição na produção de uvas (KOMÁREK et al., 2010; MACKIE et al., 2012). No RS os primeiros estudos sobre contaminação dos solos de vinhedos com Cu foram realizados no ano 2000 na região da Serra Gaúcha. A partir de então vários estudos de diagnóstico da contaminação de solos de vinhedos foram realizados, em sua maioria por participantes do grupo de pesquisas atualmente denominado Grupo de Estudos de Predição de Adubação e Potencial de Contaminação de Elementos em Solos - GEPACES (www.gepaces.com.br/index.php/en/component/users), do Departamento de Solos da Universidade Federal de Santa Maria (UFSM), em parceria com a Embrapa Uva e Vinho, outros grupos de pesquisa da UFSM e vinícolas do RS. Estes diagnósticos apontaram incrementos nos teores de Cu com o aumento da idade dos vinhedos. Também foi observado que em solos arenosos, como os da região da Campanha Gaúcha, os incrementos nos teores de Cu tendem a atingir rapidamente níveis tóxicos às plantas, pois expressiva quantidade do Cu acumulado é encontrado em formas trocáveis e solúveis, ou seja, disponível, devido à baixa capacidade de sorção destes solos (GIROTTO, 2010; MIOTTO, 2012; BUNETTO et al., 2014; GIROTTO et al., 2014; TIECHER, 2017).

O Cu é um elemento essencial às plantas, entretanto no momento da renovação de vinhedos antigos, o teor elevado do metal no solo pode causar toxidez as plantas jovens de videira e também as plantas de cobertura de solo que coabitam os vinhedos. Videiras jovens cultivadas em solos com altos teores de Cu, apresentam redução no crescimento e no acúmulo de macronutrientes, indicando toxidez pelo excesso do metal (MELO et al., 2008; 2015; BALDI et al., 2018). Além do desiquilíbrio nutricional, plantas que se desenvolvem em solos com teor elevado de Cu podem apresentar alterações morfológicas no sistema radicular, que em conjunto com o excesso de Cu no tecido vegetal ocasionam redução do crescimento e senescência das folhas em condições mais severas de contaminação, ou em espécies mais sensíveis ao metal. Além das videiras jovens, estudos realizados em ambiente controlado, com solos de vinhedos antigos do RS, também observaram sintomas de toxidez nas plantas de aveia preta (*Avena strigosa*) (GIROTTO et al., 2014; TIECHER et al., 2016).

Em videiras adultas, a ocorrência de sintomas de toxidez se mostrou pontual e quando diagnosticada não foi observada sintomatologia aguda de toxidez, mesmo com altos teores de Cu disponíveis no solo, a exemplo dos extraídos por EDTA (Miotto et al., 2014). Isso indica a necessidade de estabelecer estratégias de remediação dos solos contaminados com Cu, voltadas a videiras jovens e plantas de cobertura. A redução ou suspensão das aplicações de fungicidas que contém Cu em sua composição, caracteriza-se como medida inicial para remediação dos solos contaminados. Entretanto é fundamental o desenvolvimento de estratégias de remediação, que reduzam a biodisponibilidade do metal presente nos solos com excesso de Cu, mantendo os solos férteis e produtivos, além de reduzir o risco de contaminação ambiental pela transferência do elemento a outros ambientes.

A fitorremediação é uma estratégia de remediação *in situ* de solos contaminados com metais pesados. Com baixo custo e reduzido impacto ambiental, o emprego de plantas para recuperação de solos contaminados vem ganhando grande importância a nível mundial (ALI et al., 2013; LEGUIZAMO et al., 2017). A fitorremediação consiste no uso de plantas e sua microbiota que habita a rizosfera, para eliminar ou reduzir os efeitos do poluente no ambiente, através da degradação, complexação, sequestro ou imobilização do contaminante (PILON-SMITS, 2005). Na fitorremediação de metais pesados, destacam-se duas principais técnicas, a fitoextração, que se caracteriza pela absorção e translocação do contaminante para parte aérea, que é posteriormente colhida e a fitoestabilização, que se caracteriza pela imobilização e complexação na biomassa radicular e rizosfera (ALI et al., 2013; WAN et al., 2017).

A presença de espécies nativas do Bioma Pampa, como *Axonopus affinis*, *Paspalum notatum* e *Paspalum plicatulum*, que naturalmente coabitam os vinhedos da região da Campanha Gaúcha, apresentam lenta taxa de crescimento nas estações de outono-inverno, por predominar na composição botânica espécies estivais (QUADROS et al., 2009). Como forma de aumentar a adição de resíduos e promover maior proteção do solo, espécies anuais, a exemplo do azevém (*Lolium multiflorum*) e aveia preta (*Avena strigosa*) são introduzidas no período de outono-inverno para cobertura de solo, contribuindo na conservação do solo e ciclagem de nutrientes. A presença das plantas de cobertura, coabitando os vinhedos, pode contribuir na redução dos efeitos fitotóxicos do excesso de Cu as videiras, devido a absorção e acúmulo do Cu no tecido destas plantas e também pela exsudação de íons e compostos orgânicos, como ácidos orgânicos de baixo peso molecular, modificando a concentração de ligantes orgânicos e o valor de pH da solução do solo, que influenciam diretamente a solubilidade e distribuição das espécies solúveis de Cu (KIM et al., 2010; MEIER et al., 2012).

Mecanismos de tolerância extracelular, desencadeado por algumas espécies de plantas em condições de estresse, podem promover alterações nos atributos químicos do solo e da solução, que reduzem a biodisponibilidade do metal contaminante, por aumentar a proporção das espécies químicas complexadas na solução do solo. Estas alterações tendem a ocorrer de forma mais efetiva na rizosfera, indicando que avaliações neste ambiente podem melhorar a predição da biodisponibilidade de Cu e o potencial emprego de espécies vegetais na fitorremediação desses solos. A redução da biodisponibilidade do contaminante no ambiente rizosférico pode beneficiar outras espécies cultivadas em consórcio, indicando que o cultivo consorciado pode ser uma alternativa de fitorremediação para os solos contaminados com Cu. A evolução das espécies nativas do Bioma Pampa, em sua maioria, ocorreu sob solos quimicamente pobres e ácidos, indicando a presença de mecanismos adaptativos à condições nutricionais adversas, que podem ser expressas quando submetidas a condições de toxidez por Cu. Mas isso, ainda não é suficientemente conhecido.

Neste contexto, em que os diagnósticos dos solos de vinhedos apontam incremento nos teores de Cu com o passar dos anos de cultivo, além da suspenção ou redução das entradas de Cu no sistema de produção, torna-se necessário identificar os mecanismos de tolerância das plantas à toxidez de Cu, desencadeados por diferentes espécies e desenvolver estratégias para remediação, dos solos de vinhedos que já possuem níveis de contaminação que causam prejuízos ao crescimento das plantas. Essas ações são fundamentais para a continuidade e sustentabilidade da produção de uvas, nas regiões com vocação para a viticultura no Sul do Brasil.

2. HIPÓSTESES

I. Em concentrações tóxicas de Cu no ambiente de cultivo o *Lolium perenne* aumenta a produção e exsudação de ácidos orgânicos de baixo peso molecular, demonstrando ser um mecanismo de tolerância a toxidez de Cu.

II. A biodisponibilidade do Cu é menor na rizosfera das plantas de aveia preta, devido ao aumento na concentração de ligantes orgânicos e pH, promovido pelos compostos exsudados pelas raízes das plantas.

III. Teores elevados de Cu causam toxidez as plantas nativas do Bioma Pampa que são utilizadas como plantas de cobertura dos solos de vinhedos. As espécies mais tolerantes a toxidez de Cu possuem mecanismos que reduzem a biodisponibilidade do metal, com maior potencial de utilização para fitorremediação dos solos contaminados.

IV. O cultivo consorciado de videiras jovens com espécies nativas do Bioma Pampa diminui o potencial de fitotoxidez de Cu às videiras, por causa da sua imobilização no tecido das plantas nativas e a exsudação de íons e compostos orgânicos, que reduzem a proporção de Cu bioassimilável (Cu^{+2}) na solução do solo.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar os efeitos do excesso de Cu sobre o crescimento, estado nutricional e morfologia radicular em videiras jovens e plantas de cobertura de solo, os mecanismos de tolerância por elas desencadeados em resposta ao excesso do metal e o potencial de utilização das plantas de cobertura na fitorremediação dos solos de vinhedos contaminados com Cu.

3.2. OBJETIVOS ESPECÍFICOS

I. Avaliar os mecanismos envolvidos na mitigação da fitotoxidez de Cu e os efeitos do estresse sobre o crescimento, morfologia do sistema radicular e nutrição do azevém perene cultivado com duas fontes de quelantes do Fe na solução de cultivo.

II. Avaliar as alterações químicas promovidas pela aveia preta na rizosfera, a influência dessas plantas sobre a biodisponibilidade de Cu e o efeito de teores crescentes de Cu sobre parâmetros de crescimento, morfológicos e nutricionais na aveia preta.

III. Determinar os efeitos do excesso do Cu sobre a produção de matéria seca, morfologia radicular, estado nutricional das plantas e biodisponibilidade do Cu na solução do solo cultivado com de diferentes espécies de plantas do Bioma Pampa.

IV. Avaliar a potencial do cultivo consorciado de gramíneas nativas do Bioma Pampa, em reduzir a biodisponibilidade de Cu e os sintomas da fitotoxidez em videiras jovens, cultivadas sob diferentes níveis de contaminação do metal.

4. REVISÃO BIBLIOGRÁFICA

4.1. COBRE EM SOLOS DE VINHEDOS

O Cu é um micronutriente naturalmente encontrado em baixos teores disponíveis na maioria dos solos agrícolas do mundo, com a variação dos teores totais relacionado ao material de origem dos solos, variando de 2,0 mg Cu kg^{-1} em solos derivados de granito a 150 mg Cu kg^{-1} em solos basálticos (HUGEN et al., 2013). Avaliando os solos sob vegetação nativa, nas duas principais regiões vitivinícolas do RS, Girotto et al. (2014) observaram na camada de 0-20 cm, teor total de 47,8 mg Cu kg^{-1} no solo da Serra Gaúcha e 14,5 mg Cu kg^{-1} no solo da Campanha Gaúcha, os quais são solos originados de basalto e arenito, respectivamente (STRECK et al., 2008). A grande diferença nos teores totais de Cu entre os diferentes solos, mesmo com ausência de atividade antrópica no local, permite inferir que o teor total de Cu é um indicador limitado para monitoramento da contaminação e risco de toxidez às plantas. No entanto, a legislação brasileira possui como parâmetro de referência para contaminação de Cu teores totais superiores a 200 mg kg^{-1} (CONAMA 2009), sem considerar o material de origem e características físico-químicas do solo.

Os teores de Cu em solos de vinhedos podem ser incrementados devido a adoção de sucessivas práticas de manejo, como a adubação e as aplicações de fungicidas foliares para o controle de doenças fúngicas (MACKIE et al., 2012; BRUNETTO et al., 2016). Mesmo com condições climáticas favoráveis para a viticultura, as precipitações frequentes no Sul do Brasil no período da primavera-verão, associado a temperatura elevada, aumenta a incidência de doenças fúngicas, especialmente o míldio (*Plasmopara viticola*), tornando necessárias constantes aplicações de fungidas ao longo de cada ciclo produtivo das videiras (MIOTTO et al., 2014). Muitos dos fungicidas utilizados no manejo das doenças, à exemplo calda bordalesa [$Ca(OH)_2 + CuSO_4$] e o oxicloreto de cobre [$CuCl_2.3Cu(OH)_2$], contém em suas composições grandes quantidades de Cu, ocasionando aumento nos teores de Cu nos solos de vinhedos com o passar dos anos, podendo atingir teores que causam toxidez as videiras e plantas de cobertura de solo (BRUN et al., 2001; TOSELLI et al., 2009; CHAIGNON et al., 2009; FERNANDEZ-CALVIÑO et al., 2010; BRUNETTO et al., 2014; CAMBROLLÉ et al., 2015). A quantidade de Cu adicionada ao sistema de produção de uvas, através das aplicações de fungicidas varia em função da composição do fungicida, dosagem e frequência das aplicações, podendo alcançar em algumas condições, 30 kg Cu ha^{-1} em um ciclo de produção da cultura da videira (CASALI et al., 2008).

Incrementos nos teores de Cu em solos de vinhedos são relatados em vários países do mundo com tradição na produção de uvas, a exemplo da Itália, onde foram diagnosticados teores totais de Cu variando entre 50 a 372 mg kg^{-1} (BRETZEL e CALDERISI, 2006; DELL'AMICO et al., 2008). Na França e Espanha foram diagnosticados teores totais de Cu em solos de vinhedos variando entre 20 a 600 mg kg^{-1} (ARIAS et al., 2004; BRUN et al., 1998; FLORES-VELES et al., 1996; NÓVOA-MUÑOZ et al., 2007). No Brasil os estudos sobre contaminação dos solos de vinhedos se concentram na região Sul do país, onde foram diagnosticados teores totais de Cu variando de 20,7 a 3.216 mg kg^{-1}, com os maiores observados na região da Serra Gaúcha do RS, que é a mais antiga região produtora de uvas do país e os solos são derivados do basalto (MIRLEAN et al. 2007; NOGUEIROL et al., 2010; GIROTTO et al., 2014; MIOTTO et al., 2014; COUTO et al., 2015). A grande amplitude diagnosticada nos teores de Cu em solos de vinhedos está relacionada às características físico-químicas dos solos, a intensidade dos processos de transferências por erosão ou lixiviação e, principalmente, com a quantidade e intensidade de aplicação do metal através das aplicações de fungicidas cúpricos. Mesmo com grande amplitude nos teores de Cu observados, os estudos realizados na região Sul do Brasil demonstram incremento nos teores de Cu com o aumento da idade dos vinhedos, quando comparados a condição anterior a implantação dos vinhedos, matas ou pastagens nativas, alertando para a problemática da contaminação destes ambientes de produção (CASALI et al., 2008; BRUNETTO et al., 2014; MIOTTO et al., 2014; COUTO et al., 2015).

A alta afinidade adsortiva do Cu aos grupos funcionais de superfície de colóides minerais e, principalmente, orgânicos, ocorre devido a sua configuração eletrônica $[Ar]3d^{10}4s^{1}$, o que lhe confere alta reatividade com os grupos funcionais que contém enxofre (S) e nitrogênio (N), além dos grupos carboxílicos (COOH) e, em menores proporções, a grupos hidroxílicos (OH) e fenólicos (anel aromático OH) (McBRIDE, 1994). Como consequência da alta interação do Cu aos constituintes da fase sólida do solo, está o acúmulo na camada superficial do solo em sistemas produtivos onde o solo não é revolvido, à exemplo dos vinhedos, onde após a implantação das mudas não é preconizado o revolvimento do solo nas entre linhas (BRUNETTO et al., 2014; GIROTTO et al., 2014; DE CONTI et al., 2016). O acúmulo de Cu nas camadas superficiais potencializa o risco de transferência por escoamento superficial, transferindo o metal para cursos d'água e áreas adjacentes aos vinhedos (BRUNETTO et al., 2014).

4.2. INTERAÇÃO E BIODISPONIBILIDADE DO COBRE NO SOLO

O Cu adicionado ao solo associa-se predominantemente a fase sólida, onde é retido por ligações fisico-químicas, em diferentes graus de energia, enquanto uma pequena fração do metal permanece na solução do solo, em formas solúveis (íons hidratados, complexos e pares iônicos) ou precipitados (McBRIDE, 1994). A interação dos metais com a fase sólida do solo ocorre de diversas formas, através de complexos de esfera interna (adsorção específica), por ligações iônicas e covalentes ou de esfera externa (adsorção não específica), através de ligações eletrostáticas (McBRIDE, 1994; SPARKS, 1995). O tipo de adsorção com a fase sólida irá determinar a força pela qual o elemento está retido e a maior ou menor reversibilidade da reação (dessorção), impactando na disponibilidade do elemento.

Os elementos químicos adicionados ao solo pela atividade antrópica, como o Cu adicionado através das aplicações de fungicidas cúpricos, distribuem-se nas formas pré-existentes no solo, onde normalmente ocupam primeiramente nos sítios de ligação de maior energia. Em seguida, os íons remanescentes são redistribuídos em frações que são retidas com menor energia de adsorção ou em precipitados com maior solubilidade, o que consequentemente aumenta a biodisponibilidade e mobilidade dos elementos químicos no sistema solo (SPOSITO, 1989; BRUNETTO et al., 2014). Desta forma, as aplicações frequentes de fungicidas ao longo dos anos, podem ocasionar a saturação destes sítios adsortivos, aumentando a concentração de Cu na solução do solo, bem como o teor disponível, o que potencializa o risco de toxidez as videiras e plantas de cobertura do solo que coabitam os vinhedos, além de aumentar o risco de transferências de Cu para outros ambientes (CHAIGNON et al., 2009; MIOTTO et al., 2014; CAMBROLLÉ et al., 2015; BRUNETTO et al., 2016).

A disponibilidade e solubilidade do Cu no solo não dependem apenas do teor total deste elemento no solo, mas é governada também pelas características físico-químicas do solo, como a quantidade e tipo de argilominerais, óxidos e hidróxidos de ferro (Fe), alumínio (Al) e manganês (Mn), carbonatos e matéria orgânica, além do valor de pH e da capacidade de troca de cátions (CTC) (McBRIDE, 1994; BRADL, 2004; MIOTTO et al., 2017). Mesmo compreendendo um pequeno percentual do teor total encontrado no solo, a fração solúvel de

Cu apresenta grande importância na biodisponibilidade e potencial tóxico, por estar em equilíbrio com a fase sólida e em contato com as raízes das plantas (GIROTTO et al., 2014). As plantas e microrganismos absorvem o Cu da solução do solo, preferencialmente na forma química inorgânica, que se encontra coordenada por moléculas de água, classificada como espécie livre (Cu^{+2}) (McBRIDE, 1994). Além da espécie Cu^{+2}, o Cu pode atuar como cátion central, formando complexos e pares iônicos com vários ligantes orgânicos e inorgânicos presentes na solução do solo, com a intensidade destas interações governadas pelas características do solo e da solução, especialmente, pela concentração de compostos orgânicos dissolvidos (COD) e pelo valor de pH (NOLAN et al., 2003; KIM et al., 2010; PÉREZ-ESTEBAN et al., 2014; REN et al., 2015).

A formação de espécies químicas de Cu complexadas e pares iônicos reduz a biodisponibilidade, por reduzir a porcentagem de Cu^{+2}, consequentemente reduzindo o potencial fitotóxico do contaminante. No entanto, a presença de ligantes solúveis favorece a dessorção e a mobilidade no perfil do solo, potencializando o risco de contaminação de águas subsuperficiais (DE CONTI et al., 2016). As espécies químicas de Cu predominantes na solução dos solos agrícolas é a espécie livre e a complexada com compostos orgânicos; outras espécies complexadas com ligantes inorgânicos (OH^-, CO_3^{-2}, PO_4^-, SO_4^{-2} e NO_3^-), normalmente ocorrem em baixas proporções (NOLAN et al., 2003; MEERS et al., 2006; KIM et al., 2010; PÉREZ-ESTEBAN et al., 2014). A alta afinidade do Cu solúvel a ligantes orgânicos dissolvidos, somada a formação de complexos mais estáveis, indica que em sistemas de produção que apresentem aporte de altas quantidades de resíduos orgânicos, tende a predominar espécies de Cu complexadas na solução do solo, com baixa biodisponibilidade e potencial tóxico do metal (CROUÉ et al., 2003; PÉREZ-ESTEBAN et al., 2014; DE CONTI et al., 2016).

A biodisponibilidade e mobilidade do Cu pode ser alterada pela presença das plantas, principalmente na rizosfera, que é a interface entre o solo e a raízes das plantas (HINSINGER, 2001; BRAVIN et al., 2012). Entre as principais alterações químicas que podem ser induzidas na zona de influência das raízes, está a alteração na concentração de compostos orgânicos solúveis (COD), através da exsudação de compostos orgânicos de baixo peso molecular e pela modificação nos valores de pH, alterado com a maior exsudação de íons H^+ ou OH^- e HCO_3^- (GAHOONIA et al., 1992; JONES e DARRAH, 1994; CHAIGNON et al., 2009; KIM et al., 2010). A solubilidade e distribuição das espécies químicas de Cu é controlada principalmente pelo pH e COD na solução do solo, sendo a redução na biodisponibilidade de elementos químicos na rizosfera, consequência do aumento na proporção das espécies químicas complexadas, caracterizada como um mecanismo de tolerância a toxidez por metais, desencadeada por algumas espécies de plantas (KIM et al., 2010; BRAVIN et al., 2012). Desta forma, mecanismos de tolerância expressos no exterior das raízes, que reduzem a biodisponibilidade dos contaminantes, podem favorecer o crescimento de outras espécies cultivadas em consórcio (BRUNETTO et al., 2016; WAN et al., 2017).

4.3. ALTERAÇÕES MORFOLÓGICAS, NUTRICIONAIS E MECANISMOS DE TOLERÂNCIA DAS PLANTAS, EM RESPOSTA AO EXCESSO DE COBRE.

O adequado crescimento e desenvolvimento das plantas depende da disponibilidade equilibrada dos elementos químicos essenciais. Entre estes está o Cu, que participa de importantes processos metabólicos, como fotossíntese, respiração e metabolismo de carboidratos (YRUELA, 2005; KABATA-PENDIAS, 2011). Plantas cultivadas em solos contaminados com Cu podem absorver em excesso o metal, acumulando demasiadamente em seus tecidos, que resultam em desiquilíbrios nutricionais e respostas negativas em parâmetros bioquímicos e fisiológicos (CAMBROLLÉ et al., 2015; TIECHER et al., 2016). Em plantas mais sensíveis a toxidez de Cu, concentrações nos tecidos foliares entre 15-20 mg Cu kg^{-1} ocasionam redução do crescimento das plantas (KABATA-PENDIAS, 2011).

Altos teores de Cu no tecido das plantas ocasionam alterações fisiológicas e bioquímicas que comprometem o funcionamento do aparato fotossintético, reduzindo a assimilação de carbono (C), além de promover alterações na atividade enzimática e equilíbrio hormonal, inibindo ou retardando o alongamento, divisão e diferenciação celular, principalmente das regiões meristemáticas, que resultam em alterações morfológicas (YRUELA, 2005; BOCHICCHIO et al., 2015). Alterações na morfologia do sistema radicular são frequentemente relatadas em plantas expostas a toxidez por Cu e outros metais pesados. Entre as alterações morfológicas, destaca-se a redução no crescimento, aumento do diâmetro médio e a lignificação das raízes (AMBROSINI et al., 2015; GUIMARÃES et al., 2016; BOCHICCHIO et al., 2015). Estas alterações morfológicas podem estar relacionadas às alterações da expansão celular e no índice mitótico, reduzindo a frequência de divisão celular na região do meristema primário (JIANG et al., 2001). Normalmente a redução no alongamento das raízes é acompanhada pelo espessamento, lignificação e aumento do número de raízes laterais, possivelmente como forma de buscar zonas no solo mais adequadas para o desenvolvimento radicular (POTTERS et al., 2007; BOCHICCHIO et al., 2015).

Alterações morfológicas radiculares reduzem o volume de solo explorado pelas plantas, podendo refletir em redução na absorção de nutrientes e água, contribuindo para ocorrência de deficiências e desbalanços nutricionais, que culminam no menor crescimento das plantas (TOSELLI et al., 2009; BALDI et al., 2018). A toxidez por Cu induzir mudanças nas propriedades da membrana e função de transportadores e canais iônicos, ocasionados pelo aumento da permeabilidade da membrana não-específica, que pode ocasionar deficiência ou absorção excessiva de outros nutrientes (YRUELA, 2005; KABATA-PENDIAS, 2011; CAMBROLLÉ et al., 2015). Isso demonstra que a redução no crescimento das plantas cultivadas em ambientes com altos teores de Cu, não é apenas reflexo apenas do excesso do metal, podendo ser também combinado com efeitos indiretos da fitotoxidez do Cu, como redução no teor de Fe e P, verificados em algumas variedades de videiras por Cambrollé et al. (2015).

A clorose foliar intranerval, relacionada com a deficiência de Fe, associado ao crescimento lento das videiras jovens, são sintomas característicos da toxidez por Cu (CAMBROLLÉ et al., 2015). Estes intomas são frequentemente observados nas videiras jovens na reimplantação de vinhedos antigos erradicados, principalmente da região da Campanha do RS (MIOTTO, 2012). Além de prejudicar a formação do vinhedo, a fitotoxidez retarda o início da produção das videiras, comprometendo o retorno econômico da atividade. O acúmulo de Cu nos solos de vinhedos pode prejudicar também o crescimento das plantas de cobertura de solo, sejam elas nativas, como as espécies do Bioma Pampa, que naturalmente coabitam os vinhedos da região da Campanha do RS, ou anuais, a exemplo do azevém e aveia preta, que são implantadas nas entrelinhas das videiras durante o período de inverno, para proteção do solo. A redução na produção de matéria seca das plantas de cobertura, eleva a predisposição do solo à ocorrência de erosão hídrica e reduz a ciclagem de nutrientes no sistema, aumentando a necessidade de adição de insumos.

As plantas podem expressar mecanismos adaptativos quando expostas a condições de estresse por toxidez ou deficiência de elementos químicos (JONES, 1998; DRESLER et al., 2014). Os tipos de mecanismos de tolerância e a intensidade destes, expressos sob condições de fitotoxidez, variam entre as espécies, status nutricional, tipo e concentração do metal contaminante (MEIER et al., 2012; DRESLER et al., 2014). Um dos mecanismos de tolerância, presente em algumas plantas, consiste no aumento na exsudação de ligantes orgânicos no apoplasto e rizosfera, como forma de aumentar a complexação das espécies químicas livres de metais pesados na solução do solo, a exemplo do Cu^{+2}, evitando a absorção excessiva e transporte de íons metálicos para o interior das células, uma vez que a absorção pelas plantas e microrganismos ocorre preferencialmente através das espécies químicas livres (McBRIDE, 1994; KABATA-PENDIAS, 2011). Entre as substâncias quelantes exsudadas pelas raízes estão os ácidos orgânicos de baixo peso molecular, como fenóis, ácidos orgânicos, amino ácidos e açúcares (MONTIEL-ROZAS et al., 2016; ZAFARI et al., 2016). A presença de grupos funcionais, especialmente a carboxila (—COOH) e hidroxila (OH), confere alta reatividade das substâncias exsudadas com o Cu, alterando a distribuição das espécies químicas na solução do solo, por aumentar a proporção das espécies complexadas, consequentemente, reduzindo a biodisponibilidade do metal (KIM et al., 2010; SESHADRI et al., 2015; MONTIEL-ROZAS et al., 2016).

A exsudação de substâncias quelantes, com capacidade de reduzir a biodisponibilidade do Cu, conferem maior tolerância das plantas que expressam este mecanismo, para o crescimento em ambientes contaminados, além de maior potencial para utilização na fitorremediação dos solos de vinhedos (BRUNETTO et al., 2016). A redução da biodisponibilidade do contaminante no ambiente rizosférico pode beneficiar outras espécies cultivadas em consórcio, apontando para uma potencial alternativa de fitorremediação em solos com contaminação leve a moderadamente de metais pesados (WAN et al., 2017).

O controle da translocação do Cu absorvido entre os órgãos das plantas, acumulando o metal no sistema radicular, com baixa translocação para a parte aérea, também consiste em um mecanismo de defesa das plantas, expressado pela maioria das espécies vegetais cultivadas em teores tóxicos do metal (KABATA-PENDIAS, 2011; GIROTTO et al., 2014, CAMBROLLÉ et al., 2015). O aumento no conteúdo intracelular de ácidos orgânicos de baixo peso molecular pode ser um mecanismo de detoxificação de metais, por aumentar a complexação e a compartimentalização do metal em organelas inativas, como vacúolos, alterando a distribuição do metal via xilema na planta, reduzindo a translocação para a parte aérea onde causaria maiores danos (DRESLER et al., 2014; KISA et al., 2016). A absorção excessiva de Cu pelas plantas pode causar estresse oxidativo e aumento da produção de espécies reativas de oxigênio (EROs) (GIROTTO et al., 2013; TIECHER et al., 2016). O aumento na concentração de fenóis e da produção das enzimas superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase

(APX), são mecanismos de defesa, desencadeado em condições de toxidez e contribuem para redução do estresse oxidativo e na homeostase das EROs (CAMBROLLÉ et al., 2015; MAJDOUB et al., 2017).

4.4. FITORREMEDIAÇÃO DE METAIS PESADOS.

A fitorremediação é uma estratégia emergente na remediação de solos contaminados com metais pesados, impulsionada pelo menor custo, baixo impacto ambiental e grande aceitação pública, em relação a técnicas convencionais de remediação (NASCIMENTO et al., 2009). Esta técnica de remediação do solo consiste no uso de plantas e sua microbiota que habita a rizosfera, para eliminar ou reduzir os efeitos do poluente no ambiente, através da degradação, sequestro ou imobilização do contaminante (PILON-SMITS, 2005).

No termo fitorremediação, estão englobadas as diferentes formas de remediação dos ambientes contaminados promovidos pelas plantas, com destaque para a fitoextração e a fitoestabilização, que são as principais formas de fitorremediação de solos contaminados com metais pesados (ALI et al., 2013; BOKHARI et al., 2016), conforme apresentado na figura 1.

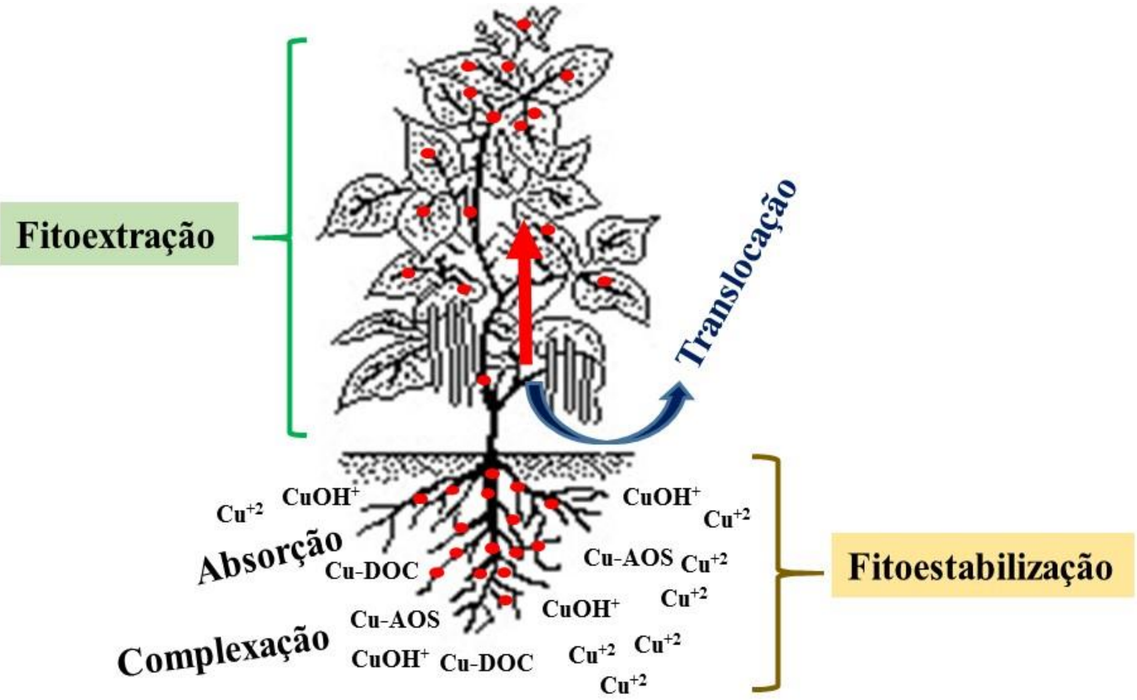

Figura 1: Principais formas de ação das plantas na fitorremediação dos solos contaminados com cobre.

A fitoextração consiste no uso de plantas para remoção de metais pesados solos, mediante a absorção pelo sistema raízes e acúmulo nos órgãos da parte aérea (RAHMAN; HASEGAWA, 2011). Para ocorrência da fitoextração é fundamental que o elemento contaminante esteja em formas biodisponíveis na solução do solo, porém não em níveis deletérios às plantas remediadoras, que a espécie vegetal possua sistema radicular abundante, elevada capacidade de produção de biomassa e de acumular na parte aérea elevadas quantidades de metal (MARQUES et al., 2009). O potencial de fitoextração de uma espécie de planta é determinado por dois fatores principais, a produção de biomassa da parte aérea e a concentração do metal na parte aérea (ALI et al., 2013). Plantas hiperacumuladoras possuem a capacidade de translocar e concentrar elevada quantidade de metais pesado nos tecidos da parte aérea, potencializando o seu emprego na fitoextração, no entanto, estas plantas geralmente apresentam crescimento lento, requerendo grande período de tempo para que haja a remoção dos contaminantes do solo (RAHMAN; HASEGAWA 2011; LEGUIZAMO et al., 2017). Após o crescimento nas áreas contaminadas, a parte aérea das plantas fitoextratoras é colhida e submetidas a técnicas reduzem o volume e concentram o metal, a exemplo da compostagem e incineração, seguido da destinação segura dos resíduos (ALI et al., 2013).

Na remediação de solos contaminados é fundamental a adoção de técnicas que reduzem o risco de transferência do contaminante para outros ambientes, através da movimentação de partículas pela erosão e/ou lixiviação no perfil do solo. A estabilização dos metais pode ser obtida através da técnica de fitoestabilização, mecanismo expresso por algumas espécies de plantas que reduzir a mobilidade e a migração dos contaminantes presentes no solo, seja através da imobilização, lignificação ou humidificação dos poluentes nos seus tecidos (WUANA; OKIEIMEN, 2011). Em solos contaminados com Cu, a fitoestabilização tende a apresentar grande importância, pois o Cu apresenta elevada interação com grupos sulfidrila de enzimas e proteínas nos apoplastos das células radiculares, acumulando o metal absorvido predominantemente no sistema radicular, com pequena translocação para parte aérea (KABATA-PENDIAS, 2011; CAMBROLLÉ et al., 2015).

Na fioestabilização, as plantas devem ser tolerantes às condições de solo, crescer rapidamente para estabelecer densa cobertura vegetal, ter sistemas radiculares bem desenvolvidos, absorver grandes quantidades do metal e acumular no sistema radicular, ser fáceis de estabelecer e ter ciclos de vida longos ou serem capazes de auto-propagar

(SANTIBÁÑEZ et al., 2008; LEGUIZAMO et al., 2017). A adaptação as condições locais, ocorrência espontânea e o ciclo perene, confere as espécies nativas do Bioma Pampa grande potencial de emprego na remediação dos solos de vinhedos contaminados com Cu na região da Campanha. Além de possibilitar a fitorremediação *in situ*, a fitorremediação pode contribuir na melhoria da qualidade do solo, por contribuir na estruturação, aumento da porosidade e infiltração de água, ciclagem de nutrientes e proteção do solo aos processos erosivos.

5. RESULTADOS

Para avaliar os efeitos fitotóxicos do Cu e os mecanismos de tolerância expressos pelas diferentes espécies de plantas de cobertura do solo e videiras jovens, foram conduzidos cinco estudos com níveis crescentes de Cu, sendo estes:

I. Tolerance mechanisms and the potential use of *Lolium perenne* L. as cover crop in alleviating copper toxicity in two iron chelators.

II. Growth and chemical changes in the rhizosphere of black oat (*Avena strigosa*) grown in soils contaminated with Cu.

III. Copper bioavailability, tolerance and growth of native grasses of South American grasslands grown in copper-contaminated soils.

IV. Intercropping of young grapevines with native grasses for phytoremediation of Cu-contaminated soils.

V. Photosynthesis and growth of young grapevines intercropped with native grasses in soils contaminated with copper.

5.1 ESTUDO I

Tolerance mechanisms and the potential use of *Lolium perenne* L. as cover crop in alleviating copper toxicity in two iron chelators [1]

Abstract

Lolium perenne can expresses mechanisms of tolerance to excess copper (Cu). Therefore, it can be used in intercropping to decrease Cu toxicity in other crops. This study aimed to assess the mechanisms involved in the mitigation of Cu phytotoxicity and the effects of stress on growth, root morphology and nutrition of ryegrass (*Lolium perenne* L.) grown with two different Fe chelators. Seedlings of *Lolium perenne*. were hydroponically grown for 14 days in controlled conditions with 4 different levels of Cu (0.2, 5.0, 25 e 50 µM). Furthermore, iron (Fe) was supplied as either Fe-EDDHA or Fe-EDTA. The pattern of root exudates was determined during plant growth. At harvesting, roots and shoots were separated. Fresh weight, dry weight, and nutrient content were assessed at this time. Root morphology parameters were measured and root contents of organic acids and total phenolic compounds were also determined. Cu toxicity promoted the expression of the tolerance mechanisms of *Lolium perenne*, that include increased exudation and root content of organic anions, which can reduce Cu bioavailability and translocation by increasing Cu complexation. Fe chelators in Cu 50 promoted changes in root phenolic compounds, citrate and fumarate contents, changing the exudation of phenolic compounds. Differences in plant growth were not observed between Fe sources, although Cu concentration in plant tissue with the use of Fe-EDTA was lower in Cu 50. The increased exudation of phenolic compounds and chelating compounds in toxic levels of Cu indicates the potential of using *Lolium perenne* in intercropping. This is because root-induced changes reduce Cu bioavailability and benefit other crops.

Keywords: Phytoremediation, ionic speciation, cover crops, vineyards.

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[1] Artigo elaborado de acordo com as normas da Revista Chemosphere.

1. Introduction

The intensification of grape production systems has increased input application for nutrient addition and pathogen management. Successive applications of copper fungicides for the control of foliar diseases have promoted increase in soil Cu contents. Bordeaux mixture ($Ca(OH)_2$+$CuSO_4$), which has a high concentration of copper (Cu) in its composition, is the most commonly used fungicide (Komárek et al., 2010; Mackie et al., 2012; Miotto et al., 2014; Baldi et al., 2018).

Although Cu is a key element for important plant metabolic processes, excessive acquisition by the roots severely compromises plant growth. This induces changes in the root system, nutritional imbalances, oxidative stress and reactive oxygen species (ROS) accumulation (Yruela, 2005; Kabata-Pendias, 2011; Cambrollé et al., 2015). This has a negative impact on plant yield and quality, in addition to increasing the risk of introducing Cu into the food chain. Cu accumulation in vineyard soils may cause toxicity to grapevines and cover crops such as *Lolium perenne*, used for soil protection and nutrient cycling (Girotto et al., 2016; Montiel-Rozas et al., 2016; Tiecher et al., 2018). *Lolium pererne* exhibits good development in several types of soils, and it can be potentially used in soils contaminated with heavy metals (Bai et al., 2015). The adaptive mechanisms triggered by plants under conditions of nutritional stress (toxicity or deficiency) are associated to the increase in exudation and root content of organic anions. This changes translocation within the plant and bioavailability of chemical elements in the rhizosphere and root apoplast (Jones 1998; Meier et al., 2012; Dresler et al., 2014).

Under heavy metal toxicity, the main mechanisms involved in detoxification are low-molecular-weight organic acids (i.e. phenolic compounds, organic acids, amino acids and sugars) (Montiel-Rozas et al., 2016; Zafari et al. 2016). The presence of carbonyl and hydroxyl groups in low-molecular-weight organic acids promotes high reactivity with metals and changes ionic species by increasing complexation (Seshadri et al., 2015; Montiel-Rozas et al., 2016). Organic acid metal complexes are far less toxic than the corresponding free metal (Cu^{+2}). This prevents entry into the symplast, because uptake by plants and microorganisms occurs preferentially in Cu^{+2} (McBride, 1994; Kim et al., 2010; Meier et al., 2012). The synthesis of metal chelating compounds (phenolic compounds, organic acids and amino acids) can also be an intercellular mechanism of detoxification of heavy metals. These compounds increase complexation and compartmentalization of metals, especially in inactive parts such as the vacuolar pool. In addition, it changes the distribution of metals in the plant via the xylem, reducing translocation to the shoots, where it would cause greater damage (Dresler et al., 2014; Kisa et al., 2016). Phenolic compounds participate in the processes of ROS homeostasis in plant cells and contribute to reducing oxidative stress generated by excess heavy metals (Michalak, 2006; Majdoub et al., 2017).

The types and intensity of detoxification mechanisms expressed by plants vary between species, nutritional status, and the type and concentration of the contaminating metal (Meier et al., 2012; Dresler et al., 2014). In studies conducted in hydroponic solutions, it is necessary that iron (Fe) be added complexed with a chelator to avoid its interaction with anionic nutrients and induce nutritional deficiencies due to the formation of precipitates. However, when chelators are used to complex Fe, this may potentially also influence the free Cu (Cu^{+2}), changing chemical species distribution, increasing complexation and reducing bioavailability (Li et al., 2018). Identifying the adaptive mechanisms of tolerance to cover crops under toxicity is key for the success in phytoremediation of contaminated environments (Montiel-Rozas et al., 2016). Species that express mechanisms of tolerance outside the roots and reduce bioavailability of the contaminant in the rhizosphere can be potentially used in phytostabilization, possibly benefitting other intercropped species (Brunetto et al., 2016; Wan et al., 2017). Intercropping is an alternative for the phytoremediation of soils slightly or moderately contaminated with heavy metals, because it also enables phytoremediation of the soil with simultaneous agricultural production, increasing efficiency in land use (Wan et al, 2016; 2017). This study aimed to assess the mechanisms involved in the mitigation of Cu phytotoxicity and the effects of stress on growth, root morphology and nutrition of *Lolium perenne* grown with two different Fe chelators.

2. Material and Methods

2.1. Plant material, treatments and growing conditions

The study was carried out in hydroponics, under controlled conditions in a climatic chamber 14/10 h light/dark, 24/19 ºC, 70% relative humidity and 250 mmol m^{-2} s^{-1} light intensity. Seeds of ryegrass (*Lolium perenne* L.) were germinated on filter paper moistened with 0.5mM $CaSO_4$ solution in darkness for 5 days (Nikolic et al., 2012). After the germination period, ryegrass seedlings were transferred into a complete full-strength nutrient solution. The composition of the hydroponic solution was as follows (mM): 2 $Ca(NO_3)_2$, 0.7 K_2SO_4, 0.1 KH_2PO_4, 0.1 KCl, 0.5 $MgSO_4$, and (µM): 1.0 H_3BO_3, 0.5 $MnSO_4$, 0.2 $CuSO_4$, 0.5 $ZnSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$, 100 Fe(III)-EDTA. The solution was continuously aerated and changed every three days (Pii et al., 2015). The experimental units consisted of 2-L polyethylene containers with 1.6 L of nutrient solution. Each pot was arranged with 10 sets of seedlings (5 seedlings per set).

After 13 days of cultivation in solution of adaptation, hydroponic solution was changed and the plants were treated with Cu (0.2; 5.0; 25 and 50 µM) added as $CuSO_4$ and two Fe chelators (Fe sources) of 100 µM Fe: Fe(III)-EDDHA or Fe(III)-EDTA. In the Fe(III)-EDDHA treatment, plant roots were kept in 0.5 mM $CaSO_4$ solution for 1 h. The composition of the hydroponic solution after Cu and Fe treatments was as follows (mM): 2 $Ca(NO_3)_2$, 0.7 K_2SO_4, 0.05 KH_2PO_4, 0.1 KCl, 0.5 $MgSO_4$, and (µM): 1.0 H_3BO_3, 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$ and 10.0 MES KOH (pH 6.0). The solution was changed every three days. After applying the treatments, the plants were grown for another 14 days (27 days from germination) in same climatic chamber. The experimental design was completely randomized with 3 replicates, and consisted of 10 sets (5 seedlings per set) in each replicate.

2.2. Plant dry weight and nutrient analysis

After 14 days of applying the treatments, three sets per treatment were sampled, washed three times in distilled water and then separated into shoots and roots. Afterwards, samples were dried at 65 ° C until constant weight. Dry weight was recorded and subsequently ground for nutrient analysis. To determine total nutrient concentrations, samples were digested in a microwave-assisted procedure (CEM MARS Xpress, CEM Corporation, NC, USA), with concentrated 65% HNO_3 and 30% H_2O_2. Nutrient concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian 720-ES; Varian, Mulgrave, Australia).

2.3. Collection and analysis of root exudates

During the growth period, root exudates were collected at two times: 3 days (1st sampling) and 14 days (2nd sampling) after treatment application. At the time of each collect, five sets of plants per treatment were removed from the nutrient solution and the roots were washed three times in deionized water. Then, the roots of each set were separately submerged into 20 mL of aerated deionized water for 4 h. The pots were wrapped with aluminum foil to keep the roots in the dark. Trap solutions were filtered at 0.20 µm, freeze-dried, resuspended in double-distilled water and methanol (60:40 v/v) and stored at -20 ° C until further analysis. The roots were weighed soon after collecting exudates.

Total chelating compounds were determined colorimetrically using a modified procedure of the spectrophotometric Chrome Azurol S (CAS) method (Shenker et al., 1995). Absorbance was measured at 585 nm and the concentration of total chelating compounds was expressed as mmoles equivalent of Ethylenediaminetetraacetic acid (EDTA). The content of total phenolic compounds in root exudates was determined using the Folin-Ciocalteu method (Folin and Ciocalteu, 1927). Absorbance was measured at 765 nm and the concentration of the total phenolic compounds was expressed as mmoles equivalent of Gallic acid.

2.4. Organic acids, amino acids and phenols in root tissue

Roots collected 14 days after the application of treatments were washed three times in deionized water and instantly frozen in N_2, and then stored at -80 ° C until the time of analysis. Subsequently, the roots were macerated with liquid N_2 and a sample of 0.2 g was extracted with 2 ml of 100% methanol (v/v) overnight (12 hours) (Valentinuzzi et al., 2015). Then, the samples were centrifuged at 14000 g for 15 minutes and the supernatant was filtered at 0.45 µm.

The determination of organic acids (OAs) was done by high performance liquid chromatography (HPLC) using a cation exchange column (Rezex ROA, Phenomenex; 300×7.8 mm), with an isocratic elution with 10 mM H_2SO_4 as carrier solution at a flow rate of 0.6 ml min^{-1}. Organic acids were detected at 210 nm using a Waters Photodiode array detector (PDA 2998, Waters Spa, Italy). Standard acids were prepared as individual stock solutions using Sigma free acids and then combined to give diluted reference standards. The organic acids were identified by comparing retention times of unknowns to pure organic acids and by standard additions (Sandnes et al., 2005).

Amino acids (AAs) were separated by HPLC prior to a pre-column derivatization with a commercial kit (AccQ:Tag, WAT052880-Waters Corporation, Italy), using a high-efficiency Nova-PakTMC18 silica based bonded column (4.6 x 250 mm, 4 µm, Waters Corporation, Italy) with a gradient elution with a flow rate of 1.0 mL/min: A = AccQ:Tag Eluent (WAT052890), B = 100% acetonitrile (HPLC grade), C = Milli-Q (waters application note for AA). The derivatized samples were detected using fluorescence detection (λex = 250 nm, λems = 395 nm, (Waters 2475, Italy) with the column condition set at 37 º C.

Total phenolic compound content in root tissue was determined according previously described for trap solution.

2.5. Root architecture

Root morphology was assessed 14 days after treatment application in three replicates. We used WinRhizo Pro 2013 software coupled to an EPSON Expression 11000 scanner equipped with additional light (TPU), with a resolution of 200 and 600 dpi for shoots and roots, respectively. We measured total root length (cm plant^{-1}), root surface area (cm^2 plant^{-1}), volume (cm^3 plant^{-1}) and average diameter (mm).

2.6. Statistical analysis

The data are expressed as mean values ± SE. Analysis of variance was done with SISVAR software, version 4.0 (Ferreira, 2011). The parameters assessed in this study were compared between Cu levels within the same Fe source (EDDHA and EDTA) and between Fe sources within the same Cu level. The means were grouped by the Scott- Knott test at 5%.

3. Results

3.1. Plant growth and root morphology

The *Lolium perenne* growth was impaired by excess Cu. This was diagnosed by decreased shoot and root dry matter yield of plants grown in Cu 25 and Cu 50 (Figure 1). The roots showed higher sensitivity to Cu toxicity, with a reduction in dry matter yield in Cu 50 of 2265 and 2058% compared to Cu 0.2 in Fe-EDDHA and Fe-EDTA, respectively. Fe source did not affect plant growth.

The plants grown in Cu 25 and Cu 50 showed lower values of total root length, root surface area and root volume (Figure 2). The lowest values of these root parameters were found in Cu 50. However, higher values of average root diameter were found in the plants grown in the highest Cu level compared to the others. At the highest Cu level, average root diameter differed between Fe sources and it was lower in Fe-EDTA (Figure 2).

3.2. Nutrient concentration

Plants grown in Cu 50 in both Fe sources showed the lowest P concentrations in shoots (Table 1). The highest K concentrations in shoots and roots were found in plants grown in Cu 25 and Cu 50. The concentrations of Ca, Mg, P and S in roots did not differ statistically between Cu levels and Fe sources. The change in macronutrient concentrations between Fe sources was limited to S concentration in shoots of plants grown in Cu 50. The highest concentration of S in shoots was found in Cu 50 with Fe-EDTA.

The highest Cu concentrations in shoots were found in plants grown in higher Cu levels (Figure 3). However, Cu concentrations in shoots did not differ statistically between Cu 25 and Cu 50 in Fe-EDTA. Shoot Cu concentration in Cu 50 in Fe-EDDHA was 70.6% higher than in Fe-EDTA (Figure 3). Absorbed Cu accumulated mainly in roots of plants grown in Cu 25 and Cu 50. Roots of plants grown in Cu 50 showed 581 and 647 times more Cu compared to those in Cu 0.2 in Fe-EDDHA and Fe-EDTA, respectively. Root Cu concentrations in Cu 25 and Cu 50 were higher in Fe-EDDHA than in Fe-EDTA.

Fe concentrations in plant shoots did not differ statistically between Cu levels in Fe-EDTA, but increased in Cu 50 in Fe-EDDHA (Figure 3). The highest concentrations of Fe, Mn and Zn in roots were found in higher Cu levels in both Fe sources. The lowest concentrations of Mn and Zn in shoots were found in Cu 50 in Fe-EDDHA, but did not differ statistically from Cu 0.2 in Fe-EDTA.

3.3. Characterization of root exudates

Root exudates released for *Lolium perenne* were collected during the cultivation period and analyzed to determine total phenolic compounds, total chelating compounds and organic acids. The highest concentrations of total phenolic compounds (expressed as Gallic acid equivalents) were found in phytotoxic levels (25 and 50 μM) 3 days after treatment application (1st sampling) (Figure 4a). This increase in total phenolic compound concentration was approximately 2.98 times in Fe-EDDHA and 5.94 times in Fe-EDTA. After 14 days of treatment application (2nd sampling), the increase in the concentration of total phenolic compounds was limited to Cu 50 (Figure 4b). At this Cu level, the concentration in Fe-EDDHA was 2.35 times higher than in Fe-EDTA.

Exudation pattern of total chelating compounds (expressed as Ethylenediaminetetraacetic acid equivalents) exhibited the same trend over total phenolic compounds, increasing concentration in plants grown in Cu 25 and Cu 50 at both collection times (Figure 4c, d). Fe sources did not significantly change the concentration of total chelating compounds in *Lolium perenne*. Exudation of organic acids could not be determined because the concentration in trap solutions was below the detection limit.

3.4. Organic acid, total phenolic compound and amino acid contents in roots

Four types of organic acids were identified in root extracts of *Lolium perenne*. Succinate showed the highest content, followed by citrate, malate and fumarate (Figure 5). Citrate content increased in Cu 50 in Fe-EDTA. At this Cu level, citrate content was 83.3% higher in comparison to Fe-EDHHA (Figure 4a).

Root succinate content did not differ statistically between Fe sources, but decreased with increasing Cu levels. The lowest contents were found in Cu 50 (Figure 5c). The reduction in succinate contents in Cu 50 compared to Cu 0.2 was 262 and 315% in Fe-EDDHA and Fe-EDTA, respectively (Figure 5c). Root malate content was slightly changed by Cu levels and Fe sources. However, root fumarate content decreased in plants grown in Cu 25 and Cu 50 in relation to the lower Cu levels (Cu 0.2 and Cu 5.0). Root fumarate contents in Cu 5.0, Cu 25 and Cu 50 were higher in Fe-EDDHA compared to the same Cu levels in Fe-EDTA. Total phenolic compound content in root extracts followed the same trend found in the concentration of exudates of the 2nd sampling. The highest contents of root phenolic compounds were found in Cu 25 and Cu 50. Roots grown in Cu 50 in Fe-EDDHA showed a 28.15% increase in total phenolic compound content in comparison to Fe-EDTA (Figure 5e).

The physiological changes caused by excess Cu affected amino acid content in the roots of *Lolium perenne*. There was an increase in serine and glycine contents in Cu 50 compared to Cu 0.2 in both Fe sources (Supplemental Fig. 1). The highest root alanine and leucine contents were found in Cu 50 in Fe-EDTA, but contents did not differ between Cu levels in Fe-EDDHA. On the other hand, cysteine and valine contents reduced in the highest Cu levels in both Fe sources (Supplemental Fig. 2). In Cu 5.0 and Cu 25, higher root serine content was found in Fe-EDDHA compared to Fe-EDTA. In Cu 50, higher root glycine content was found in Fe-EDDHA compared to Fe-EDTA. The highest root alanine content was found in Cu 50 in Fe-EDTA.

3.5. Principal component analysis

Principal component analysis (PCA) of variables Cu levels and Fe sources explained 70.1% of the variability of the data in first two components (Figure 6). The first component (PC1) explained 53.43 % of the variation and showed separation of Cu levels 0.2 and 5.0 µM from Cu 25 and 50 µM. This is largely negatively determined by dry matter yield, and accumulation of macronutrients and Mn and Zn. PC1 has strong positive loadings on phenolic compound concentration in roots and exudates, Cu accumulation, average root diameter and chelating compound exudates. On the other hand, the second component (PC2) explained 16.67% of the variation. PC2 was negatively determined by the accumulation of Fe and K and positively determined by organic acid concentration in roots. There was a small separation between Fe sources in Cu 50.

4. Discussion

The decrease in root and shoot dry matter yield of *Lolium perenne* grown in Cu 25 and Cu 50 (Figure 1a, b) demonstrates the toxic effect of excess Cu on plant growth (Cambrollé et al., 2015 Girotto et al., 2016; Baldi et al., 2018). This may occur because excess Cu induces physiological and biochemical changes which inhibit cell elongation and division (Kabata-Pendias, 2011; Ambrosini et al., 2015). Furthermore, excess Cu in tissue may compromise the proper functioning of the photosynthetic apparatus, consequently reducing the rate of CO_2 fixation, caused by increased levels of lipid peroxidation, chlorophyll degradation and concentration of reactive species of oxygen (ROS) (Cambrollé et al., 2015; Girotto et al., 2016).

Higher Cu levels reduced root length, surface area and volume, but increased average root diameter of plants grown in Cu 50, regardless of the Fe source (Figure 2). Root morphological changes may be part of a hormonal response or imbalance induced by excess Cu, modifying or inhibiting cell multiplication, especially in the meristematic regions (Potters et al., 2009; Bochicchio et al., 2015). The increase in average root diameter may be attributed to changes in the division and organization of cortical cells, increasing the area of the cortex and diameter (Ambrosini et al., 2015). The decrease in root growth may be related to higher mechanical resistance. This is a result of the accumulation of phenolic compounds in endodermal cells, which reinforce the cell walls and form a physical barrier against the entry and distribution of heavy metals in the vascular cylinder. Thus, there is reduced transport to the shoots, where severe damage can be done (Michalak, 2006; Ambrosini et al., 2015). Changes in the morphological and anatomical structure of the roots tend to compromise cover crop growth in vineyards. By limiting water and nutrient uptake, especially in chemically poor soils and in regions with low rainfall, there is reduced soil protection and nutrient cycling (Bai et al., 2015; Girotto et al., 2016).

Shoot Cu concentration of plants grown in higher Cu levels (Figure 3) was above the range considered adequate for most plant species (5-15 mg kg^{-1} of DM - Adrees et al., 2015). The high Cu concentration in tissue probably explains the decrease in plant growth, evidenced by the inverse correlation between dry matter yield and Cu accumulation in PCA (Figure 6a). Excess Cu may affect the performance of membrane carriers and ion channels. This may reduce selective capacity and increase non-specific permeability of the membrane, which causes nutritional imbalance in environments contaminated with heavy metals (Cambrollé et al., 2015).

Most of the Cu absorbed in Cu 25 and Cu 50 accumulated in roots, and there was little translocation to shoots (Figure 3). The accumulation of Cu in roots is a tolerance mechanism triggered by several species when exposed to toxicity conditions as a way to prevent and/or reduce translocation of excess Cu to shoots (Cambrollé et al., 2015; Girotto et al., 2016). This accumulation in roots may be associated with the strong interaction of Cu with sulfhydryl groups of enzymes and proteins found in root cell apoplasts, preventing its movement to the symplast (Yruela, 2005). It may also be associated to the intracellular compartmentalization of Cu in compartments with low metabolic activity, such as the vacuole of the root cells where Cu is complexed with organic compounds or phosphate ions (Dresler et al., 2014; Baldi et al., 2018). This hypothesis of the formation of Cu-P precipitates is supported by reduced P contents in shoots in Cu 50, even if root P contents did not differ between Cu levels (Table 1). This indicates that excess Cu compromises nutrient distribution between plant organs, inducing nutritional imbalance. It also shows that increased P availability/uptake tends to increase the tolerance of cover crops to excess Cu (Guimarães et al., 2016).

The decrease in Cu concentration in shoots and roots in Cu 50 with the addition of Fe-EDTA (Figure 3) was probably due to the complexation of Cu^{+2} by EDTA in the culture solution, thus reducing availability. This is because Cu uptake by plants and microorganisms occurs preferentially in free form (Cu^{+2}), which is the hydrated divalent species (McBride, 1994). The lower complexing potential of EDDHA probably contributed to the maintenance of Cu^{+2} contents in the culture solution, favoring Cu uptake in higher Cu levels (Cu 25 and Cu 50). In growing soybean in Cu-toxic solutions with four Fe chelators, Li et al. (2018) found an increase in root elongation rates with the use of Fe-EDTA, demonstrating a reduction in toxicity by the influence on metal speciation.

The increase in exudation of total phenolic compounds and chelating compounds in higher Cu levels (Figure 4) indicates that *Lolium perenne* express external root detoxification mechanisms at toxic levels. These organic compounds exuded by the roots have hydroxyl and carbonyl groups, which promotes a high capacity to form complexes with metal ions in the soil solution, reducing Cu^{+2} concentration and bioavailability (Seshadri et al., 2015). This change in the bioavailability through the exudation of organic ligands under conditions of toxicity or deficiency is more expressive in the rhizosphere, which is a thin soil layer around the roots (Hinsinger et al., 2009). Plant adaptation to environmental stress was reported by Bravin et al. (2012), who verified the limitation of Cu bioavailability in rhizosphere of wheat grown in acidic soils contaminated by Cu. The increase in the exudation of phenolic compounds is associated with root content, which was verified in PCA (Figure 6).

Phenolic compounds are considered secondary metabolites in higher plants and play an important role in plant adaptation to the environment and in overcoming stress conditions (Kısa et al., 2016). Phenolic compound content increased in roots of plants exposed to higher Cu levels (Figure 5e). This change in phenolic metabolism possibly occurred in response to the deleterious effects of excess Cu. This contributes to Cu chelation and accumulation in root cells and reduces translocation to shoots, where it would cause greater disturbances to the plant, because of the important physiological processes that occur in this organ, such as photosynthesis (Ambrosini et al., 2015; Cambrollé et al., 2015). Kısa et al. (2016) reported increase of total phenolic compound contents with the application of Cu doses in maize. Phenolics are part of the plant non-enzymatic antioxidant system. As excess Cu induces oxidative damage by producing toxic free radicals, phenolics can attenuate these damages by participating in ROS homeostasis in plant cells, as it is a mechanism for H_2O_2 scavenging (Michalak, 2006). Majdoub et al. (2017) found correlation between phenolic concentration and antioxidant activity in different plant species exposed to excess Zn.

Organic acids participate in important plant biochemical processes, such as energy generation, amino acid biosynthesis, cation transport, maintenance of osmotic potential and reduction of heavy metal stress (Jones, 1998; Dresler et al., 2014). The ability and stability of the formation of complexes between organic acids and metal ions depends on their dissociation properties, number, proximity of the carboxylic groups and type of metal ion (Jones, 1998).

The organic acids detected in roots of *Lolium perenne* were citrate, malate, succinate and fumarate (Figure 5). Several factors may affect the composition and content of organic acids, such as species, nutrition and abiotic and biotic stresses, as they promote changes in the Calvin cycle, where the production of organic acids takes place (Montiel-Rozas et al., 2016). Changes in citrate and fumarate content between Fe sources may be related to nutritional changes in micronutrients, especially Cu concentration. The highest citrate content in Fe-EDTA in Cu 50 probably contributed to the reduction of root and shoot Cu contents compared to Fe-EDDHA, even with higher fumarate content in Fe-EDDHA. This is because the stability of the Cu-citrate complex is higher than Cu-fumarate complex (Borges et al., 2005). Increased citrate contents in roots of plants exposed to high Cu levels were found in young grapevines (Baldi et al., 2018), maize (Dresler et al., 2014) and *Imperata condensata* (Meier et al., 2012).

The extracellular role of organic acids in the detoxification of heavy metals is related to the reduction in root uptake, by complexing metals in the apoplast and rhizosphere, preventing entry into the symplast (Meier et al., 2012; Montiel-Rozas et al., 2016). Carboxylic acids can act in the detoxification of complexed heavy metals within the cells, especially in inactive parts such as the vacuolar pool. Thus, this contributes to Cu compartmentalization, in addition to changing Cu distribution in the plant via the xylem (Dresler et al., 2014). In addition to toxicity conditions, the metabolism of organic acids can be modified in response to nutritional deficiencies, increasing bioavailability through the exudation of organic compounds that promote the solubilization and chelation of chemical elements (Pii et al., 2015). The reduction of succinate and fumarate with increasing Cu levels possibly occurred by the allocation of C to generate phenolic compounds and citrate in Fe-EDTA (Figure 5).

The accumulation and exudation of organic anions in response to toxic Cu concentrations in the culture solution probably contributed to reduced plant growth. Changes in carbon metabolism, which directs the fixed C to these compounds, reduces the amount allocated to forming new structures and limits growth. The accumulation and exudation of amino acids is also a protective mechanism that can be triggered by some plant species in adverse environmental conditions, given its ability to act as a ligand, which promotes the complexation of elements (Pii et al., 2015). The synthesis and accumulation of amino acids under heavy metal stress conditions can be a strategy to store energy during limited growth and photosynthesis, in addition participating in the stabilization of enzymes (Zafari et al., 2016). The reduction of cysteine content in the highest Cu level is probably related to the increase in root content and the exudation of phenolic compounds, because it participates in the synthesis of these compounds which function as antioxidants (Ali et al., 2006). Cu toxicity increased glycine content. Zafari et al. (2016) also found an increase in glycine accumulation in plants subjected to lead (Pb) stress. This suggests the association of this amino acid with the biosynthesis of phytochelatin and glutamate, both involved in detoxification. Pii et al. (2015) found an increase in glycine content in cucumber plants subjected to Fe deficiency. These results show that glycine is associated with the adaptive mechanisms to adverse conditions of toxicity or deficiency of chemical elements.

5. Conclusions

Cu toxicity promoted the expression of the tolerance mechanisms of *Lolium perenne*, that include increased exudation and root content of organic anions, which can reduce Cu bioavailability and translocation by increasing Cu complexation.

Fe chelators in Cu 50 promoted changes in root phenolic compounds, citrate and fumarate contents, changing the exudation of phenolic compounds. Differences in plant growth were not observed between Fe sources, although Cu concentration in plant tissue with the use of Fe-EDTA was lower in Cu 50.

The increase in the exudation of phenolic compounds and chelating compounds in toxic levels of Cu indicates the potential of using *Lolium perenne* in intercropping, because it reduces Cu bioavailability by root-induced modifications and benefits other crops.

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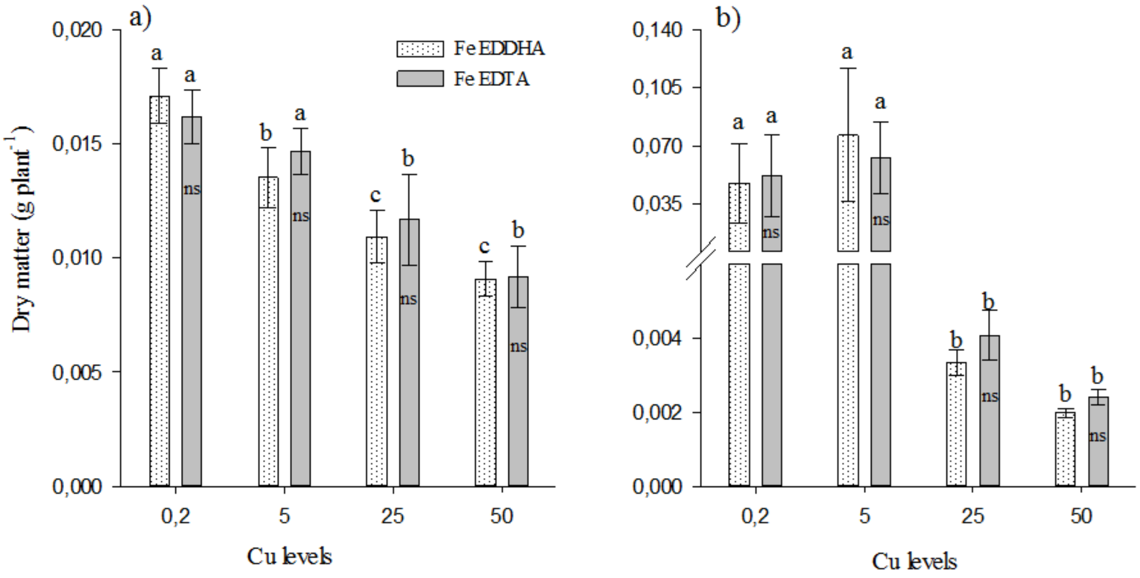


Figure 1. Dry matter yield of *Lolium perenne* exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} non-significant; * significant (F test $p<0.05$) difference between Fe source at the same Cu level.

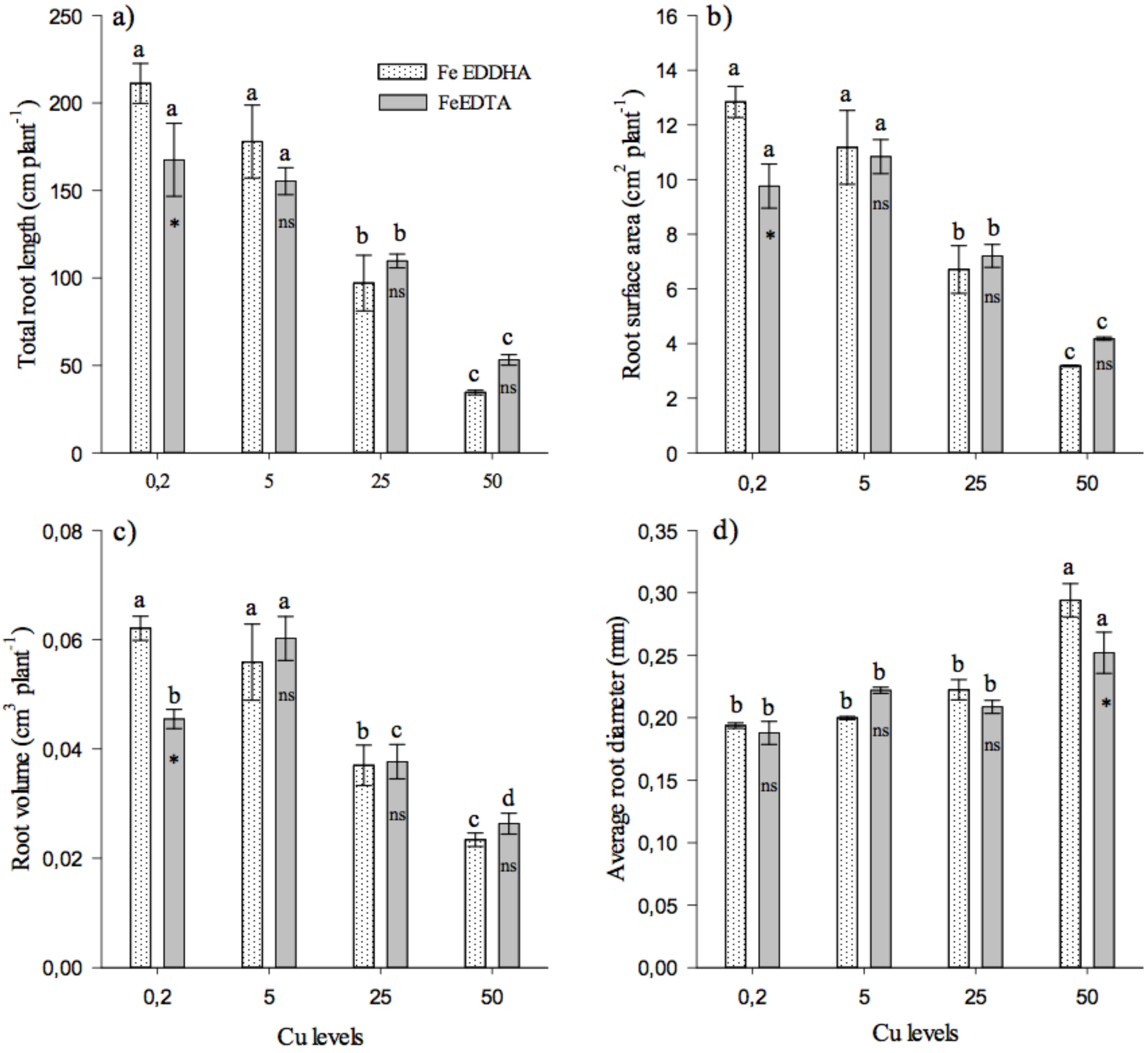


Figure 2. Root morphological parameters of *Lolium perenne* exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} non-significant; * significant (F test $p<0.05$) difference between Fe source at the same Cu level.

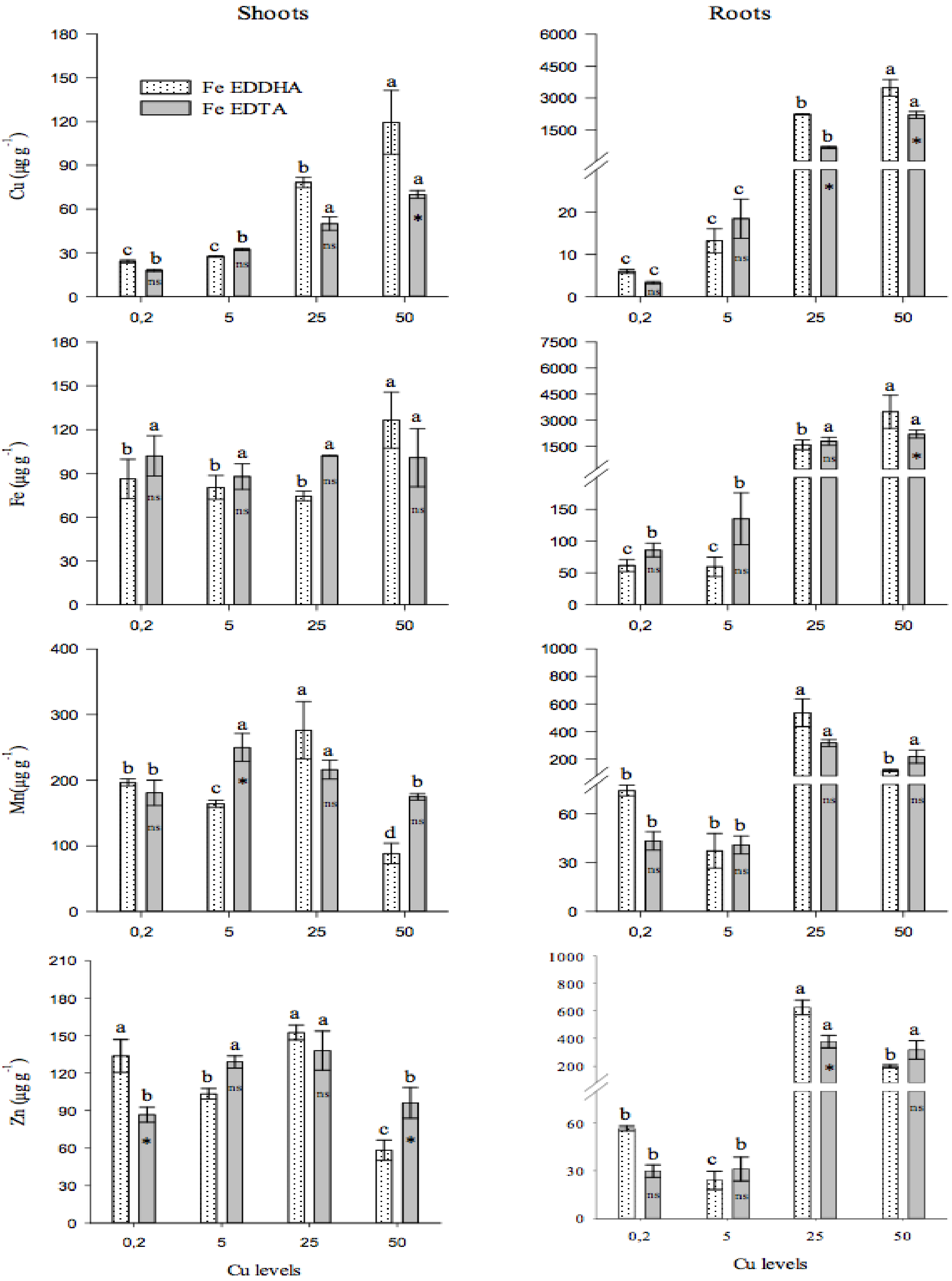


Figure 3. Micronutrient contents in shoot and root of *Lolium perenne* exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} non-significant; * significant (F test $p<0.05$) difference between Fe source at the same Cu level.

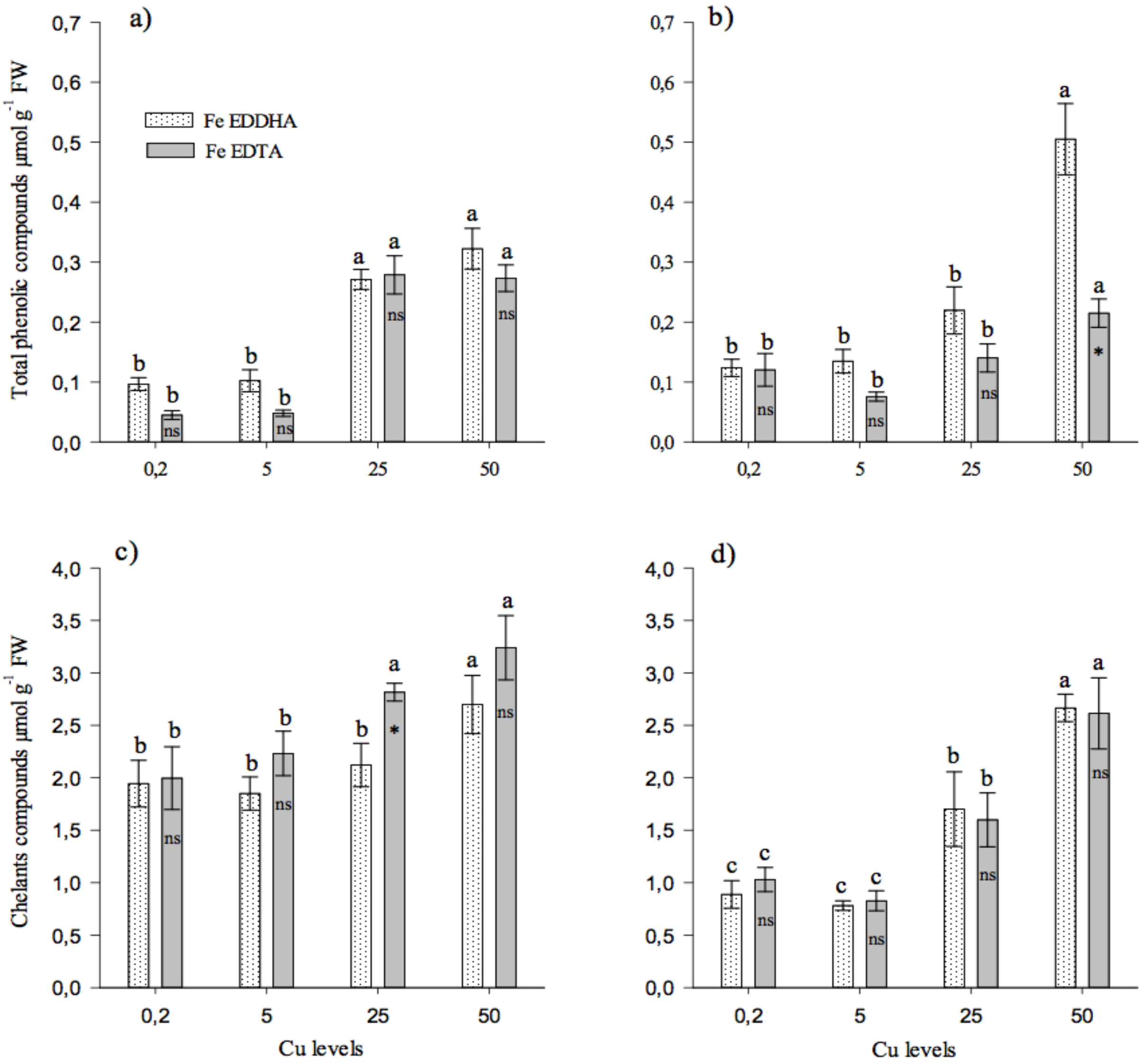


Figure 4. Concentration of total phenolic compounds and chelating compounds 3 days (a and c) and 14 days (b and d) after exposure of *Lolium perenne* to increasing Cu levels, in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p < 0.05$). ^{ns} non-significant; * significant (F test $p < 0.05$) difference between Fe source at the same Cu level.

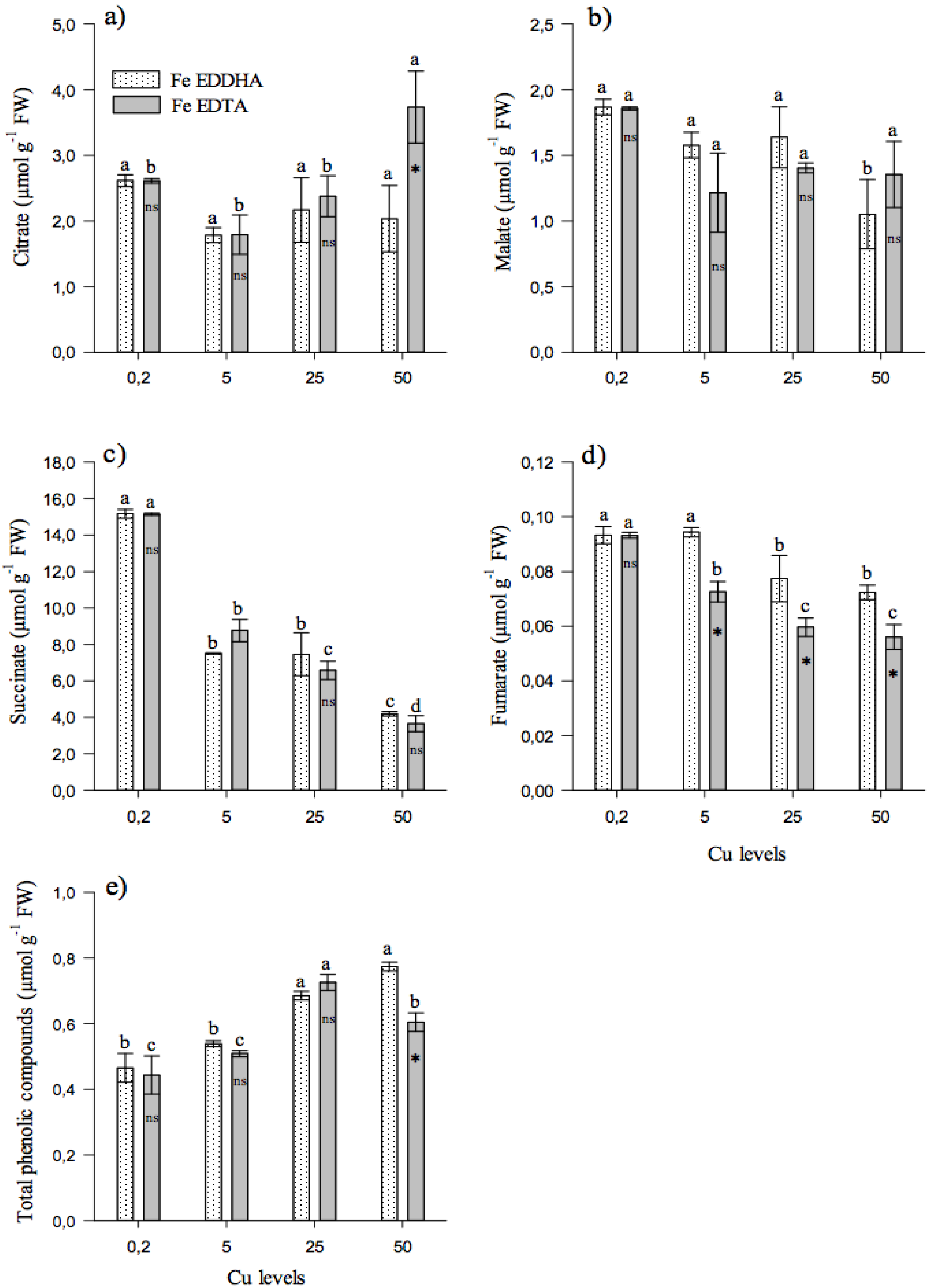


Figure 5. Accumulation of organic acids and total phenolic compounds in *Lolium perenne* roots exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} non-significant; * significant (F test $p <0.05$) difference between Fe source at the same Cu level.

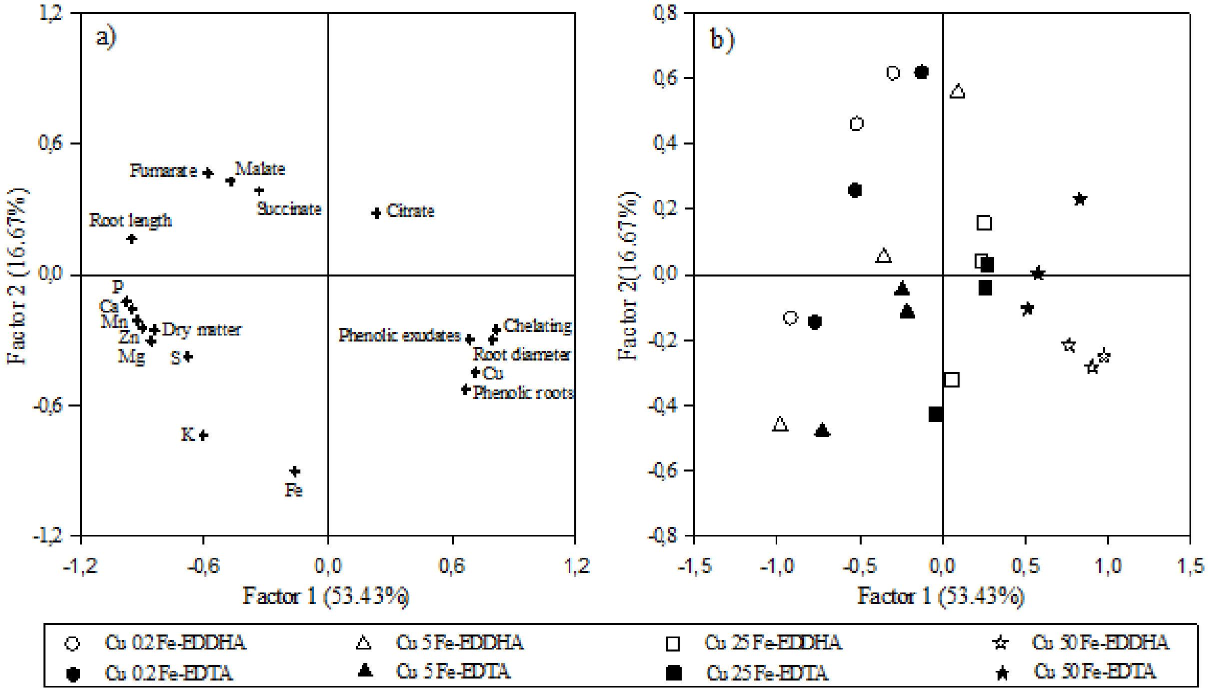
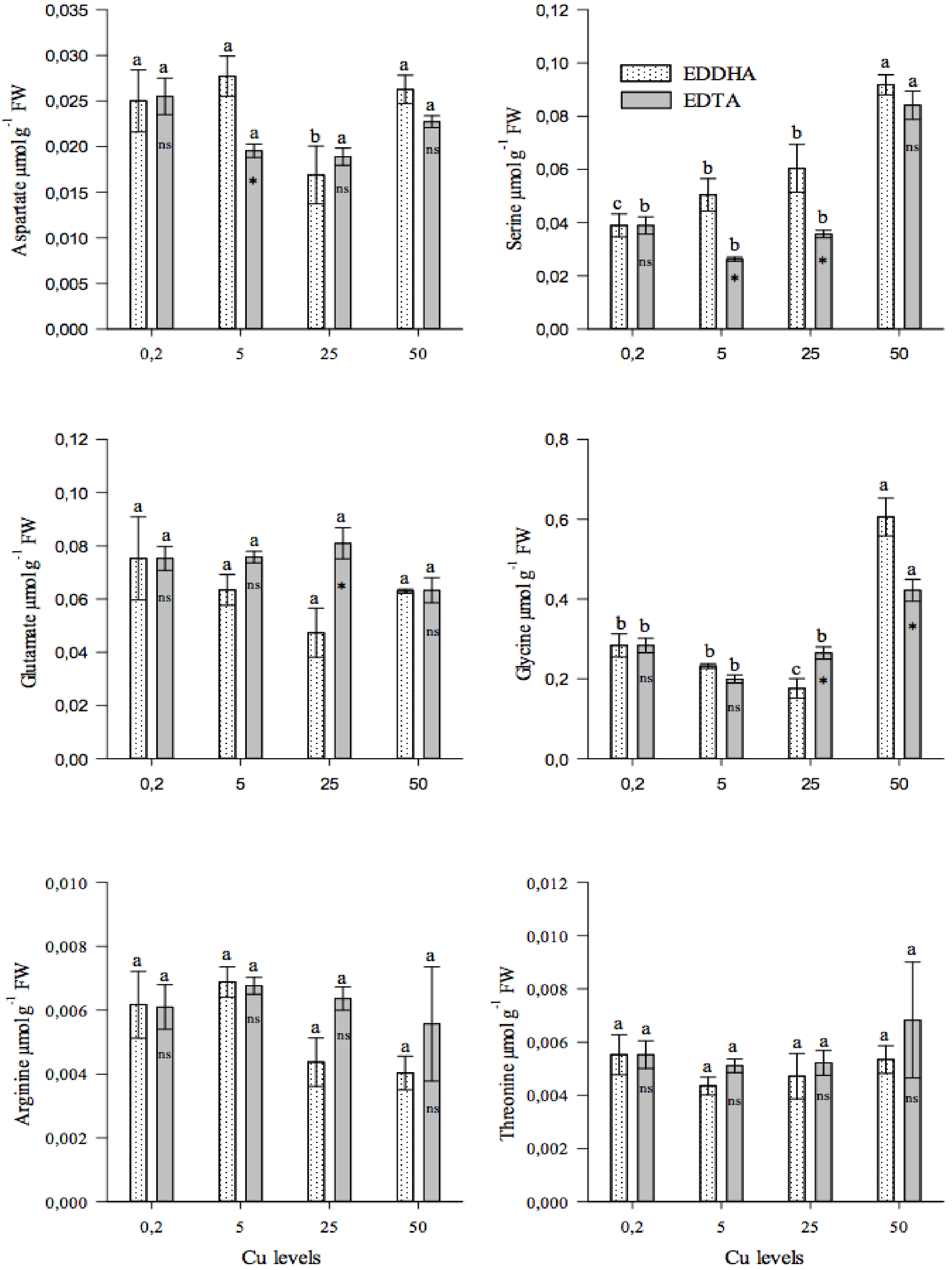


Figure 6. Scatter plot of principal component analysis (PCA) of the total dry matter (shoot + root), root morphological parameters, accumulation of nutrients, phenolic compounds and chelating compounds in exudates and accumulation of organic acids and phenolic compounds in root tissue of *Lolium perenne* exposed to increasing Cu levels for 14 days, in two Fe sources.

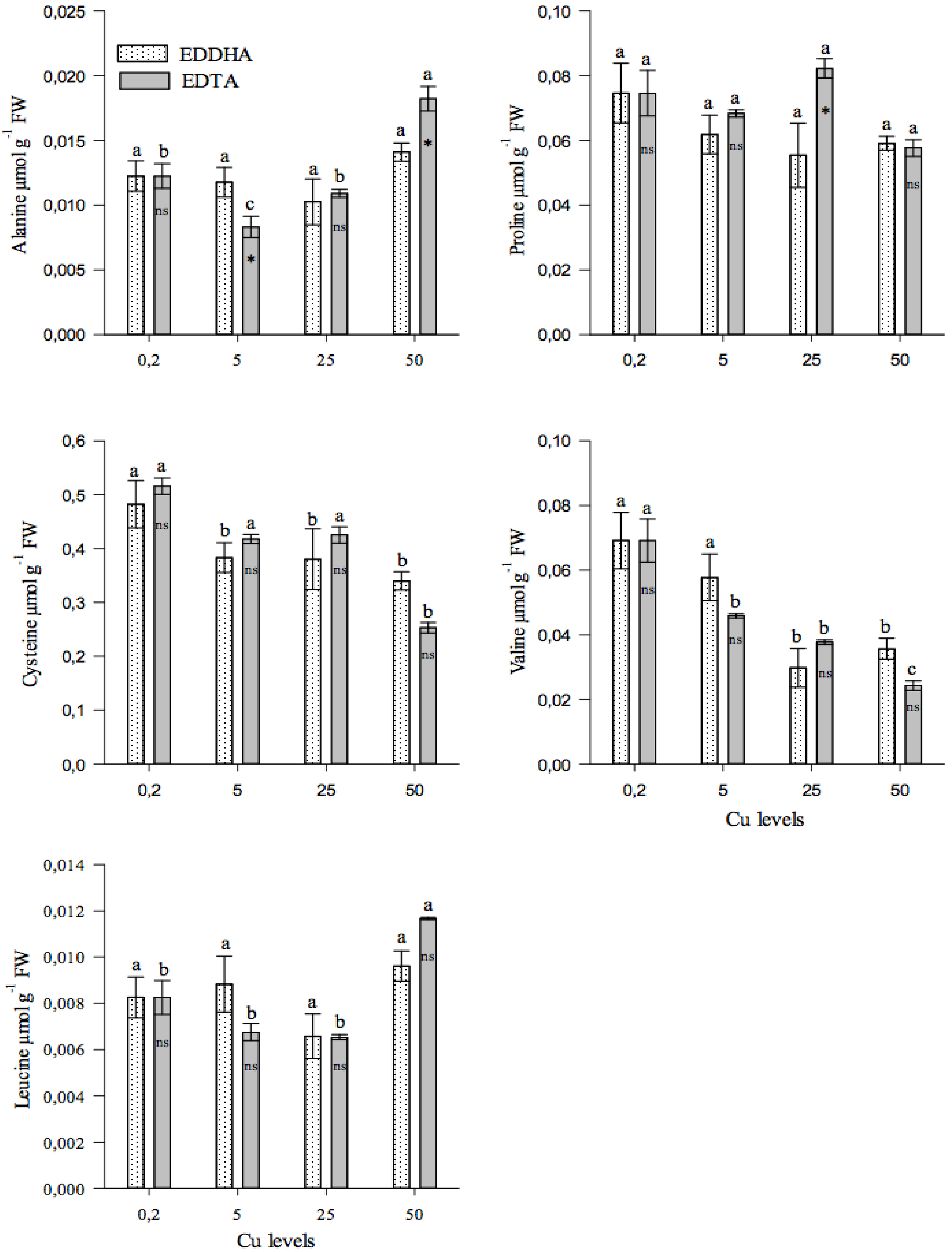
Table 1. Macronutrient contents in shoot and root of *Lolium perenne* exposed to increasing Cu levels for 14 days in two Fe sources.

Element	Fe Source	Cu levels			
		0.2	5.0	25	50
		Shoot			
Ca (mg g^{-1})	EDDHA	6.13 a(1)	5.62 a	6.64 a	5.74 a
	EDTA	6.06 a^{ns}	6.64 a^{ns}	6.40 a^{ns}	5.04 a^{ns}
K (mg g^{-1})	EDDHA	8.54 b	13.03 b	17.02 a	20.49 a
	EDTA	9.18 b^{ns}	11.66 b^{ns}	16.20 a^{ns}	20.93 a^{ns}
Mg (mg g^{-1})	EDDHA	1.75 b	1.74 b	2.34 a	2.05 a
	EDTA	1.85 a^{ns}	2.02 a^{ns}	2.03 a^{ns}	1.76 a^{ns}
P (mg g^{-1})	EDDHA	3.72 a	3.56 a	3.28 a	2.28 b
	EDTA	3.44 a^{ns}	3.44 a^{ns}	3.37 a^{ns}	2.12 b^{ns}
S (mg g^{-1})	EDDHA	2.50 a	2.36 a	3.41 a	3.03 a
	EDTA	2.15 b^{ns}	2.17 b^{ns}	3.29 b^{ns}	6.95 a*
		Root			
Ca (mg g^{-1})	EDDHA	1.04 a	0.46 a	1.41 a	1.51 a
	EDTA	0.88 a^{ns}	0.24 a^{ns}	1.27 a^{ns}	1.82 a^{ns}
K (mg g^{-1})	EDDHA	9.61 b	3.73 b	22.81 a	16.39 a
	EDTA	10.07 b^{ns}	2.70 b^{ns}	22.63 a^{ns}	21.68 a^{ns}
Mg (mg g^{-1})	EDDHA	0.48 a	0.16 a	0.76 a	0.64 a
	EDTA	0.41 a^{ns}	0.11 a^{ns}	0.68 a^{ns}	0.59 a^{ns}
P (mg g^{-1})	EDDHA	1.64 a	0.57 a	2.44 a	2.07 a
	EDTA	1.59 a^{ns}	0.40 a^{ns}	2.84 a^{ns}	1.97 a^{ns}
S (mg g^{-1})	EDDHA	6.35 a	6.63 a	2.44 a	2.24 a
	EDTA	3.19 a^{ns}	1.12 a^{ns}	1.79 a^{ns}	2.47 a^{ns}

(1) Means followed by the same letter do not differ between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} non-significant; * significant (F test $p < 0.05$) difference between Fe source at the same Cu level.



Supplement 1. Total amino acid (AA) concentration in roots of *Lolium perenne* exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} = non-significant; * significant (F test $p<0.05$) difference between Fe source at the same Cu level.



Supplement 2. Total amino acid (AA) concentration in roots of *Lolium perenne* exposed to increasing Cu levels for 14 days, in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p<0.05$). ^{ns} non-significant; * significant (F test $p<0.05$) difference between Fe source at the same Cu level.

5.2 ESTUDO II

Growth and chemical changes in the rhizosphere of black oat (*Avena strigosa*) grown in soils contaminated with copper

Lessandro De Conti, Carlos A. Ceretta, Tadeu L. Tiecher, Lincon O. S. Silva, Adriele Tassinari, Luiza M. Somavilla, Tanja Mimmo, Stefano Cesco, Gustavo Brunetto

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Growth and chemical changes in the rhizosphere of black oat (*Avena strigosa*) grown in soils contaminated with copper [2]

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Abstract

Copper based pesticides are used to protect vineyards from fungal infections. Plants like black oats (*Avena strigosa* Schreb) can promote chemical changes in the rhizosphere, reducing copper (Cu) bioavailability in contaminated soils. The objective of this study was to evaluate how copper additions would affect growth, morphology and nutrient uptake by black oats and how the plants affect the chemical composition in rhizosphere and bulk soil. The soil was collected in grassland of southern Brazil. The soil was air-dried, adjusted pH and added phosphorus and potassium amendments, and then it was incubated. Three Cu levels were established in the soil with the addition of 0, 40 and 80 mg Cu kg^{-1}. The experimental design consisted of pots containing 8 plants with 10 kg of soil. Rhizosphere (2 kg of soil) and bulk (8 kg of soil) separated by a 30 µm nylon membrane. Black oat plants were grown for 54 days. The soil and solution were chemically characterized throughout cultivation for Cu speciation. At 54 days after emergence, the soil was sampled and proceeded chemical analysis and plants were collected to determine yield dry matter, morphological parameters and nutrient concentration. Black oat plants induce increase of pH and dissolved organic carbon in the rhizosphere. These root-induced processes increase the percentage of complexed chemical species and decrease free Cu^{+2} in soil solution, decreasing Cu toxicity. However, soil contamination with Cu induces morphological changes and nutritional imbalances. Black oats could thus be planted along with vineyards, for such increasing protect the soil and promote nutrient cycling, as well as reduce the free Cu available fraction due to the root-induced modifications in the rhizosphere.

Keywords: bioavailability, chemical speciation, heavy metals, phytotoxicity.

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[2] Artigo elaborado de acordo com as normas da Revista Ecotoxicology and Environmental Safety.

1. Introduction

More than 90% of the national wine production in Brazil is concentrated in the Southern region of the country; mostly in the state of *Rio Grande do Sul* (Flores and Medeiros, 2013). Due to the specific regional climatic conditions – *i.e.* high temperature and humidity- the incidence of fungal diseases is generally high in spring and summer requiring frequent fungicide applications (especially copper-based) in order to ensure plant and fruit protection. This phenomenon is not limited to Brazil, its occurs in other regions affecting other viticultural areas (Komárek et al., 2010; Miotto et al., 2014; Babcsányi et al., 2016). As a consequence of this intensive agronomic practice, copper (Cu) concentration in soil is gradually increasing reaching, in some cases, toxic Cu levels to plants (Miotto et al., 2014; Girotto et al., 2014; Cambrollé et al., 2015; Tiecher et al., 2016). Even though Cu is essential for important metabolic processes (Marschner, 2011), its higher uptake by roots compromises severely the growth of plants inducing morphological changes and nutritional imbalances (Kabata-Pendias, 2011; Cambrollé et al., 2015) with negative impacts to plant yield and quality of the fruit. The incidence of this phenomenon is constantly growing not only in terms of geographical area but their symptoms also increase in severity (Komárek et al., 2010; Mackie et al., 2012; Miotto et al., 2014; Babcsányi et al., 2016; Baldi et al., 2018).

With the exception of grasses, which take up Cu-phytosiderophores complexes, the Cu sources used by roots for nutrient acquisition are mainly the ionic ones (Cu^{2+} and/or Cu^{+} (Marschner, 2011; Brunetto et al., 2016). In soil, the magnitude of this pool is influenced by the total soil concentration of the micronutrient itself as well as by physicochemical characteristics such as clay types, organic matter levels, soil pH and cation exchange capacity (CEC) (McBride, 1994; Bradl, 2004). In the rhizosphere, the processes underlying the soil-root-microorganism interactions can further affect Cu availability (Hinsinger, 2001; Bravin et al., 2012; Terzano et al., 2015). In particular, the root exudation of low-molecular-weight organic ligands with high affinity for Cu (such as citric acid, malic acid, fumaric acid, oxalic acid and succinic acid), phytosiderophores and phenolic compounds, can affect the free Cu^{2+} fraction in soil (Jones and Darrah, 1994; Chaignon et al., 2009; Kim et al., 2010; Brunetto et al., 2016). Since the distribution of Cu species in soil solution is controlled mainly by pH and dissolved organic carbon (DOC), a triggered release of root exudates and/or microbial degradation of soil organic matter can increase the percentage of Cu species complexed with these ligands (Cu-DOC). Complexed Cu may reduce bioavailability with a consequent reduction of the phytotoxic potential in contaminated soils, increasing the mobility of the metal in the soil profile (Chaignon et al., 2009; Kim et al., 2010; De Conti et al., 2016).

Plants grown in soils contaminated with heavy metals may express mechanisms that are adaptive to stress conditions (Brunetto et al., 2016). The increase in the exudation of chelating substances and rhizosphere pH are changes that reduce Cu bioavailability, favoring Cu^{2+} complexation (Bravin et al., 2012; Meier et al., 2012). On the other hand, acidification of the rhizosphere can significantly contribute to increase the extent of the available Cu pool (Tomasi et al., 2009).

Cover crops such as black oats are commonly grown in old vineyards, because this agronomic practice mainly aimed at conserving soil and/or protecting it from erosion, particularly during grapevine dormancy in autumn and winter, plants lose their foliage which increases soil erosion (Colugnati et al., 2003; Barlow et al., 2009; Babcsányi et al., 2016). Oat-induced modifications in the root environment may thus have a large effect on Cu bioavailability, reducing phytotoxicity throughout cultivation. This study aimed evaluate how copper additions would affect growth, morphology and nutrient uptake by black oats and how the plants affect the chemical composition in rhizosphere and bulk soil.

2. Material and Methods

2.1. Characterization of soil and treatments

The experiment was conducted using a Typic Hapludalf soil (Soil Survey Staff, 2006) collected at depth of 0-20 cm in an area of uncultivated grassland (30º47'23.7 "S and 55º22'7.3 " W) with the following characteristics: 54 g kg^{-1} clay, 52 g kg^{-1} silt, 894 g kg^{-1} sand and 9 g kg^{-1} organic matter (OM). The area was located in the city of Santana do Livramento, state of Rio Grande do Sul, southern Brazil. After sampling, the soil was air-dried, homogenized and passed through a 2 mm mesh sieve. Soil pH was adjusted by adding a mixture of calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$) with a 2:1 ratio at a concentration of 0.57 g kg^{-1} soil. The soil was consequently incubated for 35 days at a constant moisture of 80% maximum water holding capacity (MWHC). Afterwards, 40 mg kg^{-1} of phosphorus (P) and 100 mg kg^{-1} of potassium (K) soil were applied to the soil by adding triple superphosphate and potassium chloride, respectively. Moisture was restored to 80% MWHC and incubation continued for another 25 days.

Treatments consisted of three Cu levels; soil with natural Cu concentration, representative of the condition prior to the implantation of vineyards (Cu 0) and with the addition of 40 and 80 mg Cu kg^{-1} (Cu 40 and Cu 80), which are levels normally observed in vineyards of approximately 15 and 30 years of grapevine cultivation, respectively (Miotto et al., 2014). The addition of Cu occurred 50 days after the application of the corrective, by applying a solution of Cu sulfate ($CuSO_4.\ 5H_2O$ – PA reagent, Vetec). Subsequently, the soil was incubated an additional 45 days under the same conditions described above. Three times a week throughout the incubation period, the soil was mixed thoroughly and weighed to evaluate moisture levels; distilled water was added to maintain the MWHC at 80%.

2.2. Experimental design and crops

The experiment was conducted in a completely randomized design with four replications. The experimental units consisted of 11-L pots filled with 10 kg of soil. A soil portion of 2 kg was separated by a 30 µm nylon membrane and considered as rhizosphere (Figure 1). To simulate rhizosphere conditions, the 2-kg-soil portion was seeded with oat plants. Fifteen oat seeds (*Avena strigosa* Schreb.) were sown in the rhizosphere compartment of each pot in June 2015. At five days after emergence (DAE), 8 plants were kept per pot (32 plants per treatment). Plants were grown for 54 days. At 6 and 32 DAE, nitrogen (N) was applied to the entire surface area of the pot using a urea solution at a dose of 50 and 30 mg N kg^{-1} soil, respectively. This dosage of N was based on previous experiments with grasses (Girotto et al., 2014; Tiecher et al., 2016).

2.3. Extraction, analysis and speciation of the soil solution

Soil solution was extracted the day prior to the sowing of black oat (1st sampling), at 19 (2nd sampling) and at 53 DAE (3rd sampling) using rhizon mini-lysimeter for metals (DOM). Rhizon samplers were installed at a depth of 2 to 12 cm, one in the rhizosphere and another in the bulk soil compartment (Figure 1b). Soil moisture was maintained at 70% MWHC during cultivation with daily weighings and addition of distilled water to maintain soil moisture. The day prior to the extraction of the soil solution, moisture was raised to 95% MWHC. The solution was sampled 16 hours after irrigation by creating a vacuum with a 60 mL syringe. On the same day of the collection, we determined pH and dissolved organic carbon (DOC) concentration, which were measured spectrophotometrically at 560 nm after digestion with 0.4 N potassium dichromate at 60 °C for 4 h (Silva and Bohnen, 2001). Total concentration of aluminum (Al), calcium (Ca), magnesium (Mg), copper (Cu), zinc (Zn), manganese (Mn), potassium (K), iron (Fe) and phosphorus (P) were determined by inductively coupled plasma atomic emission spectroscopy (ICP Perkin-Elmer, USA); and the concentrations of nitrate (NO_3^-), sulfate (S) and chlorine (Cl) by ion chromatography (S135 Ion Chromatography system, Germany).

Ionic speciation of the solution was determined by Visual Minteq software (version 3.0 - Gustafsson, 2013) using the total soluble Al^{+3}, Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Mn^{+2}, K^+, Fe^{+2}, PO_4^-, NO_3^-, SO_4^{-2}, Cl^-, DOC and pH of the soil solution (n=4, Supplementary material, Figure 2). The formation of metal complexes with DOC was evaluated using the Gaussian DOM model (Grimm et al., 1991). The formation of inorganic soluble complexes was assessed using the standard equilibrium constants of Visual Minteq software developed by Smith et al. (2003). We thereby obtained the percentage distribution of all species of Cu in the soil solution.

2.4. Dry matter production and chemical analysis in plant tissue and soil

At 54 days after emergence (DAE), plant shoots were cut close to the soil surface. Then, we determined the fresh biomass mass using a microbalance (three decimal) and dividing it into two portions: one was kept for the determination of dry matter (DM) and nutrient analysis while the second one was used to determine the leaf area. The roots were separated from the soil by hand, washed in running tap water to remove soil and weighed. Afterwards, the root system of two plants was sampled, weighed and placed in distilled water for future assessment of the root architecture. The remaining root mass was washed in 0.012 mol L^{-1} EDTA solution to remove nutrients outside the roots and consequently in distilled water for subsequent determination of DM and nutrient concentration. Shoots and roots were then oven-dried at 65 ° C until reaching constant weight to assess DM.

Dried shoots and roots were ground in a Wiley mill and digested with HNO_3-$HClO_4$ (Embrapa, 1997) to determine the concentration of Cu, Zn, Fe, Mn, Ca, Mg, P and K. Cations were determined by atomic absorption spectrophotometer (AAS) and P by colorimetry (Murphy and Riley, 1962). For nitrogen (N) analysis, the tissue were digested with H_2SO_4-H_2O_2 and the extract was distilled following the Kjeldahl method (Tedesco et al., 1995).

After collecting the plants, the soil of each compartment (rhizosphere and bulk soil) was homogenized, sampled, air-dried and stored. We measured pH in water (1:1 v/v), exchangeable Ca^{+2}, Mg^{+2} and Al^{+3} (extracted with 1 mol L^{-1} KCl), available P and K (extracted with Mehlich-

1: 0.05 mol L^{-1} HCl + 0.0125 mol L^{-1} H_2SO_4) (Tedesco et al., 1995) in both soils. The available concentrations of Cu, Zn, Fe and Mn were extracted by EDTA (0.01 mol L^{-1} EDTA + 1 mol L^{-1} $C_2H_7NO_2$ at pH 7.0) as described by Chaignon et al. (2003) and determined by AAS.

2.5. Root architecture and leaf area

Leaf area and root morphology were assessed using WinRhizo Pro 2013 software, coupled to an EPSON Expression 11000 scanner equipped with an additional light (TPU) (Regent Instruments, Quebec, Canada) (Danjon et al., 2000), with a resolution of 200 and 600 dpi for shoots and roots, respectively. We measured root surface area (cm^2), volume (cm^3) average diameter (mm) and the percentage distribution of roots for each diameter range.

2.6. Statistical analysis

The data were tested for normality and homogeneity of variance through Lilliefors and Shapiro-Wilk tests. Afterwards, the data were submitted to analysis of variance through SISVAR software, version 4.0 (Ferreira, 2011). The chemical characteristics of soil were compared between Cu levels in the same soil compartments, and between compartments (rhizosphere and bulk soil) with the same level. The means were grouped by the Scott- Knott test at 5% probability.

3. Results

3.1. Nutrient concentration and speciation in the soil solution

As expected, Cu concentration increased in both, bulk and rhizosphere soil solution, with increasing Cu application in all three samplings (Figure 2a). The concentration of soluble Cu was significantly lower in the solution withdrawn from the rhizosphere compartment than the one from the bulk soil, yet only in soil treated with 40 (Cu 40) and 80 (Cu 80) mg Cu kg^{-1} and at the third sampling. In the third sampling, Cu concentration in the rhizosphere and bulk soil solution increased in comparison to the second sampling at Cu 80, which also occurred in the bulk soil solution at Cu 40. In the same environment, additions of Cu 40 and Cu 80 decreased soil pH in all samplings compared to the soil without Cu addition (Cu 0), both in the rhizosphere and bulk soil (Figure 2b). Black oat plants lead to an increase of rhizosphere pH in the third sampling, with an increase of 26, 49 and 12% at Cu 0, Cu 40 and Cu 80, respectively.

In the second sampling, rhizosphere pH increased only at Cu 0 (Figure 2b). The pH decreased in the third sampling compared to the second sampling in both environments at Cu 80, and in the bulk solution at Cu 40. The DOC concentration (Figure 2c) was slightly modified by the cultivation of black oats in the second sampling, but increased 213, 256 and 70% in the rhizosphere solution compared to the bulk soil solution at Cu 0, Cu 40 and Cu 80, respectively, in the third sampling (Figure 2c). At this sampling, DOC concentrations decreased in the bulk soil solution in relation to previous collections at all Cu levels.

The predominant chemical Cu species in soil solution were Cu^{+2} and Cu-DOC in all treatments, comprising between 81 and 98% of soluble Cu (Figure 3). The distribution of Cu species showed no significant changes between the environments and Cu levels of the first sampling. In the second sampling, there was an increase in Cu^{+2} in treatments with Cu addition compared to Cu 0, which was accompanied by a reduction of Cu-DOC. The changes induced by oat plants resulted in the reduction of Cu^{+2} and increase of Cu-DOC at all Cu levels, compared to bulk soil in the third sampling (Figure 3). In this sampling, $CuOH^+$ percentage was 17.8 and 3.3% in the rhizosphere of Cu 0 and Cu 40. $CuOH^+$ showed a percentage greater than 1% in the first and second samplings only at Cu 0. At Cu 40 and Cu 80 showed the highest percentages of $CuSO_{(aq)}$, comprising between 0.9 and 1.6% of soluble Cu. The bulk soil solution showed an increase in the percentage of Cu^{+2} in the third sampling, compared to previous samplings, comprising 55% of Cu in solution at Cu 0, 72% at Cu 40 and 77% at Cu 80.

3.2. Chemical characteristics of soil

Available P and K increased in the rhizosphere with increasing rate of Cu added to soil (Table 1). In the bulk soil, the lowest concentration of available P was found at Cu 0 and did not differ between Cu 40 and Cu 80 (Table 1). At Cu 0 and Cu 40, there was a reduction in available P and K levels in the rhizosphere compared to the bulk soil, which was not observed at Cu 80. The concentration of available Cu increased linearly with increasing rate of Cu added to the soil, which was also observed for Mn, increasing 41 and 88% in the rhizosphere, and 42 and 73% in bulk soil, at Cu 40 and Cu 80, respectively (Table 1). Available Fe concentration was lower in the rhizosphere soil than in the bulk soil at all Cu levels. On the other hand, comparing the different treatments, there was an increase only in the bulk soil at Cu 80.

The presence of the plants did not change the available Zn concentration in the soil of all treatments, without any difference between the rhizosphere and bulk soil. The rhizosphere of Cu 0 showed a lower available Zn concentration compared to soils spiked with Cu (Table 1). We observed the highest pH values and exchangeable Mg concentration in the rhizosphere at all rates of Cu added to soil. The concentration of exchangeable Ca showed the same behavior, except for Cu 80, where no difference between the rhizosphere and bulk soil could be observed. The pH of the rhizosphere and bulk soil decreased with increasing rate of Cu added to soil. However, the concentration of exchangeable Al was higher only at Cu 80.

3.3. Growth, morphological parameters and nutrient concentration

The growth of black oat was impaired by excess Cu concentration, diagnosed by reduced dry matter production of shoots with increasing rate of Cu added to soil. At Cu 40 and Cu 80, black oat produced only 26.65 and 7.28% of dry matter produced at Cu 0 (Figure 4a). The leaf area of black oat plants also decreased with increasing rate of Cu added to soil (Figure 4b).

The development of the root system of black oats was also negatively affected by increasing rate of Cu added to soil (Figure 4a). Dry matter production of the root system was reduced by 226 and 777% at Cu 40 and Cu 80, respectively. Excess Cu also altered the morphology of the root system, reducing root surface area by 122 and 595%, and root volume by 160 and 714%, at Cu 40 and Cu 80, respectively (Figure 5b, c). Copper addition reduced average root diameter, although statistical difference was not observed between Cu 40 and Cu 80. The percentages of fine roots with diameters between 0 and 0.2 mm and thick roots with diameters larger than 0.8 mm decreased with increasing rate of Cu added to soil. The percentage of roots with diameters between 0.2 and 0.4 mm increased with addition of Cu 40 and Cu 80, compared to Cu 0 (Figure 5d, e). The percentage of roots with diameters between 0.4-0.6 and 0.6-0.8 mm was slightly affected by rate of Cu addition.

Phosphorus concentration in shoot tissues of black oat plants was reduced by 24 and 46% when grown in soils with rate of Cu addition of Cu 40 and Cu 80, respectively. Concentrations of Ca, Mg, Zn and Mn in shoots increased with increasing rate of Cu added to soil. Manganese concentration in shoots even increased by 498 and 985% at Cu 40 and Cu 80, respectively (Table 2). Copper and Fe concentrations in shoots increased with Cu addition compared to Cu 0, but did not statistically differ between Cu 40 and Cu 80. Copper concentration in roots of black oat increased by 2078 and 4712% with rate of Cu addition of Cu 40 and Cu 80, respectively (Table 2). The highest Mn concentration in root tissue was observed at Cu 80, while iron concentration decreased in roots of plants grown in soil with rate of Cu addition of Cu 40 and Cu 80, compared to the one determined at Cu 0.

The accumulation of macro and micronutrients in shoot biomass of black oat plants reduced with increasing Cu rate added to soil, except for Ca where Cu 0 did not differ from Cu 80. Mn in shoots exhibited greater accumulation at Cu 40 (Table 3). In the roots, the accumulation of nutrients followed the same trend, reducing with increasing Cu added to soil, except for Cu, where the highest accumulation occurred at Cu 40 and Cu 80 (Table 3).

4. Discussion

Regular and prolonged use of copper-based fungicides such as Bordeaux mixture ($Ca(OH)_2+CuSO_4$) to protect grapevine plants from disease (like downy mildew *Plasmopara viticola*) can cause a long-term accumulation of Cu in vineyard soils, reaching concentrations higher than those required for healthy plant growth (Pietrzak and McPhail, 2004; Komarek et al., 2010; Cambrollé et al., 2015). However, the total concentration of a metal is not a good predictor of effects to plants because a very limited fraction of Cu remains in soil solution (Sauvé et al., 2000; Nolan et al., 2003), while the majority reacts with the functional groups of the reactive particles of both the mineral phase and the organic fraction (Brunetto et al., 2014; Miotto et al., 2014; Pérez-Esteban et al., 2014). Initially, just after the addition, Cu adsorption involves the most avid adsorptive sites, the carboxylic groups (COOH), and consequently, even if in smaller proportions, hydroxyl (OH) and phenol (aromatic ring OH) groups of organic compounds (McBride, 1994). Once adsorption sites are saturated the soluble Cu fraction increases. Nonetheless, this soluble Cu is in equilibrium with the Cu absorbed to the solid phases. In this study, the rate of Cu addition of 40 and 80 mg Cu kg^{-1} to the soil led to an increased Cu soil solution concentration (Figure 2). This indicates that these two Cu concentrations - that can occur after 15 or 30 years of continued vineyard cultivation - are able to almost completely saturate the adsorption sites of the soil. Interestingly, similar behavior was also described in vineyards after prolonged cultivation of this crop (Brunetto et al., 2014; Girotto et al., 2014). It should be stated that high Cu concentrations in soil solution represent not only a risk of plant toxicity, but also an environmental concern due to the mobility along the soil profile of this Cu fraction (Ren et al., 2015; Babcsányi et al., 2016).

Data concerning the Cu concentration of soil solution (Figure 2) show that, prolonging the period of plant growth and thus the root/soil contact (3rd sampling), a significant decrease of the available Cu in the rhizosphere of soils treated with Cu 40 and Cu 80 occurred compared to the bulk soil. In contrast, the soluble Cu remains mostly stable or even increases in the bulk soil. These effects could be ascribed to several processes taking place concurrently in the rhizosphere (Terzano et al., 2015). One example can be represented by Cu acquisition by roots of oat plants (Tables 2, 3); as expected, this process is at the expense of the available fraction of the metal in soil solution and it is obviously more effective in the rhizosphere (Figure 2). It is interesting to note that a very high Cu concentration was accumulated at the root level, while only a limited concentration was allocated at the shoot level when plants grown in soils treated with Cu 40 and Cu 80 (Tables 2, 3). These phenomena of accumulation of Cu absorbed in the root system have also been described for other crops (Chaignon and Hinsinger, 2003; Girotto et al., 2014; Cambrollé et al., 2015; Tiecher et al., 2016) and could be part of a strategy to limit Cu toxicity in plants. In fact, while a very different concentration of Cu was measured at the root level by plants grown in the two conditions of contamination (Cu 40 or Cu 80), a similar Cu concentration was measured at the shoot level of plants exposed to both Cu treatments (Table 2). This indicates that the reduction of the accumulation of Cu in the shoots with increasing rate of Cu added to soil occurred exclusively due to the lower biomass production of black oats (Table 3). This result suggests how plants control their Cu concentration (homeostasis) between different organs under phytotoxicity conditions (Marschner, 2011; Girotto et al., 2014): for instance, a reduced concentration of P in shoots with increasing Cu levels in soil, even with reduced dry matter production, is most likely linked to the formation of Cu-P precipitates in the roots, reducing translocation via the xylem (Brown et al., 1995; Arriagada et al., 2009). However, roots of plants grown in soil with no Cu addition (Table 3) showed similar P concentrations. Nonetheless, an inhibitory effect on biomass accumulation was evident in this study and it was particularly severe in plants exposed to the highest Cu levels (Figure 4 and 5). This phenomenon is in line with that also described by other authors (Cambrollé et al., 2015; Tiecher et al., 2016; Baldi et la., 2018) and, in our specific case, confirms the phytotoxicity of the Cu levels measured in the plant tissues. In any case, based on our results and the fact that old grapes lack any symptoms of Cu toxicity when co-cultivated with cover crops (Miotto et al., 2014), it is evident that the pool of Cu acquired by black oat plants represents a fraction removed from the rhizosphere and no longer available for the acquisition process of other plants.

Regarding the root, it is important to highlight that the impaired root apparatus development due to Cu toxicity (Figure 5) could have a negative effect on the capability of plants in both exploring soil and acquiring water and nutrients (Kabata-Pendias, 2011; Marschner, 2011). These effects might further influence the availability of nutrients (including Cu) within the rhizosphere. In this respect, our data show a clear decrease at the shoot level of the P concentration and accumulation as a consequence of the Cu contamination of soils, as also observed in grapevine plants by Cambrollé et al. (2015). Interestingly, the phenomenon seems to be proportionally related to the concentration of available Cu in soil (Tables 2, 3 vs Table 1). The low mobility in soil of this macronutrient (mostly related to diffusion processes) and the limited development of the root (Figure 5) could, at least partially, be on the basis of the observed effect.. In addition to P, increased soil contamination with Cu (Cu 40 and Cu 80) reduced the accumulation of N, K, Mg, Zn and Fe (Table 3). This reduction in nutrient accumulation could be linked to lower biomass production, even though some of these nutrients increased concentration with increasing rate of Cu addition In particular, the Cu-Mn exchange on the adsorption sites of the soil colloids due to the higher adsorption energy of Cu (Sposito, 1989) could be responsible of the increased Mn availability (Table 1) and, thus, concentration in black oat shoots. Although Mn is an essential element for plant growth, it adversely affects growth for most plants at levels above 400 mg kg^{-1} in plant tissues (Kabata-Pendias, 2011). In this study, Mn concentration in the shoots was higher than the critical level when the highest Cu rate was applied, which highlights the potential of combined Cu/Mn toxicity in acidic soils. Furthermore, black oat plants exhibited symptoms of interveinal chlorosis (data not shown), which are characteristic for Cu toxicity (Cambrollé et al., 2015), as well as Fe deficiency. Furthermore, Cu toxicity might even induce Fe deficiency in some plants (Waters and Armbrust, 2013). In fact, in Fe deficient conditions, plants trigger the uptake of Fe^{2+}, but also of other divalent cations such as Cu^{2+}, Zn^{2+} and Mn^{2+} (Yruela, 2005; Marschner, 2011) mediated by the transmembrane iron-regulated transporter (IRT) for FeII (Yruela, 2005). This probably contributed to the reduction in Fe accumulation in the biomass of the shoots and roots with increasing rate of Cu in soil (Table 3).

It is well known that rhizosphere pH may differ from that of the bulk (Marschner, 2011). The increase or decrease of pH in the rhizosphere can consistently influence nutrient availability since pH is the main driver of mobilization/immobilization processes of nutrients (Hinsinger et al., 2009). In this study, black oat plants induced an increase in pH (Figure 2). Rhizosphere alkalinization could also be ascribed to the preferential acquisition of nitrate by black oat roots: the uptake (co-transport with protons) and then the assimilation process increase the rhizosphere pH. Consequently, the de-protonation of functional groups present on the surface of reactive soil particles in the rhizosphere increases the negative charges and, therefore, the electrostatic adsorption of Cu (McBride 1994), thus limiting Cu availability in soil solution (Figure 2).

In addition to rhizosphere alkalinization, the concentration of DOC can play an important role in governing the distribution of chemical species of Cu in soil solution (Li et al., 2013; De Conti et al., 2016), thus having an impact on its acquisition by roots. It is well known that Cu has a very high affinity for dissolved organic carbon (Bravin et al., 2012; Pérez-Esteban et al., 2014). The origin of these organic compounds could be ascribed to the mineralization process of soil organic matter and/or to root exudation (Pii et al., 2015). Concerning the latter, it is estimated that around 5 to 25% of C fixed by plant photosynthesis is released in the rhizosphere as root exudates (Mwafulirwa et al., 2016). In fact, results of this study show that the fraction of Cu complexed by DOC increased in the rhizosphere of black oat plants (Figure 3). The complexation of free Cu can be considered a process that hinders the plant uptake of the metal since plants and microorganisms take up preferentially the hydrated divalent form of Cu (Cu^{+2}) (McBride, 1994; Martinez and McBride, 1999). A similar phenomenon has been described by De Conti et al. (2016) in corn and black oat cultivation in a Typic Hapludalf soil subjected to prolonged applications of Cu-containing pig slurry. Furthermore, the high concentration of pH-dependent charges of DOC can benefit from the pH alkalinization of the rhizosphere solution in limiting the availability of free Cu. It is interesting to note that a degradation of this C source by microorganisms could favor the limitation in Cu availability *via* a Cu co-precipitation with aluminium-(hydr)oxides (Terzano et al. (2017). Yet, these effects have been observed only in a calcareous soil.

In addition to changes in the concentration of DOC between rhizosphere and bulk soil solution in the third sampling (Figure 2), qualitative modifications of the composition of this carbon pool cannot be excluded. In fact, the lower available Fe concentration in rhizosphere soil (Table 1) and in accumulated in the tissues (Table 3) together with the presence of chlorotic symptoms at the leaf level (data not shown) suggest an induced latent Fe shortage in black oat plants exposed to Cu 40 or Cu 80 mg kg^{-1}. It is well known that grasses such as black oats are able to enhance the release of organic ligands (phytosiderophores, organic acids and phenolics) with high affinity for metals in order to cope with nutrient stress (Mimmo et al., 2014). Since these compounds are active also towards Cu, it could be hypothesized that they can also contribute to the limitation of Cu availability to plants. Similarly, earlier studies (Bravin et al. 2012) reported that wheat grown in a Cu contaminated acidic soil limited Cu bioavailability in the rhizosphere.

5. Conclusions

Black oat plants induce several modifications within the rhizosphere, particularly in terms of pH and dissolved organic carbon. These root-induced processes increase the percentage of complexed chemical species and decrease free Cu^{+2} in soil solution, decreasing Cu toxicity. However, soil contamination with Cu induces morphological changes and nutritional imbalances.

Black oats could thus be planted along with vineyards, for such increasing protect the soil and promote nutrient cycling, as well as reduce the free Cu available fraction due to the root-induced modifications in the rhizosphere.

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Table 1. Available and exchangeable concentrations of macro and micronutrients determined in rhizosphere (R) and bulk soil (B) where black oats were grown in soils with increasing Cu concentration.

Soil chemical parameters	Location	Rate of Cu (mg kg^{-1})		
		0	40	80
Available P by Mehlich-1(mg kg^{-1})	B	14.62 B	18.04 A	18.75 A
	R	12.12 C*	15.42 B*	19.69 A^{ns}
Available K by Mehlich-1 (mg kg^{-1})	B	41.50 B	89.50 A	99.25 A
	R	19.50 C*	58.25 B*	105.75 A^{ns}
Available Cu by EDTA (mg kg^{-1})	B	2.42 C	31.55 B	61.55 A
	R	6.09 C*	32.24 B^{ns}	62.56 A^{ns}
Available Zn by EDTA (mg kg^{-1})	B	1.07 A	1.28 A	1.31 A
	R	0.99 B^{ns}	1.19 A^{ns}	1.32 A^{ns}
Available Mn by EDTA (mg kg^{-1})	B	21.42 C	30.45 B	37.00 A
	R	18.64 C*	26.35 B*	35.06 A^{ns}
Available Fe by EDTA (mg kg^{-1})	B	12.36 B	13.28 B	15.18 A
	R	10.08 A*	9.21 A*	10.91 A*
Exchangeable Ca (cmol$_c$ kg^{-1})	B	1.04 A	0.94 A	0.94 A
	R	1.24 A*	1.15 A*	0.87 B^{ns}
Exchangeable Mg (cmol$_c$ kg^{-1})	B	0.63 A	0.63 A	0.67 A
	R	1.06 A*	1.09 A*	0.90 B*
Exchangeable Al (cmol$_c$ kg^{-1})	B	0.03 B	0.02 B	0.05A
	R	0.01 B^{ns}	0.01 B^{ns}	0.03 A*
pH$_{H2O}$ (1:1)	B	6.18 A	5.46 B	5.16 C
	R	6.88 A*	6.05 B*	5.50 C*

(1) Means followed by the same letter (row) do not differ between Cu rate in the same environment by Scott-Knott test ($p < 0.05$). ^{ns} non-significant.; * significant (F test $p < 0.05$) difference between environments (column) at the same Cu rate.

Table 2. Content of macro and micronutrients in shoot and root biomass of black oat plants grown in soils with increasing Cu concentration.

	Rate of Cu (mg kg^{-1})					
Nutrient	0	40	80	0	40	80
	Shoot			Root		
N (g kg^{-1})	27.95 c(1)	33.78 a	29.99 b	7.97 c	14.02 b	17.03 a
P (g kg^{-1})	2.42 a	1.96 b	1.66 c	1.19 a	1.40 a	1.21 a
K (g kg^{-1})	38.61 a	41.78 a	37.42 a	10.45 a	14.28 a	13.66 a
Ca (g kg^{-1})	5.11 c	6.98 b	8.74 a	3.86 a	2.69 c	3.17 b
Mg (g kg^{-1})	7.2 c	8.66 b	10.94 a	8.32 a	7.34 a	6.94 a
Cu (mg kg^{-1})	8.34 b	14.67 a	16.23 a	19.65 c	428.13 b	945.54 a
Zn (mg kg^{-1})	61.35 c	116.22 b	131.31 a	33.42 c	61.59 b	117.30 a
Fe (mg kg^{-1})	83.46 b	144.15 a	138.45 a	2156.94 a	1661.76 b	1291.44 b
Mn (mg kg^{-1})	41.22 c	246.57 b	447.15 a	98.85 b	149.13 b	317.91 a

(1) Means followed by the same letter do not differ between Cu rate in the same plant organ by Scott-Knott test ($p < 0.05$).

Table 3. Accumulation of macro and micronutrients in shoot and root biomass of black oat plants grown in soils with increasing Cu concentration.

Nutrient	Rate of Cu (mg kg^{-1}) 0	40	80	0	40	80
		Shoot			Root	
N (mg pot^{-1})	460.40 a(1)	148.15 b	35.92 c	62.92 a	33.91 b	15.32 c
P (mg pot^{-1})	39.86 a	8.60 b	1.99 c	9.39 a	3.39 b	1.09 c
K (mg pot^{-1})	635.99 a	183.24 b	44.82 c	82.50 a	34.54 b	12.29 c
Ca (mg pot^{-1})	30.61 a	10.47 b	30.47 a	30.47 a	6.51 b	2.85 c
Mg (mg pot^{-1})	118.60 a	37.98 b	13.10 c	65.69 a	17.75 b	6.24 c
Cu (mg pot^{-1})	0.137 a	0.064 b	0.019 c	0.155 b	1.04 a	0.851 a
Zn (mg pot^{-1})	1.01 a	0.510 b	0.157 c	0.264 a	0.149 b	0.106 c
Fe (mg pot^{-1})	1.37 a	0.632 b	0.166 c	17.03 a	4.02 b	1.16 c
Mn (mg pot^{-1})	0.679 b	1.08 a	0.536 c	0.780 a	0.361 b	0.286 b

(1) Means followed by the same letter do not differ between Cu rate in the same plant organ by Scott-Knott test ($p < 0.05$).

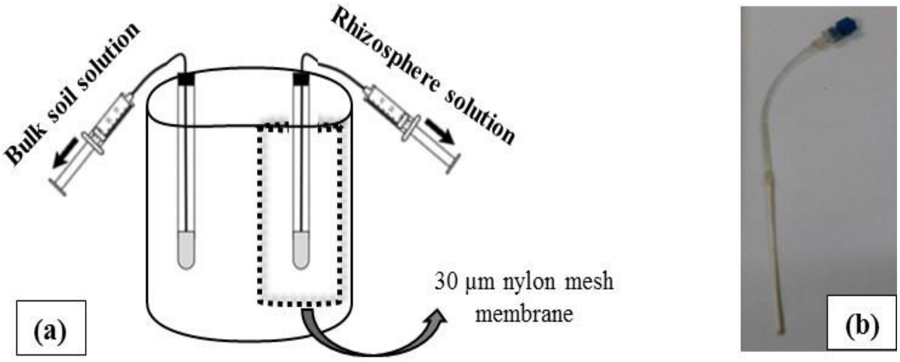

Figure 1. Schematic representation of the pots used to simulate rhizosphere and bulk soil. The rhizosphere compartment was separated by a 30 µm nylon membrane (a). In each soil compartment Rhizon samplers (b) were inserted to sample the soil solutions throughout the experiment. Black oats plants were sown in the rhizosphere compartment.

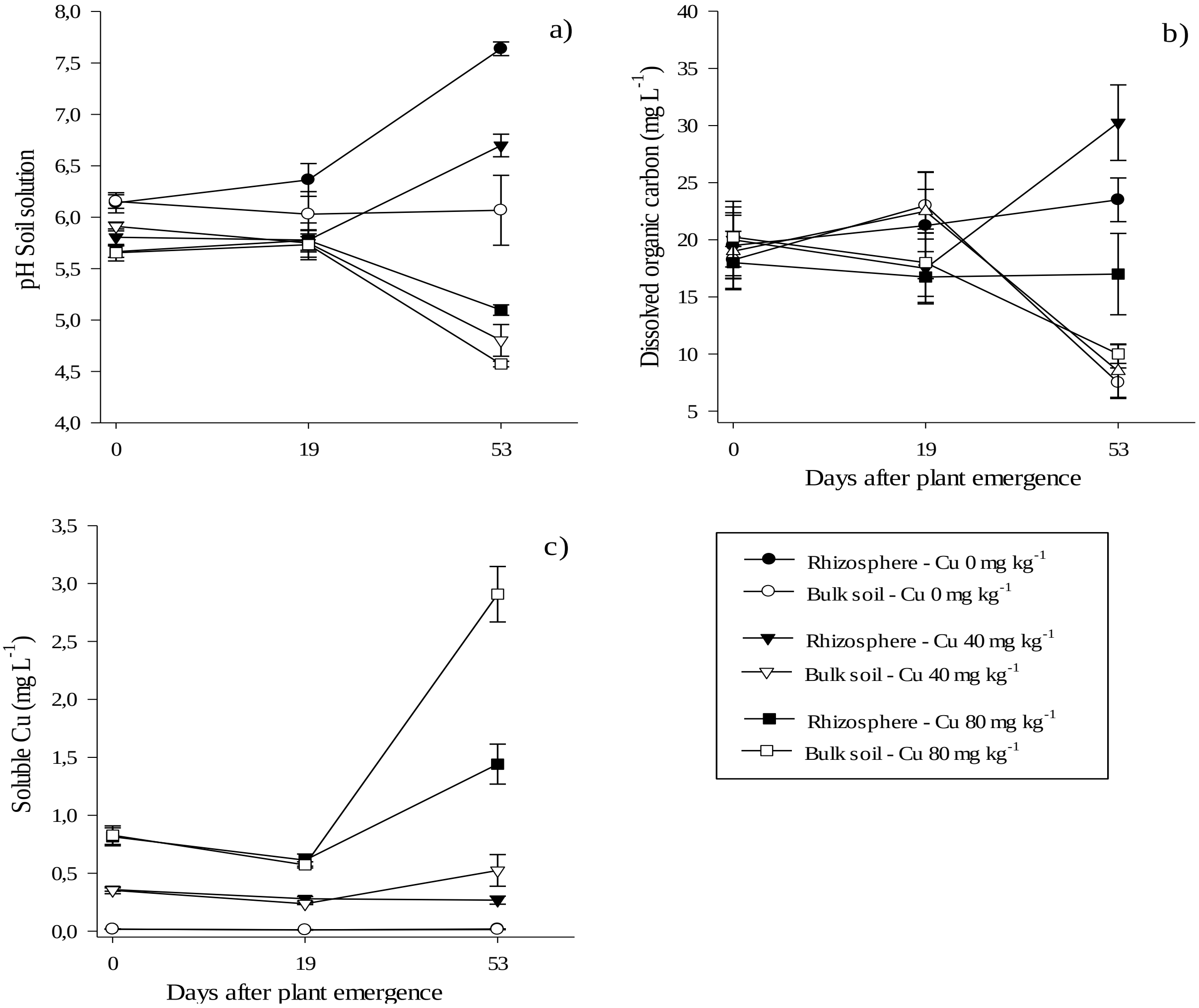

Figure 2. Chemical characteristics of rhizosphere and bulk soil solution withdrawn during the cultivation of black oats grown in soils with increasing Cu concentration. Vertical bars indicate standard errors of the means (n=4).

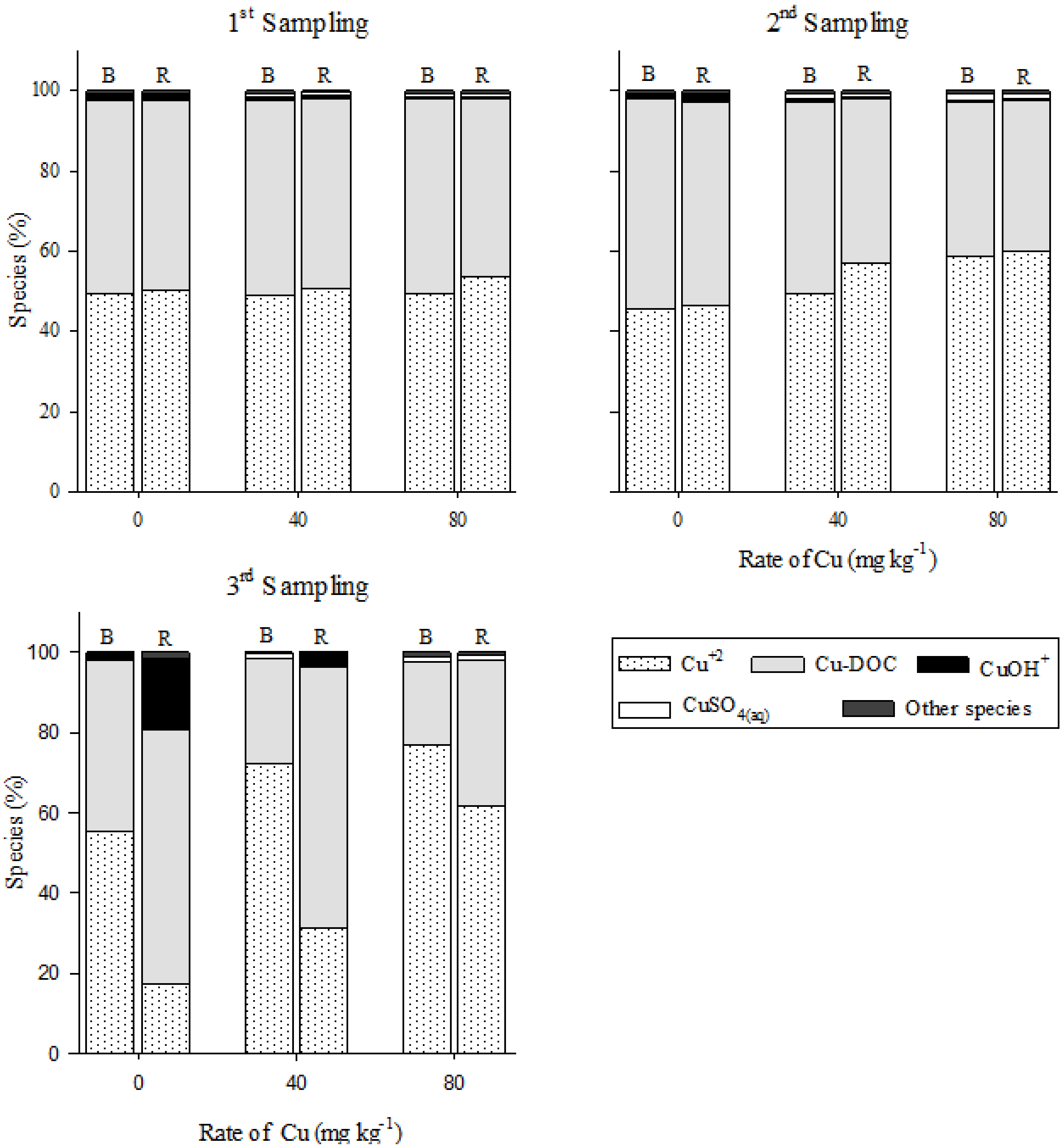

Figure 3. Distribution of Cu chemical species in rhizosphere (R) and bulk (B) solution in 3 samples taken during cultivation of black oats grown in soils with increasing Cu concentration.

Other species correspond to a $CuNO_3^+$, $CuHPO_{4(aq)}$, $CuCl^+$ and $Cu(OH)_{2(aq)}$..

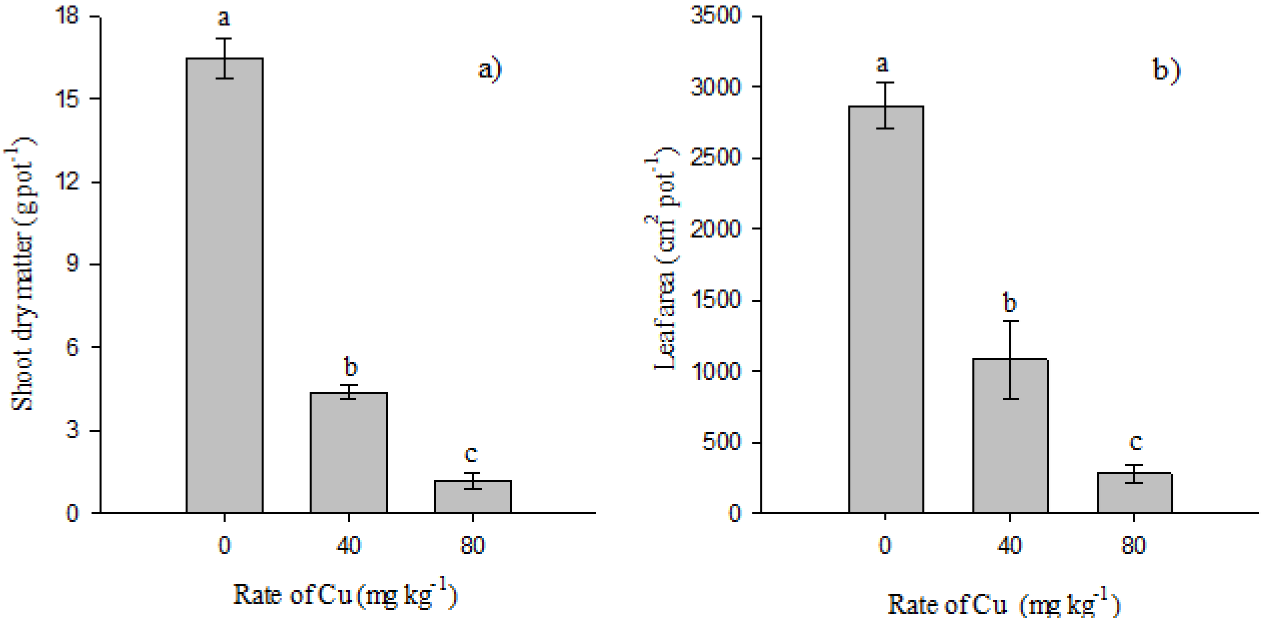

Figure 4. Shoot dry matter production (a) and leaf area (b) of black oats grown in soils with increasing Cu concentration. Means followed by the same letter in the column do not differ by Scott-Knott test ($p < 0.05$).

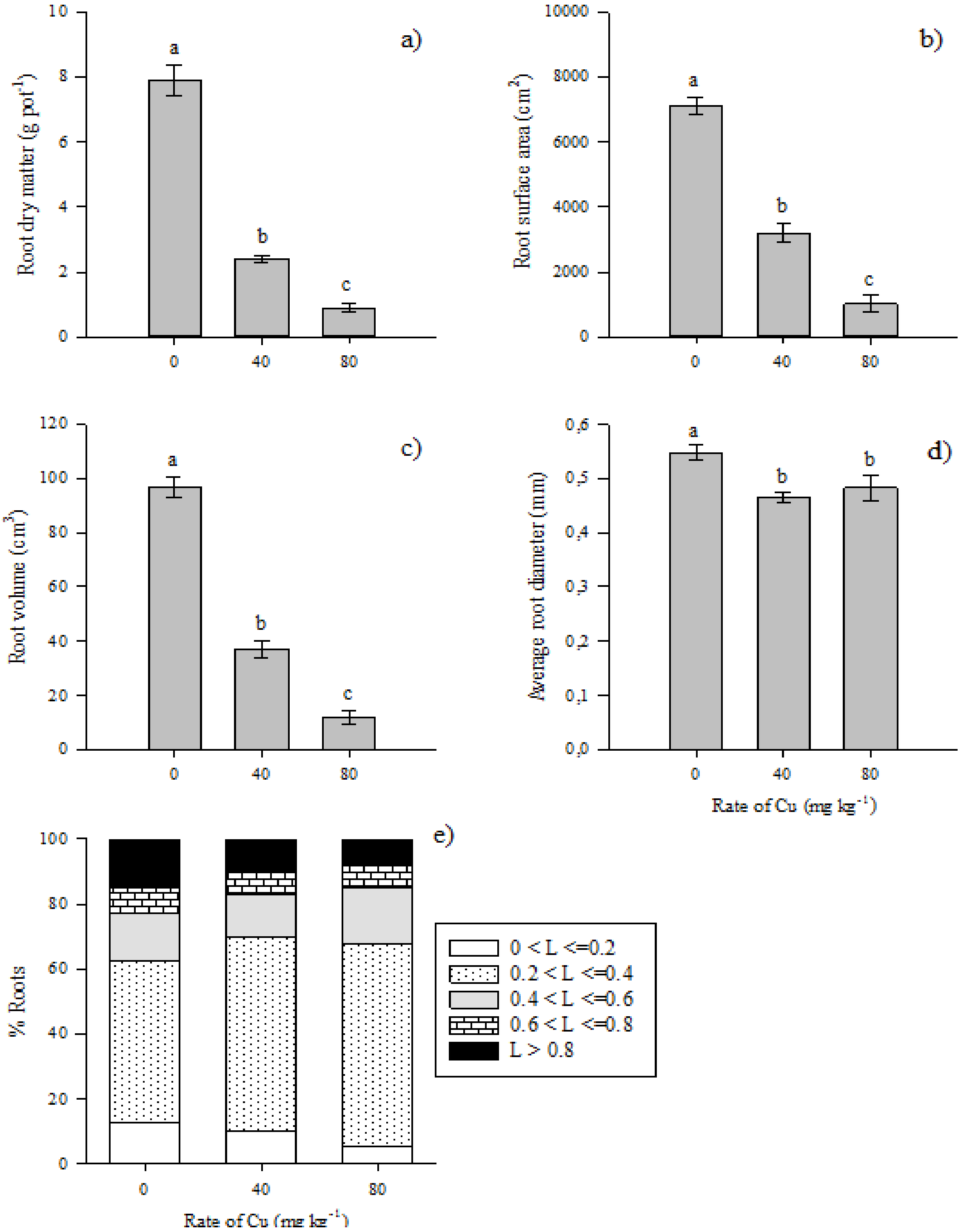

Figure 5. Root dry matter production (a), surface area (b), volume (c), average diameter (d) and percentage distribution of roots for each diameter range (e) of black oats grown in soils with increasing Cu concentration. Means followed by the same letter in the column do not differ by Scott-Knott test ($p < 0.05$).

Supplement 1. Chemical characteristics of rhizosphere (R) and bulk (B) soil solution in 3 samples taken during the cultivation of black oat.

Cu Rate	Location	Al	Ca	Mg	Mn	Zn	K	Fe	PO_4^{-3}	Cl	NO_3^-	SO_4^{-2}
		mg L^{-1}										
1st Sampling												
0	B	0.048	221.8	195.6	0.007	0.019	298.0	0.008	1.227	84.2	308.8	13.1
	R	0.047	251.4	222.6	0.005	0.023	303.4	0.007	1.112	83.3	309.7	14.9
40	B	0.053	222.9	202.2	0.064	0.021	284.6	0.007	1.139	72.4	322.9	65.6
	R	0.052	255.9	217.5	0.063	0.021	283.6	0.006	1.040	61.7	204.6	49.8
80	B	0.051	229.2	209.2	2.325	0.034	331.8	0.006	1.096	81.9	369.7	59.0
	R	0.049	247.3	217.0	2.328	0.035	279.0	0.006	1.051	60.9	257.0	65.3
2nd Sampling												
0	B	0.047	239.5	213.5	0.224	0.055	260.4	0.004	0.870	84.4	436.3	14.0
	R	0.048	235.6	217.7	0.090	0.012	162.9	0.003	0.672	93.3	270.7	10.8
40	B	0.051	283.9	243.3	0.088	0.121	278.5	0.004	0.816	107.3	582.4	90.1
	R	0.052	295.4	253.3	0.071	0.028	227.6	0.003	0.837	66.8	315.9	55.5
80	B	0.054	333.6	267.2	0.699	0.130	276.7	0.004	0.840	85.6	458.4	102.0
	R	0.051	321.9	263.5	0.119	0.061	250.6	0.004	0.864	58.1	351.2	65.4
3rd Sampling												
0	B	0.027	108.2	110.1	0.078	0.027	26.9	0.009	0.795	15.0	110.6	2.2
	R	0.038	72.2	69.2	0.016	0.007	0.59	0.007	0.401	12.6	0.7	0.3
40	B	0.089	267.6	183.5	3.062	0.131	162.3	0.008	1.079	50.9	127.5	46.5
	R	0.061	186.9	112.1	0.222	0.016	24.4	0.004	0.646	18.6	79.4	4.4
80	B	0.223	330.6	273.6	16.93	0.288	321.5	0.008	1.060	57.8	503.3	70.1
	R	0.076	315.0	254.2	5.45	0.124	282.1	0.007	0.920	63.8	331.5	67.6

5.3 ESTUDO III

Copper bioavailability, tolerance and growth of native grasses of South American grasslands grown in copper-contaminated soils [3]

Abstract

Native grasses may present mechanisms of tolerance to excess copper (Cu), enhancing their use in the phytoremediation of these environments. The aim of this study was to evaluate the distribution of Cu chemical species in soil solution, growth, morphological and nutritional parameters of native grasses of South American grasslands grown in sandy soil with increasing Cu levels. The soil used in the experiment was collected in natural grassland in southern Brazil. The samples were air-dried; acidity, phosphorus and potassium levels were corrected and samples were then incubated. We used three Cu levels - natural level (Dose 0) and the addition of 40 and 80 mg kg^{-1} Cu (Dose 40 and 80). The soil (4 kg) was placed in 5 L pots. In August, three seedlings of *Axonopus affinis*, *Paspalum notatum* and *Paspalum plicatulum* were transplanted per pot and cultivated for 121 days. We collected soil solution throughout cultivation using Rhizon (MOM) lysimeters. In the solution, the concentration of the main cations, anions, dissolved organic carbon, pH were analyzed and ionic speciation was carried out. At 121 days after transplanting, the plants were collected to determine dry matter, morphological parameters and nutrient concentration in the roots and shoots. *Paspalum plicatulum* is a grass species of South American grasslands with great potential to be used as cover crops in vineyards with high levels of Cu. These native grass species can be used as phytostabilizing plants in Cu-contaminated soils, by accumulating the absorbed Cu predominantly in the roots, thus benefiting grapevines grown intercropped.

Keywords: Phytoremediation, ionic speciation, native plants, cover crops, vineyards.

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[3] Artigo elaborado de acordo com as normas da Revista Chemosphere.

1. Introduction

The contamination of soils with heavy metals occurs in various places around the world and it is especially due to anthropogenic activities, such as industrial activity, mining and agricultural production (Ali et al., 2013; Boechat et al., 2017; Leguizamo et al., 2017; Wan et al., 2017). The application of cupric fungicides such as Bordeaux mixture ($Ca(OH)_2 + CuSO_4$), which has a high concentration of copper (Cu), causes the increase of Cu levels in soils over the years of cultivation, as diagnosed in vineyard soils of several traditional grape growing countries (Komárek et al., 2010; Miotto et al., 2014; Cambrollé et al., 2015; Brunetto et al., 2016; Girotto et al., 2016). The increase in Cu levels in the soil can cause toxicity to grapevines or cover crop species that co-habit the vineyards. This is due to the fact that heavy metals persist in the soil for long periods, and may or may not be in soluble forms (Miotto et al., 2014; Girotto et al., 2016; Oustriere et al., 2016).

Although Cu is an essential element, involved in important metabolic processes, excessive absorption can cause negative responses to physiological levels, causing morphological changes and reduced plant growth (Michaud et al., 2008; Kabata-Pendias, 2011; Guimarães et al., 2016). The accumulation of Cu in shoot biomass of plants may impair the functioning of the photosynthetic apparatus, reducing carbon fixation (C) and inducing changes in the functioning of ion carriers and channels, which may lead to loss of selective capacity, resulting in nutritional imbalances (Yruela, 2005, Cambrollé et al., 2015, Tiecher et al., 2016).

The expansion of grapevine cultivation in southern Brazil, which is the main wine region of the country and one of South America's leading wine regions, occurred in areas of South American grasslands, mainly used for raising cattle. The absence of soil tillage after the installation of the vineyards and the soil seed bank of these species, allow the presence of the native vegetation co-habitating the vineyards, promoting nutrient cycling and soil conservation. The great biodiversity of the South American grasslands allows the occurrence of a large number of grass species, estimated in more than 450 species (Brazil, 2017), with frequent occurrence of *Axonopus affinis, Paspalum notatum, Paspalum plicatulum* (Quadros et al., 2009). The evolution of the native species of South American grasslands occurred for the most part on acidic and chemically poor soils, indicating the presence of adaptive mechanisms to the conditions of low nutrient levels and the presence of toxic elements (Pallarés et al., 2005). These tolerance mechanisms may favor growth in soils contaminated with heavy metals, such as Cu, enhancing the use in phytoremediation (Sainger et al., 2011; Leguizamo et al., 2017). The spontaneous occurrence of native species and high adaptability to the environment potentiate their use in phytoremediation in relation to introduced species, since in addition to lower cost, they have less impact on the ecological and environmental dynamics of the site (Sainger et al., 2011; Leguizamo et al., 2017). The maintenance of the native vegetation co-habitating the vineyards can help control the nutritional balance, increasing the quality and contributing in the specific viticultural terroir of the region (Muscas et al., 2017).

Phytoremediation is the use of plants to reduce the concentration or the toxic effects of pollutants in the soil (Ali et al., 2013, Boechat et al., 2017). This technique can be used to remove heavy metals from the soil by Cu absorption and accumulation in the biomass, called phytoextraction or phytoaccumulation. There is more efficiency when translocation and accumulation occurs in the shoots, due to the greater difficulty in removing the root biomass of the plants (Sainger et al., 2011, Ali et al., 2013). The reduction of the mobility and bioavailability of heavy metals in the soil with plant use through root sorption, precipitation and complexation is characterized as phytostabilization (Ali et al., 2013; Leguizamo et al., 2017).

Some plants promote changes in the bioavailability of soil elements when subjected to nutritional stress conditions caused by toxicity or deficiency, altering the concentration dissolved organic carbon (DOC), by the exudation of organic compounds or modifying the pH by the exudation of H^+, OH^- and HCO_3^- ions (Jones 1988; Chaignon et al., 2009; Kim et al., 2010; Brunetto et al., 2016). The bioavailability of chemical elements (such as Cu) in the soil is influenced by the solubility and distribution of the chemical species in the soil solution, because Cu absorption by plant roots and microorganisms occurs preferentially in the free ionic form, which is coordinated by water molecules, classified as free specie (Cu^{+2}) (McBride, 1994). The solubility and distribution of Cu chemical species is mainly controlled by pH and DOC in the soil solution. This means that plants capable of promoting changes in these chemical attributes modify the bioavailability and phytotoxic potential of Cu (Bravin et al., 2012; Pérez-Esteban et al., 2014; De Conti et al., 2016). The aim of this study was to evaluate the distribution of Cu chemical species in soil solution, growth, morphological and nutritional parameters of native grasses of South American grasslands grown in sandy soil with increasing Cu levels.

2. Material and Methods

2.1. Soil characterization and treatments

The soil used in the experiment was a Typic Hapludalf Soil (Soil Survey Staff, 2006), collected at 0-20 cm in a natural grassland area with no anthropogenic activity (30º47'23.7"S and 55º22'7.3"W). The physical and chemical characterization of the soil is shown in Table 1. The area is located in the city of Santana do Livramento, state of Rio Grande do Sul, southern Brazil. After sampling the soil was air-dried, homogenized and passed through a 2 mm mesh sieve. To correct acidity, we applied a mixture of calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$), with a 2:1 ratio at a dose of 0.57g kg^{-1} of soil. After applying the corrective, the soil was incubated for 35 days with moisture of 80% of the maximum water holding capacity (MWHC). Afterwards, we applied 40 mg P kg^{-1} and 100 mg K kg^{-1} of soil by adding triple superphosphate and potassium chloride, respectively. Moisture was restored to 80% of MWHC and incubation continued for another 25 days.

Treatments consisted of a soil with natural Cu level, representing the condition prior to the installation of the vineyards (Dose 0) and with addition of 40 and 80 mg kg^{-1} Cu (Dose 40 and 80), which are levels normally found in vineyards with approximately 15 and 30 years of grapevine cultivation, respectively (Miotto et al., 2014). The addition of Cu occurred 50 days after the application of the corrective, by applying a solution of $CuSO_4.5H_2O$. Subsequently, the soil was incubated again for 45 days under the same conditions described previously. Three times a week throughout the incubation period, the soil was tilled and weighed to evaluate moisture content. We added distilled water whenever necessary to maintain the MWHC at 80%.

2.2. Experimental design and crops

The experiment was conducted in a completely randomized design with three replicates. The experimental units consisted of 5 L pots with 4 kg of soil. In August 2015, three seedlings of *Axonopus affinis, Paspalum notatum* and *Paspalum plicatulum* were transplanted per pot. The native grasses were collected three months before in a natural pasture area, and then multiplied by means of pre-culture with nutrient solution in sand in order to obtain uniform seedlings. The complete nutrient solution used to irrigate the plants in pre-culture consisted of (mg L^{-1}): 149.80 NO_3^-; 24.80 of $H_2PO_4^-$; 39.27 SO_4^{2-}; 41.31 Mg^{2+}; 288.72 Ca^{2+}; 234.60 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, supplied through irrigation three times a day (15 minutes each).

At the time of the installation of the experiment the plants were carefully removed from the pre-culture substrate and the root system was washed with distilled water. The plants were grown for 121 days after transplanting, with the addition of nitrogen (N) at 16 and 63 days, through a urea solution at a dose of 12 and 20 mg N kg^{-1} of soil, respectively, applied on the soil surface.

2.3. Extraction, analysis and speciation of the soil solution

On the day before the transplanting of the seedlings (1st sampling), and on the 73rd (2nd sampling) and 119th (3rd sampling) day after transplanting, the soil solution was extracted using Rhizon MOM mini-lysimeters. The extractors were installed in the center of the pot at depth of 2-12 cm. Soil moisture was maintained at 70% of the MWHC during cultivation, with daily weighing and addition of distilled water to maintain soil moisture. The day before the extraction of the solution of each sampling, moisture was raised to 95% of the MWHC. The extraction was done after 16 hours of irrigation, with the application of a vacuum using 60 mL polystyrene syringes. Subsequently, we measured pH and dissolved organic carbon (DOC) content spectrophotometrically at 560 nm after digestion with 0.4 N potassium dichromate at 60°C for 4 h, as described by Silva and Bohnen (2001). Total contents of aluminium (Al), calcium (Ca), magnesium (Mg), copper (Cu), zinc (Zn), manganese (Mn), potassium (K), iron (Fe) and phosphorus (P) were determined by inductively coupled plasma atomic emission spectroscopy (ICP Perkin-Elmer, USA); contents of ammonia (NH_4^+) and nitrate (NO_3^-) were determined by colorimetry at 660nm and 540 nm, respectively (SANplus, Skalar, Breda, Holanda); and the contents of sulfur (S) and chlorine (Cl) were determined by ion chromatography (S135 Ion Chromatography system, Germany).

Ionic speciation of the solution was determined by Visual Minteq software (version 3.0 - Gustafsson, 2013) using total soluble cations (Al^{+3}, Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Mn^{+2}, K^+ and Fe^{+2}), anions (PO_4^-, NO_3^-, SO_4^{-2} and Cl^-), DOC and pH of the soil solution (n=3). The formation of metal complexes with DOC was evaluated using Gaussian DOM model (Grimm et al., 1991). The formation of inorganic soluble complexes was assessed using the standard equilibrium constants of Visual Minteq software developed by Smith et al. (2003). We obtained the percentage distribution of all species of Cu in the soil solution.

2.4. Dry matter yield and chemical analysis in plant tissue

At 121 days after the seedlings were planted, the shoots were cut close to the soil surface and reserved for dry matter (DM) determination and nutrient analysis. The roots were separated from the soil by hand, washed in running water to remove soil and weighed. Then, the root system of one plant per pot was placed in distilled water for subsequent evaluation of root architecture. The remaining root mass was washed in 0.012 mol L^{-1} EDTA solution, followed by washing in distilled water for subsequent determination of DM and nutrients levels. To determine DM, shoot and root samples were dried in an oven with forced air at ± 65°C to constant weight.

The samples of DM were ground and the tissue was digested with HNO_3-$HClO_4$ (Embrapa, 1997). We determined the contents of Cu, Zn, Fe, Mn, Ca, Mg, P and K in the extract. The determination of cations was done in atomic absorption spectrophotometer (AAS) and P by colorimetry (Murphy & Riley, 1962).

2.5. Root architecture, bioconcentration and translocation factors

The morphological characterization of the roots was obtained by scanned images, using WinRhizo Pro 2013 software, coupled to an EPSON Expression 11000 scanner equipped with additional light (TPU), with a definition of 600 dpi. We determined total root length (cm), surface area (cm^2), volume (cm^3), average diameter (mm) and the percentage distribution of roots in diameter bands.

The bioconcentration factor (BCF) (Equation 1) and translocation factor (TF) (Equation 2) were calculated as follows (Ali et al., 2013):

BCF = Cu concentration in roots/initial Cu concentration in soil Equation 1

TF = Cu concentration in shoots/Cu concentration in roots Equation 2

2.6. Statistical analysis

The data were tested for normality and homogeneity of variance through the Lilliefors and Shapiro-Wilk tests. Afterwards, the data were submitted to analysis of variance through Sisvar software, version 4.0 (Ferreira, 2011). The solution attributes as well as growth, morphological and nutritional parameters were compared between Cu doses in the same plant species and between the species within the same dose. The means were grouped by the Scott-Knott test at 5% probability.

3. Results

3.1. Soil solution

The concentration of Cu in the soil solution increased with increasing Cu dose, regardless of the cover crop species, at all the samplings (Table 2). At dose 80, the cultivation of *P. plicatulum* reduced the concentration of soluble Cu in the 3rd sampling in relation to *A. affinis* and *P. notatum*. The highest pH values were found in the solution of soil grown with *P. plicatulum*. However, the pH value of the soil solution with dose 80 in the 2nd sampling and dose 0 in the 3rd sampling did not differ from the *P. notatum* species (Table 2). In the 3rd sampling the pH of the soil solution reduced with the addition of Cu, and the highest pH values were found at dose 0, except for *A. affinis*, which did not differ from dose 40, but with a higher pH value than dose 80.

The DOC concentration was lower with *A. affinis* cultivation compared to the other species at all Cu doses in the 2nd sampling and also at dose 0 and dose 80 in the 3rd sampling (Table 2). *P. notatum* and *P. plicatulum* showed a decrease in DOC concentration with the addition of Cu in the 3rd sampling, while *A. affinis* exhibited no change in DOC concentration between the doses in this sampling.

The predominant chemical species of Cu in the soil solution were Cu^{+2} and Cu-DOC in all treatments, comprising between 76 and 98% of soluble Cu (Figure 1). The distribution of Cu species did not show significant changes between treatments in the 1st sampling. The highest percentages of Cu^{+2} in the 2nd and 3rd sampling for all doses occurred with the cultivation of *A. affinis*, which presented increase of 421, 21 and 5% more Cu^{+2} in relation to *P. notatum* and 983, 554 and 42% more Cu^{+2} in relation to *P. plicatulum* for dose 0, 40 and 80, respectively.

The reduction in Cu^{+2} percentage was accompanied by an increase in Cu-DOC percentage. The percentage of Cu^{+2} increased with increasing Cu dose in the cultivation of *P. notatum* and *P. plicatulum*. On the other hand, the percentage of Cu^{+2} remained constant between the doses in the cultivation of *A. affinis* (Figure 1). The $CuOH^{+}$ species presented a higher percentage in the 2nd sampling (dose 0) with the cultivation of *P. plicatulum*, comprising 21.7% of soluble Cu. In addition to this species, $CuOH^{+}$ constituted 5.15 and 1.3% of soluble Cu at dose 0 and 40 of the solution of soil grown with *P. notatum*. The $CuSO_{(aq)}$ species exhibited percentages higher than 1% only at doses 40 and 80 in the 1st sampling and dose 80 in the 2nd sampling.

3.2. Growth, accumulation and translocation of Cu

The growth of native grasses was reduced by excess Cu, diagnosed by the reduction in shoot and root dry matter yield at dose 80 (Figure 2a, b). Dry matter yield of the shoots of *P. notatum* reduced with increasing Cu dose added to the soil, while *A. affinis* and *P. plicatulum* showed no reduction in dry matter yield of shoots and roots at dose 40. The highest dry matter yield, with the exception of the roots at dose 0, was observed in *P. plicatulum* with increases of 994 and 468% in shoot dry matter yield and 2273 and 357% in root dry matter yield, in relation to *A affinis.* and *P. notatum*, respectively, at dose 80 (Figure 2a, b).

The highest accumulation of Cu in dry matter occurred in *P. plicatulum* grown in soils with Cu addition and the highest amount of Cu accumulated in shoots at dose 40 and roots at dose 80 (Figure 2c, d). *A. affinis* presented lower Cu accumulation at dose 0 in relation to the other species. However, it did not change in accumulation compared to *P. notatum* in soils with Cu addition, except in roots at dose 80, which was lower.

The bioconcentration factor (BCF) and translocation factor (TF) reduced in all species of native grasses with the Cu addition (Figure 2e, f). BCF greater than 1 was observed in all species only at dose 0. *A. affinis* presented a BCF of 0.87 at dose 80, which was higher than the other species (Figure 2e). TF was lower than 1 for all doses and species, reducing with increasing Cu doses in *P. notatum* and *P. plicatulum*. The lowest TF was found at dose 80 in *P. plicatulum* (Figure 2f).

3.3. Root morphology and nutrient levels

Root morphology was altered by excess Cu, verified by reduced root length, surface area and volume in all species at dose 80 (Figure 3a, b, d). *P. notatum* decreased in length, surface area and volume with increasing Cu dose, while reduction was restricted to dose 80 in *A. affinis* and *P. plicatulum*. Among the cover crops, *P. plicatulum* showed the highest root length and surface area at all Cu doses and also the highest root volume in soils with Cu addition. The addition of Cu to the soil did not alter the average root diameter of *A. affinis* and *P. notatum*, but the addition of dose 80 increased the average root diameter of *P. plicatulum*

(Figure 3c). *P. plicatulum* had the lowest average root diameter at dose 0 and 40, while *A. affinis* had the lowest average root diameter at dose 80.

Toxicity from excess Cu altered the root system partition in the different diameter classes, reducing the percentage of roots with diameter greater than 0.8 mm with increasing Cu dose in *A. affinis* (Figure 3e). For *P. plicatulum*, there was an increase in the percentage of roots with diameter greater than 0.8 mm with increasing Cu dose. Most of the root system of *P. plicatulum* consists of fine roots, with approximately 80% in diameter classes between $0 \geq 0.2$ mm and $0.2 \geq 0.4$ mm at doses 0 and 40. However, at dose 80, there was a decrease in this proportion because of the reduction of the percentage of roots with a diameter between $0 \geq 0.2$ mm with increasing Cu dose (Figure 3e). In *P. notatum*, we observed a small change in the distribution of the root diameter classes at different Cu doses.

Excess Cu promoted a change in the status of other nutrients in the native cover crops, as shown by P levels in shoot tissue and roots of *A. affinis* and *P. notatum*, which presented the lowest levels at dose 80. Contrarily, *P. plicatulum* showed the highest P level at dose 80 compared to the other Cu doses in both shoots and roots (Table 3). At dose 80, P level in the roots of *P. plicatulum* was 139 and 178% higher than those of *A. affinis* and *P. notatum*. The highest Ca levels in shoot and roots in all native cover crops were found at dose 80.

The Cu levels in tissue increased with increasing doses in all cover crops, with increases in shoots and roots of 236 and 566% in *A. affinis*, 179 and 249% in *P. notatum*, and 164 and 233% in *P. plicatulum* for dose 40 and 80, respectively (Table 3). The Cu levels in shoots did not change between the species grown at dose 0 and 40. However, *A. affinis* showed the highest level at dose 80, followed by *P. notatum*. In roots, *A. affinis* presented the highest Cu level between the species at dose 40, but did not differ from *P. plicatulum* at dose 80 (Table 3). The Fe and Mn levels increased with increasing Cu dose in *A. affinis*. This species presented the highest Fe and Mn levels in shoots compared to the other species grown with Cu addition. The increase in Mn level in *A. affinis* in relation to *P. notatum* and *P. plicatulum* at dose 80 was 402 and 589% in shoots and 506 and 1194% in roots. The Fe and Mn levels in shoot tissue and roots did not differ statistically between the Cu doses in *P. plicatulum*.

4. Discussion

The Cu added anthropically to the soil is adsorbed initially to functional groups of mineral and organic particles with high energy, thus remaining only a small fraction of the metal in the soil solution (Nolan et al., 2003; Girotto et al., 2014). With the saturation of the most avid adsorptive sites, Cu concentration in soil solution increases, increasing the risk of toxicity to plants and contamination of water resources by the transfer of the metal (Babcsányi et al., 2016). Increases in the concentration of soluble Cu were diagnosed in soils of old vineyards in southern Brazil, due to a history of cupric fungicide applications, indicating risk of toxicity to grapevines and cover plants (Girotto et al., 2014).

The high pH values in the solution of soil grown with *P. plicatulum* demonstrate the ability of this species to induce changes in the chemical characteristics of the soil solution, modifying the bioavailability of the chemical elements (Table 2). The increase in pH probably occurred because of the release of OH^-/HCO^3 by plant roots as a tolerance mechanism to excess metal or as a way to maintain the electrochemical balance of the plant with the absorption of anions (Gahoonia et al., 1992). Increasing pH promotes the deprotonation of the functional groups on the surface of the colloids, increasing the negative charges in the solid phase and consequently the electrostatic adsorption of cations, thus reducing Cu concentration in the soil solution (McBride, 1994).

The grown of plants and application of Cu doses altered the distribution of chemical species of soluble Cu, with the largest changes observed in the 3rd sampling (Figure 1). The predominance of Cu-DOC in the soil solution with *P. plicatulum* cultivation possibly contributed to the increased plant growth of this species in relation to the others, given the greater ability to reduce the bioavailability of the contaminant, since Cu^{+2} is preferentially absorbed by plants and microorganisms (McBride, 1994; Martínez & McBride, 1999). Oustriere et al. (2016) attributed the reduction of Cu^{+2} in the soil solution for Cu reaction with DOC after the biochar and compost amendments. In addition to the concentration, plants can modify the DOC composition through the root exudation of soluble organic compounds, such as organic acids and phenolic compounds, which have functional clusters, especially carboxylic acid, which promotes a high capacity of metal cation complexation, reducing the free fraction in solution (Kim et al., 2010, Dresler et al., 2014). The lower pH and concentration of the organic ligands (DOC) in the 3rd sampling were possibly reflected in the predominance of the Cu^{+2} species in *A. affinis* cultivation, since DOC concentration and pH are the main factors that govern the distribution of chemical species of metals in soil solution (Li et al., 2013, De Conti et al., 2016). At higher pH the percentage of Cu^{+2} is reduced by the increase in the percentage of $CuOH^+$ and also because it contributes to the increase of Cu-DOC. This is because the organic compounds have pH-dependent charges and with increasing pH there is increase in the negative charges of the functional groups of these compounds, increasing complexation and consequently Cu-DOC ratio in soil solution (Pérez-Esteban et al., 2014).

Among the possible adaptive mechanisms for nutrient absorption at low concentrations and reduction of the availability of potentially toxic elements are the exudation of complexing organic compounds and the increase in rhizosphere pH, which may also reduce toxicity by heavy metals (Pallarés et al., 2005, Malta et al., 2016). These mechanisms can also be expressed when plants are grown in soils with high Cu levels, explaining the reduction in Cu bioavailability observed in *P. plicatulum* and *P. notatum* at dose 0 and 40, since soils in southern Brazil naturally present low Cu levels (Miotto et al., 2014). Induction in the exudation of malic acid in response to high levels of Cu and Al was observed by Nian et al. (2002) on young wheat plants.

The reduction in growth of all species at dose 80 shows the phytotoxic effect of Cu at high concentrations (Figure 2a, b). Excessive absorption of Cu by plants generally induces reduced pigment levels, especially chlorophylls (Chl *a*, Chl *b*), compromising the efficiency of the photosynthetic apparatus and reducing the rate of CO_2 fixation, possibly causing the reduction in dry matter yield observed in the different species (Cambrollé et al., 2015).

The highest accumulation of Cu by *P. plicatulum* indicates greater potential of this species to be used in the phytoremediation of Cu in vineyard soils. This could be by phytoextraction, through the removal of shoot biomass or by phytostabilization, with the reduction in bioavailability by the increase of the complex chemical species in soil solution, or by immobilization of the metal in the biomass, because it is a perennial species and has high Cu accumulation in the roots (Figure 2c, d). The main factors that determine the potential of plants for use in phytoremediation are the adaptability to the environment, high biomass yield and high metal concentration in the biomass (Li et al., 2010). Leguizamo et al. (2017) stress the importance of using native herbaceous plants for phytoremediation of areas contaminated with heavy metals, avoiding the introduction of exotic species. This is because these introduced species may affect the ecological and environmental dynamics of the environment. The author suggests 41 native or endemic species with potential for phytoremediation of heavy metals for further research.

The BCF and TF are criteria used to estimate the potential use of plant species in phytoremediation and one of the criteria for the identification of metal-hyperaccumulating species, which need to have coefficients higher than 1 (Leguizamo et al., 2017). The low bioconcentration and translocation factors found in plants grown in soils with Cu addition may have occurred because the plants have tolerance mechanisms that reduce bioavailability and consequently absorption (Figure 1). Boechat et al. (2017) also observed translocation factors <1 in *Mucuna deeringiana* grown in multi-metal contaminated soils from a gold mining area. The high accumulation of Cu in roots, with little translocation to shoots (Table 3), contributed to reduce BCF and TF. The accumulation of Cu in roots is a tolerance mechanism triggered by several species as a way of preventing and/or reducing the translocation of excess Cu to shoots (Cambrollé et al., 2015; Girotto et al., 2016). The isolated evaluation of BCF and TF provides limited information on the phytoremediation potential of these species, as verified at dose 80 for *P. plicatulum*, which presented lower TF and BCF compared to *A. affinis*, but had the highest accumulation of Cu in shoots and roots (Figure 2c, d), which indicates high capacity for Cu phytostabilization. This highlights the need to quantify the accumulation by plants in order to select species with higher potential for phytoremediation of environments contaminated with Cu.

Excess Cu can impair protein activity and promote hormonal imbalances, retarding or inhibiting cell division and elongation (especially in meristematic regions), promoting morphological changes in the root system (Potters et al., 2009; Bochicchio et al., 2015). Changes in root morphology that restrict the volume of explored soil may induce deficiency or excessive absorption of other nutrients (Yruela, 2005; Ambrosini et al., 2015). Changes in root morphology caused by excess Cu were also found in other grass species, such as oat (Guimarães et al., 2016) and wheat (Michaud et al., 2008). The increase in root diameter in plants grown with high levels of Cu, as seen in *P. plicatulum*, was found in species more resistant to the metal, while more sensitive species showed a decrease in root diameter (Zhang et al., 2014). The increase in average root diameter may be a consequence of changes in the division and organization of cortical cells, increased cortex area and consequently root diameter (Ambrosini et al., 2015).

The reduction of the volume of soil explored by the roots can affect plant nutrition, mainly reducing the absorption of poorly mobile nutrients in soils. This was observed in the reduction of P levels in *A. affinis* and *P. notatum*, which probably occurred due to the element having diffusion as a main supply mechanism, indicating indirect losses by Cu toxicity, as it limits the absorption of nutrients and water (Kabata-Pendias, 2011). Although the formation of precipitates between Cu and P in roots may contribute to the attenuation of toxicity by the immobilization of the metal in the vacuole, it impairs the redistribution of P to other parts of the plant, altering the nutritional status and probably contributing to the reduction in dry matter yield (Brown et al., 1995; Arriagada et al., 2009). When grown in toxic concentrations of Cu, Cambrollé et al. (2015) found reduced P levels in grapevine leaves. The increase in P level at dose 80 in *P. plicatulum*, which was contrary to the other species, is possibly an adaptive mechanism to the excess Cu triggered by the plant, since P has an important role in controlling the accumulation and transport of Cu inside the plant (Ke et al., 2007).

The variation in Cu levels in shoots between the native grasses at dose 80 demonstrates greater homeostasis in *P. plicatulum* under phytotoxic conditions. Excessive absorption of Cu contributed to the reduction of plant growth, since shoots levels of Cu between 20-100 mg kg^{-1} of dry matter are considered toxic or excessive (Kabata-Pendias, 2011). Excess Cu induces physiological changes, such as increased levels of lipid peroxidation and chlorophyll degradation, causing internerval chlorosis (common symptom of Cu phytotoxicity), reducing the photosynthetic efficiency and consequently the fixation of C (Li et al. 2012; Tiecher et al., 2016).

Excess Cu affects the activity of membrane transporters and ion channels, which may reduce selective capacity, increasing membrane non-specific permeability, resulting in nutritional imbalances (Cambrollé et al., 2015). This physiological imbalance probably explains the increase in Mn levels in the tissue of *A. affinis* and *P. notatum* (Table 3). The substitution of Mn of the soil colloid exchange sites by Cu, given the higher adsorption energy, possibly contributed to increase the availability of Mn in the soil (Sposito, 1989). Although Mn is an essential element and participates in important processes such as photosynthesis, oxidation-reduction reactions, electron transport and also component of enzymes, it adversely affects growth for most plants at levels above 400 mg kg^{-1} in plant tissue (Kabata-Pendias, 2011). In this study, *A. affinis* grown in soil with Cu addition at dose 80 exhibited 818.68 mg kg^{-1} of Mn in shoots (Table 3), pointing to potential of joint toxicity in vineyard soils. The maintenance of Mn levels in *P. plicatulum*, even with increasing Cu dose added to the soil, probably contributed to increased growth in relation to the other species, indicating the presence of mechanisms that maintain the nutritional balance in phytotoxic conditions.

5. Conclusions

Paspalum plicatulum is a native species of South American grasslands with great potential to be used as cover crops in vineyards with high levels of Cu, among the species studied, because it has a greater capacity to reduce Cu bioavailability and maintain nutritional balance, which resulted in higher dry matter yield and Cu accumulation. Native grasses of South American grasslands can be used as phytostabilizing plants in Cu-contaminated soils, by accumulating the absorbed Cu predominantly in the roots, benefiting grapevines grown intercropped.

The reduction in dry matter yield of native grasses of South American grasslands used as cover crops with the addition of 80 mg Cu kg^{-1} indicates reduced protection and nutrient cycling in the soils of old vineyards, increasing the risk of soil degradation.

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Table 1. Physical and chemical characteristics of the soil at 0.0-0.20 m in a Typic Hapludalf soil of South American grassland.

	Natural grassland
Clay (g kg^{-1})	54
Sand (g kg^{-1})	894
Silt (g kg^{-1})	52
Organic matter (g kg^{-1})	9.0
pH_{H2O} (1:1)	5.2
Exchangeable Al (mg kg^{-1})	0.4
Available Cu by EDTA (mg kg^{-1})	0.7
Available Zn by EDTA (mg kg^{-1})	0.9
Available K by Mehlich-1 (mg kg^{-1})	66.4
Available P by Mehlich-1 (mg kg^{-1})	3.6
Available Fe by EDTA (mg kg^{-1})	5.9
Available Mn by EDTA (mg kg^{-1})	15.4
Exchangeable Ca (mg kg^{-1})	0.5
Exchangeable Mg (mg kg^{-1})	0.2
CEC_{ef}^{*}, $cmol_c$ kg^{-1}	1.4
$CEC_{pH\ 7.0}^{**}$, $cmol_c$ kg^{-1}	3.2

$^{*}CEC_{ef}$ = Ability to effectively exchange cations; $^{**}CEC_{ph\ 7.0}$ = Cation exchange capacity the pH 7.0.

Table 2. Chemical characteristics of the soil solution withdrawn during the grown of three native species of cover crops in soils with increasing Cu levels.

Solution chemical parameters	Cu doses	Plant species		
		A. affinis	*P. notatum*	*P. plicatulum*
			1st Sampling	
pH	0	5.41 aA(1)	5.42 aA	5.74 aA
	40	5.29 aA	5.28 aA	5.18 bA
	80	5.24 aA	5.29 aA	5.32 bA
Dissolved organic carbon (mg L^{-1})	0	28.33 aA	29.33 aA	25.67 aA
	40	25.33 bA	25.33 aA	26.33 aA
	80	31.00 aA	28.67 aA	29.00 aA
Soluble Cu (mg L^{-1})	0	0.021 cA	0.019 cA	0.017 cA
	40	0.357 bA	0.386 bA	0.331 bA
	80	0.984 aA	1.016 aA	0.943 aA
			2nd Sampling	
pH	0	5.43 bC	5.88 aB	7.56 aA
	40	5.67 aB	5.65 aB	6.78 bA
	80	5.29 bB	5.91 aA	6.03 cA
Dissolved organic carbon (mg L^{-1})	0	16.67 bB	25.00 aA	25.67 aA
	40	19.33 bB	26.33 aA	23.00 aA
	80	22.67 aB	27.00 aA	27.67 aA
Soluble Cu (mg L^{-1})	0	0.014 cA	0.014 cA	0.016 cA
	40	0.427 bA	0.479 bA	0.366 bA
	80	1.296 aB	1.520 aA	1.137 aB
			3rd Sampling	
pH	0	5.74 aB	7.21 aA	7.12 aA
	40	5.61 aC	6.14 bB	6.68 bA
	80	5.19 bB	5.34 cB	6.70 bA
Dissolved organic carbon (mg L^{-1})	0	19.7 aC	43.0 aA	32.7 aB
	40	18.5 aB	27.9 bA	18.2 bB
	80	18.2 aB	23.3 cA	21.8 bA
Soluble Cu (mg L^{-1})	0	0.018 cA	0.030 cA	0.014 cA
	40	0.360 bA	0.331 bA	0.276 bA
	80	1.214 aA	1.189 aA	0.774 aB

(1) Means followed by the same lowercase letter do not differ between Cu doses within the same species (column) and means followed by the same capital letter do not differ between species within the same Cu dose (row) by the Scott-Knott test ($p < 0.05$).

Table 3. Macro and micronutrient levels in plants of *Axonopus affinis*, *Paspalum notatum* and *Paspalum plicatulum* grown in soils with increasing Cu levels.

Nutrient	Cu doses	Plant species					
		A. affinis	*P.not*atum	*P.plicatulum*	*A. affinis*	*P.not*atum	*P.plicatulum*
		Shoot			Root		
P (g kg^{-1})	0	1.44 aB(1)	1.76 bA	0.71 bC	1.57 aA	0.69 bB	0.42 cC
	40	1.52 aB	2.12 aA	0.70 bC	1.54 aA	1.12 aB	0.57 bC
	80	1.09 bA	0.91 cB	1.09 aA	0.82 bB	0.64 bC	1.14 aA
K (g kg^{-1})	0	17.34 aA	16.54 bA	8.14 bB	13.29 aA	7.39 cB	4.27 bC
	40	14.96 aB	21.69 aA	8.54 bC	11.48 aB	13.99 bA	5.32 bC
	80	17.60 aA	15.53 bA	15.40 aA	6.56 bC	18.13 aA	9.59 aB
Ca (g kg^{-1})	0	7.15 bA	5.52 bB	4.48 bB	4.73 bA	2.32 cB	4.62 bA
	40	7.87 bA	4.99 bB	4.57 bB	5.22 bA	3.94 bB	5.18 bA
	80	18.26 aA	7.16 aB	6.59 aB	8.28 aA	5.22 aC	6.97 aB
Mg (g kg^{-1})	0	8.79 bA	8.89 aA	7.80 aA	10.96 aA	4.51 bB	5.03 aB
	40	9.92 bA	7.67 aB	8.17 aB	12.27 aA	6.52 aB	6.40 aB
	80	16.81aA	7.25 aC	9.32 aB	7.72 bA	7.59 aA	7.63 aA
Cu (mg kg^{-1})	0	12.88 cA	17.88 cA	15.64 cA	68.84 cA	41.92 cA	41.68 cA
	40	30.44 bA	32.08 bA	25.72 bA	429.3 bA	182.76 bB	240.64 bB
	80	72.88 aA	44.48 aB	36.44 aC	599.6 aA	385.40 aB	594.56 aA
Zn (mg kg^{-1})	0	44.32 aA	26.12 bB	19.24 bB	41.84 aA	23.72 cA	21.80 bA
	40	56.80 aA	59.76 aA	28.56 bB	61.04 aB	98.12 aA	49.16 aB
	80	50.64 aB	58.52 aA	43.44 aB	65.24 aA	75.12 bA	66.24 aA
Fe (mg kg^{-1})	0	348.64 cA	143.04 bB	90.72 aB	886.6 bB	1996.6 aA	971.4 aB
	40	565.44 bA	175.16 bB	153.88 aB	1323 bB	2268.2 aA	1472.9 aB
	80	905.28 aA	315.48 aB	143.32 aC	1928 aA	1308.2 bA	1281.8 aA
Mn (mg kg^{-1})	0	144.48 cA	56.36 bB	123.00 aA	175.5 cA	108.36 bA	117.80 aA
	40	219.24 bA	173.28 aB	137.88 aB	402.2 bA	199.35 bB	132.44 aB
	80	818.68 aA	203.68 aB	138.88 aC	1657 aA	327.68 aB	138.76 aC

(1) Means followed by the same lowercase letter do not differ between Cu doses within the same species (column) and means followed by the same capital letter do not differ between species within the same Cu dose (row) by the Scott-Knott test ($p < 0.05$).

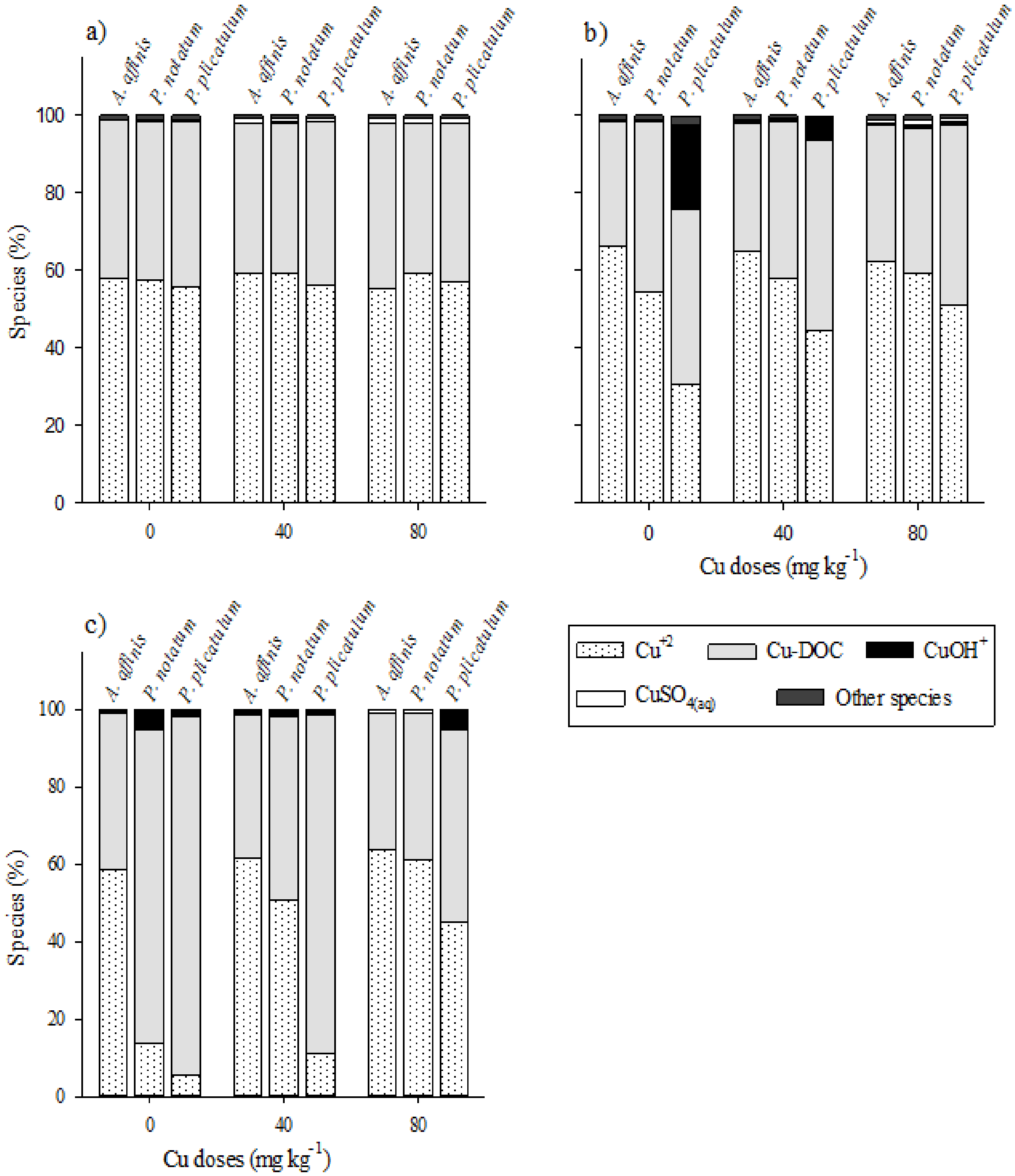

Figure 1. Distribution of chemical species of Cu in the solution of soil grown with three native species of cover crops at the 1st sampling (a), 2nd sampling (b) and 3rd sampling (c) during growth in soils with increasing Cu levels. Other species correspond to $CuNO_3^+$, $CuHPO_{4(aq)}$, $CuCl^+$, $CuNH_3^{+2}$ and $Cu(OH)_{2(aq)}$.

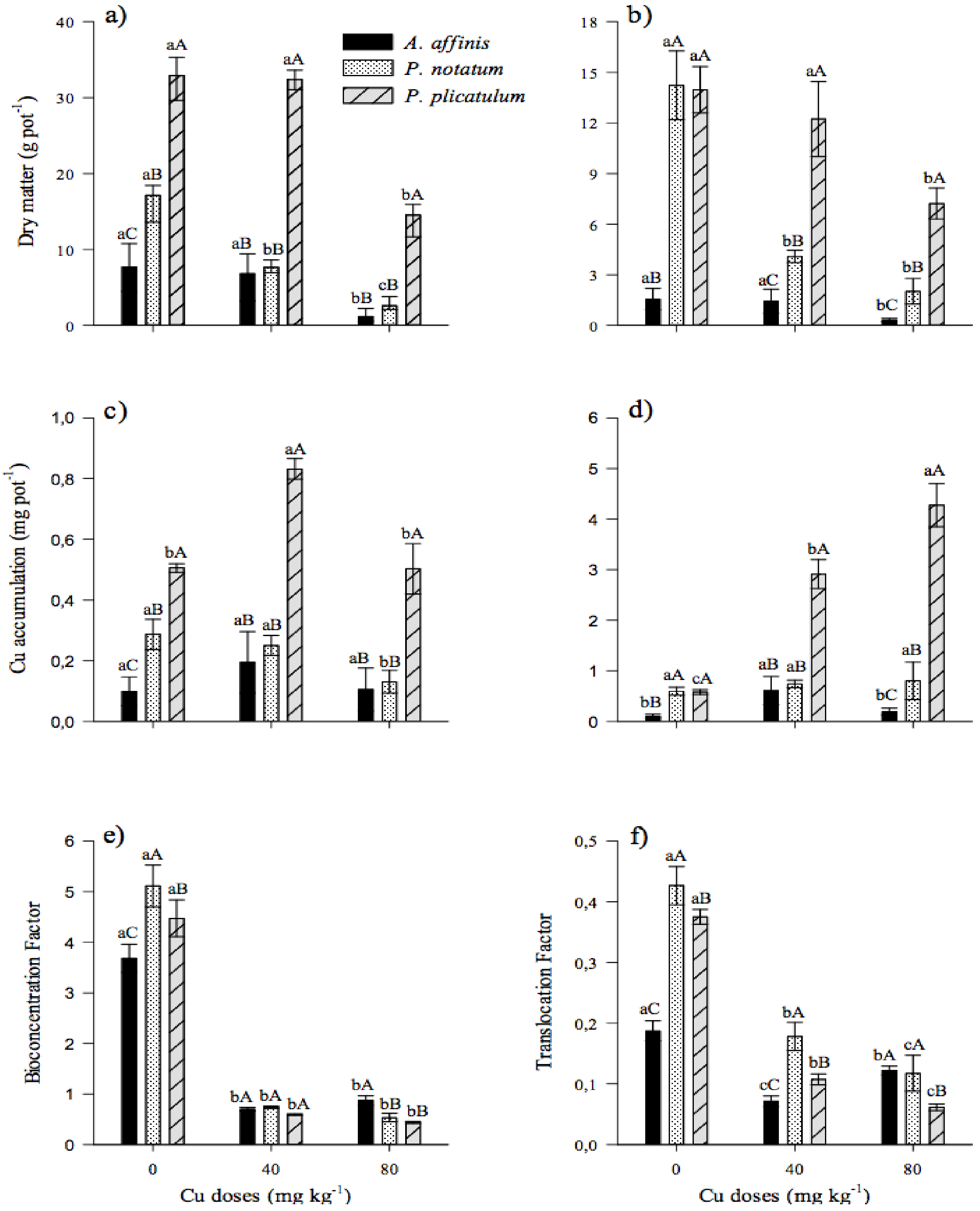

Figure 2. Dry matter yield of shoots (a) and roots (b). Copper accumulation in the biomass of shoots (c) and roots (d). Bioconcentration factor (e) and translocation factor (f) of three native species of cover crops grown in soils with increasing Cu levels. Different lowercase letters indicate difference between Cu doses within the same species and different capital letters indicate difference between species within the same Cu dose by the Scott-Knott test ($p < 0.05$).

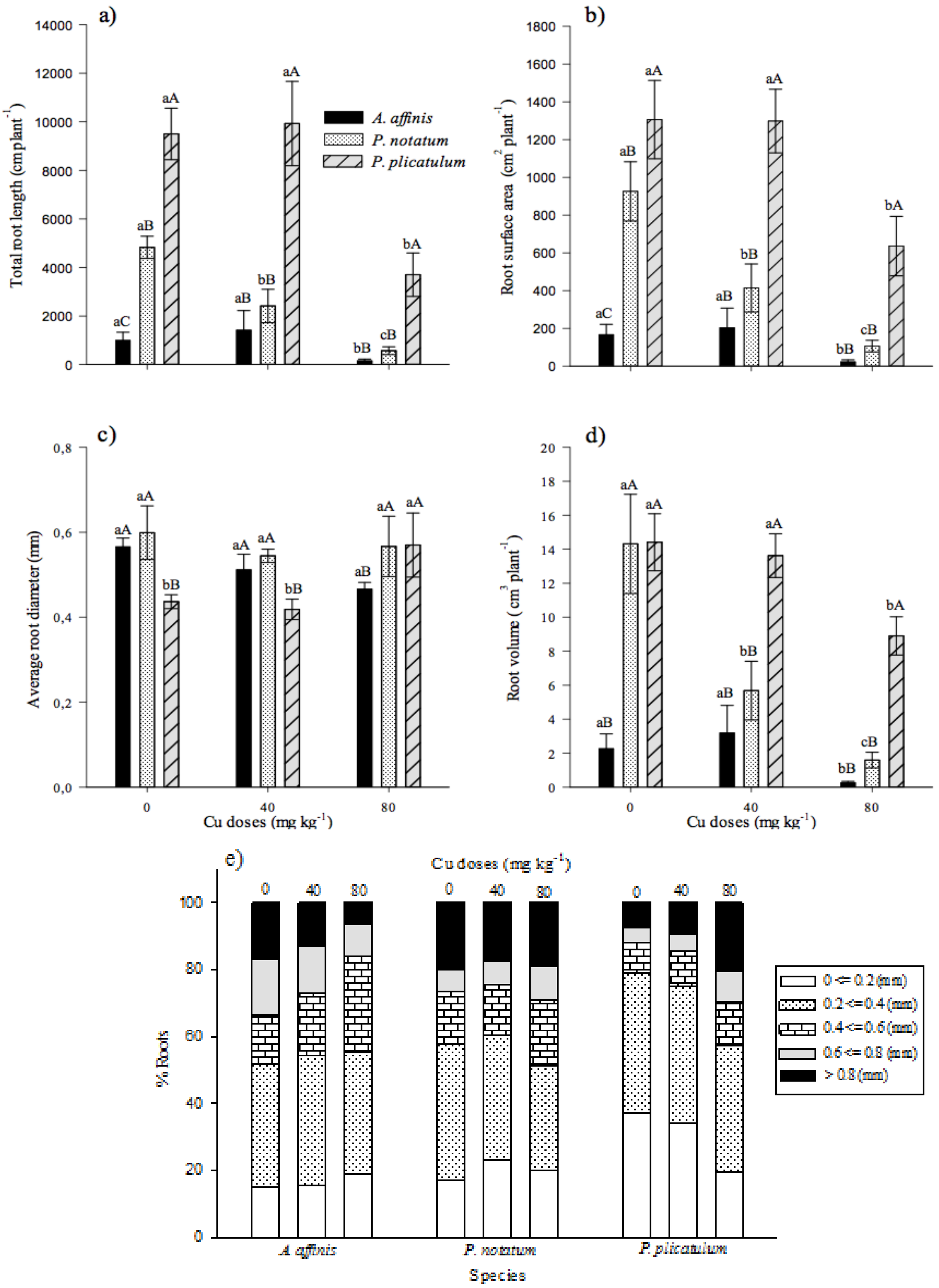

Figure 3. Total root length (a), root surface area (b), average root diameter (c), root volume (d) and percentage of roots in different diameter classes (e) of three native species of cover crops grown in soils with increasing Cu levels. Different lowercase letters indicate difference between Cu doses within the same species and different capital letters indicate difference between species within the same Cu dose by the Scott-Knott test ($p < 0.05$).

5.4 ESTUDO IV

Intercropping of young grapevines with native grasses for phytoremediation of Cu-contaminated soils [4]

Abstract

Intercropping may be a strategy for phytoremediation of vineyard soils with high copper (Cu) content. This agronomic practice increases the complexation of Cu^{2+} in the soil solution, reducing its bioavailability, favouring grapevine growth and contributing to soil conservation and nutrient cycling. The study aimed to evaluate the contribution of South American native grasses in limiting Cu availability and toxicity in soils cultivated with vine plants. The soil used in the experiment was collected in a field of natural grassland with no history of cultivation in Southern Brazil. The samples were air-dried; acidity, P and K levels were corrected and samples were then incubated. We used three Cu levels - natural content (Dose 0) and the addition of 40 and 80 mg Cu kg^{-1} of soil (Dose 40 and 80). At each Cu dose, grapevine was grown in three cropping systems: monocropping (Grapevine), intercropping with *Paspalum plicatulum* (Grapevine + *Paspalum plicatulum*) and intercropping with *Axonopus affinis* (Grapevine + *Axonopus affinis*). In intercropping, two grass seedlings were transplanted into each experimental unit, 35 days prior to the transplanting of the grapevines. Throughout the cultivation period, the soil solution was collected with Rhizon samplers. We analyzed the concentration of the main cations, anions, dissolved organic carbon and pH in the solution, and we carried out ionic speciation. At 70 days after planting, we sampled the grapevines to determine dry matter, morphological parameters and nutrient concentration in the roots and shoots. The intercropping of *Paspalum plicatulum* and *Axonopus affinis* was efficient in promoting the growth of young grapevines at moderate and low levels of Cu-contaminated soils by reducing the Cu bioavailability due to an increase in pH and in the concentration of dissolved organic carbon in the soil solution. The phytotoxic effects of Cu on root morphology in young grapevines were reduced by intercropping in moderately Cu-contaminated soils (dose 40). This

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[4] Artigo elaborado de acordo com as normas da Revista Chemosphere (Pré-aceito para publicação em 11 de julho de 2018).

indicates that maintaining native grasses in young vineyards may be a strategy for phytoremediating Cu-contaminated soils and obtaining a grape production system with reduced interventions in the original environment. However, the excess of Mn uptake and the drop of P contents in grapevine tissues caused by Cu toxicity, even if attenuated in the intercropped systems, need to be taken into account for a balanced nutritional state of grapevines.

Keywords: copper availability, *Vitis vinifera*, ionic speciation, native plants, heavy metal, cover crops.

1. Introduction

It is well known that anthropic activities, especially in the agricultural context, has caused heavy metals accumulation in the environment, giving rise to soil pollution in different parts of the world (Ali et al., 2013; De Conti et al., 2016; Leguizamo et al., 2017; Wan et al., 2017). In this respect, one example is represented by the application of copper(Cu)-based fungicides aimed at controlling pest diseases of orchards and vineyards, particularly massive in regions with frequent rainfall and high temperatures during the productive plant cycle (Komárek et al., 2010; Miotto et al., 2014; Cambrollé et al., 2015; Baldi et al., 2018). Therefore, it is very common to find soils characterized by a very high content of this metal (Brunetto et al., 2016) with evident impacts on crop performance. In fact, though Cu is an essential element for important metabolic processes in plants (Marschner, 2011); yet, when the ionic form is present in high concentrations in the soil solution, excessive root-acquisition of the metal can occur, inducing toxicity phenomena in crops. The consequences are stunted crop-growth with limitation in yield and quality, root morphological changes and nutritional imbalance (Yruela, 2005; Cambrollé et al., 2015; Oustriere et al., 2016). These phenomena, i.e. metal accumulation in soil and toxicity for plants, are well described for both vineyard soils and grapevines plants. However, it is interesting to note that also other plants co-habitating these soils suffer from the same nutritional disorder with very similar symptoms (Ambrosini et al., 2015; Girotto et al., 2016; Tiecher et al., 2016). From the agricultural point of view, this problem is even worse when replanting new vines limiting the success of the vineyard renewal (Miotto et al., 2014; Brunetto et al., 2016; Baldi et al., 2018). For this reason, where the problem is already present, from a medium to a long-term perspective that still guarantees grapevine cultivation in these areas vocated to viticulture, the need to set agronomic practices limiting Cu availability in soil, is urgent (Brunetto et al., 2017). Certainly, all the agronomical measures limiting the use of Cu-based agrochemicals for plant-defence programs can prevent the further worsening of the problem.

In the recent decades, the expansion of viticulture in the state of Rio Grande do Sul, currently responsible for 90% of Brazil's wine production (Flores & Medeiros, 2013), has interested mainly natural grasslands (in the Pampa Biome) in southern Brazil as well as in Uruguay and Argentina. It is interesting to note that botanical investigations conducted in these areas have shown the presence of about 3000 plant species, with remarkable diversity of grasses (more than 450 species) (Brazil, 2017). Within these latter, *Axonopus affinis* and *Paspalum plicatulum* are the most ubiquitous native plant species (Quadros et al., 2009). It is interesting to note that both species evolved in acidic and chemically poor-soils and possess adaptive mechanisms to optimize nutrient acquisition processes and to limit the effects of Al^{3+} toxicity (Pallarés et al., 2005). In particular, the absence of crop rotation in vineyards and their seed availability as cover crops, have further favoured their diffusion in the above-mentioned areas.

It is well demonstrated that the features of the soil surrounding the roots (pH, humidity, redox state, microbial community, etc.), i.e. rhizosphere, can be massively modified by root activity as a consequence of several different environmental conditions like, for instance, nutrient deficiency/toxicity (Hinsinger et al 2009). In this respect, roots release a plethora of exudates (e.g. protons, soluble organic compounds such as organic acids, amino acids, phenolic compounds, etc). Due to their impact on pH and to their metal-complexing properties (thus named ligands), they considerably affect also the elements availability for plant uptake (Chaignon et al., 2009; Dresler et al., 2014; De Conti et al., 2016; Chen et al., 2017, Mimmo et al., 2014). With respect to Cu and particularly when its concentration in soil is high, it has been hypothesized that the Cu complexation process, mediated by these ligands released by roots and at the expenses of the ionic (Cu^{+2}) pool usable by plants and microorganisms (McBride, 1994; Kabata-Pendias, 2011), could limit the extent of the metal acquisition by roots. In other words, this root-mediated phenomenon can be considered as a plant attempt to stabilize the metal outside the root limiting thus its availability and, in turn, its plant uptake. It is interesting to note that when this mechanism occurs in the rhizosphere of intermingled roots of two intercropped plant species (one tolerant and one not tolerant), it is evident that both can benefit of it (Brunetto et al., 2016; Wan et al., 2017), independently of the relative contribution on metal stabilization. In fact, the agronomic practice of intercropping is hypothesized to be a valid phytoremediation approach for soils slightly or moderately contaminated with heavy metals (Wan et al., 2016). In particular for the case of Cu, the functionality of adaptive mechanisms to Al^{3+} toxicity in *Axonopus affinis* and *Paspalum plicatulum* plants makes them particularly interesting (Sainger et al., 2011; Leguizamo et al., 2017). In addition, the use of this mild practice to control and limit Cu availability in soils would not only guarantee vine cultivation in vocated areas (Wan et al., 2017), but also the achievement of quality parameters intrinsically linked to the cultivation environment such as the viticulture terroir (Muscas et al., 2017). However, up to now the mechanisms underlying the process at the soil level remain to be clarified, and a crucial premise for an extensive field exploitation of this agronomic technique (intercropping) for the mitigation of the soil Cu-toxicity in vine plants.

On the basis of these premises, the present work aimed at evaluating the contribution of native grass species in the limitation of Cu availability in soil cultivated with vine plants. For this purpose, plants of the rootstock Paulsen 1103 (*Vitis vinifera* cv.), alone or intercropped with *Axonopus affinis* or *Paspalum plicatulum* plants, have been grown in pots containing a Typic Hapludaf soil. In order to mimic the soil contamination via Cu-based agrochemicals after 15 or 30 years of their applications, two batches of soil were contaminated with 40 or 80 mg kg^{-1} Cu, respectively. Results of soil solution analysis and those of plants tissues are discussed in terms of redistribution of the metal among the soil fractions and its impact on plant health.

2. Material and Methods

2.1. Soils

The study was carried out using a Typic Hapludalf Soil (Soil Survey Staff, 2006) collected at 0-20 cm in an area of uncultivated native grassland (30º47’23.7’’S and 55º22’7.3’’W) with naturally low concentration of Cu. Table 1 shows the soil physical and chemical characteristics. The area is located in the city of Santana do Livramento, state of Rio Grande do Sul, which is part of the Pampa Biome in southern Brazil. After collection, the soil was air dried, homogenized and passed through a 2 mm mesh sieve. To correct acidity, we applied a mixture of calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$), with a ratio of 2:1, at a dose of 0.57 g kg^{-1} of soil. The soil was then incubated at 80% maximum water holding capacity (MWHC) for 35 days. Afterwards, we applied 40 mg P kg^{-1} and 100 mg kg^{-1} K of soil by adding triple superphosphate and potassium chloride, respectively. The moisture was restored to 80% of the MWHC and incubation continued for another 25 days. This soil was considered as control soil (Cu dose 0), representative of the condition prior to the installation of the vineyards. This soil was further spiked either with 40 and 80 mg kg^{-1} Cu (dose 40 and 80), which are levels typically found in vineyards under grapevine cultivation for approximately 15 and 30 years, respectively (Miotto et al., 2014). Cu was added 50 days after the application of the soil amendment, by applying a solution of $CuSO_4$ $5H_2O$. Subsequently, the soil was incubated again for 115 days under the same conditions described above. The soil was mixed thoroughly and weighed three times a week throughout the incubation period to evaluate moisture content; distilled water was added when necessary to maintain the MWHC at 80%.

2.2. Experimental design and crops

The experiment was conducted in a completely randomized design with three replicates. The experimental units consisted of pots containing 7 kg of soil. At each Cu dose, grapevine was grown in three cropping systems: monocropping (Grapevine), intercropping with *Paspalum plicatulum* (Grapevine + *Paspalum plicatulum*) and intercropping with *Axonopus affinis* (Grapevine + *Axonopus affinis*) (Figure 1). In November, we transplanted one grapevine (*Vitis vinifera* cv. Paulsen 1103) plant per pot and grown for 70 days. In treatments with intercropping, the two South American native grasses were transplanted into each pot, 35 days prior to the transplanting of the grapevines. Grapevine plants were obtained by tissue culture explants multiplied *in vitro*, with acclimatization and rooting in a greenhouse for 50 days, where seedlings were grown to a size of 30 cm.

The native grasses were collected in natural grassland and multiplied by means of preculture with nutrient solution in sand to obtain uniform seedlings of about 12 cm in height. The complete nutrient solution used to irrigate the plants in preculture consisted of (mg L^{-1}): 149.80 NO_3^-; 24.80 $H_2PO_4^-$; 39.27 SO_4^{2-}; 41.31 Mg^{2+}; 288.72 Ca^{2+}; 234.60 K^+; 0.03 Mo; 0.26 B; 0.06 Cu; 0.50 Mn; 0.22 Zn; and 4 Fe, supplied through irrigation three times a day (15 minutes each).

At the time of transplanting the grapevines and native grasses, the preculture substrate was carefully removed by washing with distilled water. At 16 and 48 days after transplanting, nitrogen (N) was applied as urea (20 and 10 mg N kg^{-1} of soil, respectively). In the intercropping treatments, the shoots of the cover crops were cut at 10 cm (height) and placed on the soil surface every 21 days, totalling three cuts throughout the grapevine cultivation period. This cover crop management aims to simulate mowing management used in commercial vineyards.

2.3. Extraction, analysis and speciation of the soil solution

Soil solution was extracted the day prior to the transplanting (1st sampling) and 69 (2nd sampling) days after transplanting using Rhizon MOM mini-lysimeter. The Rhizon MOM samplers were installed in the soil at a depth of 2 to 12 cm. Soil moisture was maintained at 70% MWHC during cultivation with daily weightings and addition of distilled water to maintain soil moisture. The day prior to the extraction of the soil solution, moisture was raised to 95% MWHC. The solution was sampled 16 hours after irrigation by creating a vacuum with the use of a 60 mL syringe. Consequently, we measured pH and dissolved organic carbon (DOC) content spectrophotometrically at 560 nm after digestion with 0.4 N potassium dichromate at 60°C for 4 h as described by Silva and Bohnen (2001). In soil solution sampled were determined contents of aluminum (Al), calcium (Ca), magnesium (Mg), copper (Cu), zinc (Zn), manganese (Mn), potassium (K), iron (Fe) and phosphorus (P) by inductively coupled plasma atomic emission spectroscopy (ICP Perkin-Elmer, USA); ammonia (NH_4^+) and nitrate (NO_3^-) were determined by colorimetry at 660nm (NH_4^+) and 540 nm (NO_3^-) (SANplus, Skalar, Breda, Holand); and sulfur (S) and chlorine (Cl) were determined by ion chromatography (S135 Ion Chromatography system, Germany) (Table 2).

Ionic speciation of the solution was determined by Visual Minteq software (version 3.0 - Gustafsson, 2013) using the total soluble cations (Al^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, K^+ and Fe^{2+}), anions (PO_4^{3-}, NO_3^-, SO_4^{-2} and Cl^-), DOC and pH of the soil (n=3). The formation of metal complexes with DOC was evaluated using Gaussian DOM model (Grimm et al., 1991). The formation of inorganic soluble complexes was assessed using the standard equilibrium constants of the Visual Minteq software developed by Smith et al. (2003). We thereby obtained the percentage distribution of all species of Cu in the soil solution.

2.4. Dry matter yield and chemical analysis in plant tissue

At 70 days after the transplanting of the grapevines, the shoots were cut close to the soil surface; the leaves were separated from the branches and stored for the determination of dry matter (DM) and nutrient analysis. The roots were separated from the soil by hand, washed in running tap water to remove soil particles, dried with paper and then weighed. Afterwards, the root system was divided into two portions: one was placed in distilled water for future assessment of the root architecture, while the second portion was washed in 0.012 mol L^{-1} EDTA solution to remove nutrients outside the roots and then in distilled water for subsequent determination of DM and nutrient concentration. Leaves, stem and roots were then dried in an oven with forced air at ± 65° C until reaching constant weight to assess DM.

Dried leaves, stem and roots were ground in a Wiley mill and digested with HNO_3-$HClO_4$ (Embrapa, 1997) to determine the concentration of Cu, Fe, Mn and P. Cations were determined by atomic absorption spectrophotometer (AAS) and P by colorimetry (Murphy and Riley, 1962).

2.5. Root architecture

The morphological characterization of the roots was obtained by scanned images, using WinRhizo Pro 2013 software, coupled to an EPSON Expression 11000 scanner equipped with additional light (TPU), with a definition of 600 dpi. We determined total root length (cm), surface area (cm^2), volume (cm^3), average diameter (mm) and root distribution in each diameter range.

2.6. Statistical analysis

The data were tested for normality and homogeneity of variance through the Lilliefors and Shapiro-Wilk tests. Afterwards, the data were submitted to analysis of variance through SISVAR software, version 4.0 (Ferreira, 2011). The chemical attributes of the soil solution, the growth, morphological and nutritional parameters were compared between Cu doses in the same cropping system and between cropping systems at the same Cu dose. The means were grouped by the Scott-Knott test at 5%.

3. Results

3.1. Soil solution

The exogenous addition of Cu in soil increased the concentration of Cu in soil solution, regardless of the cropping system, at the two samplings (Table 2). The highest concentrations of Cu were detected in the soil solutions sampled from the Grapevine + *Axonopus affinis* at dose 80 compared to the other cropping systems at the 2nd sampling. The addition of Cu led to a decrease of soil pH, being lowest at the highest Cu dose in all the cropping systems. Soil solutions sampled from the intercropped plants (in both treatments, Grapevine + *Paspalum plicatulum* and Grapevine + *Axonopus affinis*, Table 2) exhibited higher pH values than the one sampled form the monocropped plants.

In all the cropping systems, the dissolved organic carbon (DOC) concentration decreased with the addition of the highest Cu dose compared to dose 0 at the 2nd sampling. Among the cropping systems, Grapevine + *Paspalum plicatulum* promoted the highest DOC concentration, except for dose 0 and dose 80 of the 2rd sampling.

The chemical species of Cu predominating in the soil solution at all doses and cropping systems were Cu^{+2} and Cu-DOC, comprising between 93 and 99% of the soluble Cu (Figure 2). Cu^{+2} was predominant in all the treatments at the 1st sampling, when the percentage of Cu^{+2} increased with increasing Cu dose in Grapevine + *Paspalum plicatulum,* while the percentage was similar among Cu doses in the other cropping systems. At the 2nd sampling, Cu^{+2} increased with increasing Cu doses in all the cropping systems, and the highest percentages were found in monocropping.

The reduction in the percentage of Cu^{+2} at dose 0 and dose 40 throughout cultivation was accompanied by the increase in the percentage of Cu-DOC (Figure 2). Cu-DOC was predominant at the 2rd sampling in the solution of the soils under intercropping at dose 0 and dose 40. At the 2rd sampling, Grapevine + *Paspalum plicatulum* increased the proportion of Cu-DOC in approximately 84, 131 and 41% in comparison to monocropping and in 65, 81 and 47% compared to Grapevine + *Axonopus affinis* at doses 0, 40 and 80, respectively. Other species, including $CuOH^+$, $CuSO_{4(aq)}$, $CuNO_3^+$, $CuHPO_{4(aq)}$, $CuCl^+$ and $Cu(OH)_{2(aq)}$ were present in small amounts in the soil solution.

3.2. Plant growth

Excessive Cu impaired grapevine plant growth. This was diagnosed by the reduction in leaf and stem dry matter (DM) yield (Figure 3a, b). This growth reduction was alleviated when the plants were intercropped with the grass species: at dose 40 the intercropping increased leaf and stem DM yield by 362 and 523%, respectively in Grapevine + *Paspalum plicatulum* and 262 and 346%, respectively in Grapevine + *Axonopus affinis* compared to monocropping. At dose 40 Grapevine + *Paspalum plicatulum* also presented higher stem and leaf DM yield compared to the other cropping systems (Figure 3a, b). There was no significant difference in leaf and stem DM yield among the cropping systems at dose 80. Cu addition and cropping systems did not significantly change root DM yield of young grapevines (Figure 3c).

3.3. Nutrient content and root morphology

Excessive Cu affected the nutritional status of the grapevines, particularly the content of P, Fe and Mn in all plant tissues analysed (Table 3). Phosphorus leaf content resulted lowest in the grapevine plants grown in soil with the highest Cu dose, while no differences were observed in the intercropping systems among the Cu doses. Also the stem P content resulted the lowest at dose 80 in all the cropping systems, which was also verified in roots of the intercropping systems (Table 3). In roots, at dose 40 P content was 52 and 38% higher in the Grapevine + *Paspalum plicatulum* and Grapevine + *Axonopus affinis* treatments, respectively, compared to the monocropping. At dose 80, there was no difference in P contents among the cropping systems in the three organs evaluated in this study.

The highest Cu content in leaf, stem and root tissue was found at dose 40 and 80 while, as expected, the lowest Cu contents in all the plant tissues analysed were found at dose 0 (Table 3). Intercropping the grapevines with grasses reduced the Cu content of grapevine leaves and stems, particularly evident in the leaves of vines intercropped with *Paspalum plicatulum*. In roots we observed an opposite trend: root Cu content was highest in the intercropped plants compared the monocropped grapevines (Table 3).

Leaf and stem Fe content was not affected by the Cu dose, yet by the cropping system. In fact, the intercropped grapevines exhibited a lower Cu content than the monocropped ones. This phenomenon was observed also in the roots, yet only at dose Cu 0. At higher Cu doses, intercropped grapevines revealed higher Fe root contents compared to the monocropped plants (Table 3).

Monocropped grapevine leaves and stems revealed an increasing Mn content with increasing Cu dose while intercropped vines showed an increased Mn content in both stems and leaves only at the highest Cu dose (Table 3). Furthermore, monocropped grapevines exhibited higher Mn concentrations in their plant tissues than the intercropped vines: Mn content increased by 254 and 106% in stems and by 101 and 55% in roots in monocropping compared to Grapevine + *Paspalum plicatulum* and Grapevine + *Axonopus affinis* at dose 80, respectively.

Root morphology was affected by excessive Cu concentrations, verified by the reduction in length and surface area at dose 80 in all the cropping systems (Figure 4a, b). At dose 40, the largest root length and surface was observed in Grapevine + *Paspalum plicatulum*, followed by Grapevine + *Axonopus affinis* and the monocropped grapevines. However, these parameters did not present differences among cropping systems at dose 0 and 80. The addition, the average root diameter was increased at dose 80 in all the cropping systems (Figure 4c). The highest root volume in monocropped grapevines was determined at dose 0 and in Grapevine + *Paspalum plicatulum* at dose 40 (Figure 4d). The intercropping of both native grasses at dose 40 promoted an increase in root volume compared to monocropped vines.

Copper toxicity changed the partition of the root system in the different diameter classes, increasing the percentage of roots with a diameter greater than 0.8 mm and reducing the percentage of roots with diameter between $0.2<L\geq0.4$ mm (Figure 4e). In Grapevine + *Paspalum plicatulum*, these changes were significant only at dose 80 (Figure 4e). At dose 40, roots with diameter greater than 0.8 mm comprised 39% of monocropped grapevines, 33% in Grapevine + *Axonopus affinis* and 27% in Grapevine + *Paspalum plicatulum*. The ratio of finer roots ($0.0<L\leq0.2$ mm) reduced with increasing Cu dose in the monocropping treatment, while reduction was restricted to dose 80 in intercropping. The ratio of the roots between $0.4 <L\geq0.6$ and $0.6 <L\geq0.8$ mm were little affected by the addition of Cu in all the cropping systems.

4. Discussion

As expected the anthropic addition of Cu caused a sharp increase of the soluble Cu-pool in the soil, being obviously massive at the dose of 80 mg Cu kg^{-1}. This result indicates that the mineral and organic Cu-adsorptive sites have been saturated by both Cu treatments (40 and 80 mg kg^{-1}), as also described by Girotto et al. (2014) and De Conti et al. (2016). In this respect, it should be noted that the higher the level of the Cu soluble fraction, the easier the risk of toxicity for the plants, being this pool the source mainly utilized by the roots in the nutrient acquisition process (Marschner, 2011). Moreover, from an environmental and aquifer perspective, the extent of this fraction could represent, particularly when excessive, a serious concern for its mobility through surface runoff and leaching phenomena (Babcsányi et al., 2016). Interestingly, Figure 2 revealed that the magnitude of the Cu^{2+} fraction, i.e. the ionic source mainly utilized by root uptake (McBride, 1994; Martínez and McBride, 1999), the prolonging the plant growing period, a clear decrease of this fraction has been measured in the soil solution of monocropped vine plants; this effect can be reasonably ascribed to the depletion of the metal as a consequence of the root acquisition, as actually confirmed by the levels of Cu accumulation in plant tissues (Figure 3). In particular, in roots a clear relationship between the soil availability levels of Cu and the nutrient contents in the plant tissue has been observed although without a macroscopic toxic effect on the root biomass accumulation among the treatments (Figure 3 and Table 3). The root extraplasmatic pool of Cu and/or of Cu-bearing soil particles may have, at least in part, contributed to this result (Baldi et al., 2018).

The morphological changes of the root systems, although having similar values of biomass accumulation (Figure 3), clearly indicate the activation of Cu-induced stress response mechanisms (Figure 4). In this regard, it is interesting to note that an altered activity of the root meristematic regions (Potters et al., 2009; Bochicchio et al., 2015) as well as an accumulation of phenols in endoderm cells (Ambrosini et al., 2015) have been described in Cu toxicity. In particular for phenols, the process has been explained as an attempt aimed at forming a physical barrier to the entry of the metal and, then, to its allocation *via* the vascular system to the shoot (Ambrosini et al., 2015). Also the Cu accumulation in the root system is itself considered a tolerance mechanism to prevent and/or reduce an excessive translocation of Cu to the shoot (Juang et al., 2012; Girotto et al., 2016). However, once inside the plant tissues, the Cu complexation with carboxylic acids (citric, malic and oxalic), as well as the compartmentalization of the complexes in vacuoles, have been described as component of the complex tolerance response to metal toxicity (Dresler et al., 2014). In this regard an important role has been attributed also to the Cu-complexation by phosphate ions (Arriagada et al., 2009).

The higher the level of Cu availability in the growing medium, the lower is the root concentration of P. This effect on P contents in grapevine plants is not entirely new, being also described in different conditions by Toselli et al. (2009) and Cambrolle’ et al. (2015). On the other hand, Mn contents in root tissues increased with increasing Cu availability (Table 3). These nutritional imbalances seem to corroborate the idea of a role of the Cu toxicity on the membranes integrity as well as on the functionality of the membrane transporters impacting in turn on the selectivity of the nutrient acquisition process (Cambrollé et al., 2015). It should be noted that, although being Mn an essential element (important for several processes such as photosynthesis, oxidation-reduction reactions, electron transport as well as a component of enzymes, Marschner, 2011), its tissue content over 400 mg kg^{-1} impairs severely the plant growth of most plants (Kabata- Pendias, 2011). Moreover, being in the present work the Mn content particularly great in plants exposed to a soil treated with 80 mg Cu kg^{-1}, the phenomenon surely represents a further aspect of concern especially in cases of highly Cu-contaminated soils. In this respect, the Mn-Cu exchange at the sorption sites which has affects the availability of Mn in the soil solution (Sposito, 1989).

Shoot level, Cu contents in leaves and stems were not significant different among the three experimental conditions (Table 3) in spite of the massive depressive effect exerted on the biomass accumulation in these two tissues by the Cu treatments (Figure 3), similarly to what already described in the literature (Yruela, 2005; Cambrolle' et al., 2015; Oustriere et al., 2016). Overall, the results related to the monocropeed grapevines, attest that the conditions here employed well represent a realistic case study of Cu toxicity of exogenous origin in viticulture. In order to evaluate the contribution of the grapevine intercropping with spontaneous graminaceous species in the mitigation of the soil Cu toxicity, in this work grapevine plants have been grown in the pots also in the presence of *Axonopus affinis* and *Paspalum plicatulum* plants. It is interesting to note that these plant species, native of the Pampa Biome of South America, are evoluted in acidic and nutritionally-poor soils and exhibit also a particular resistance to toxic levels of Al^{3+} (Pallares et al., 2005). Results here presented seem to confirm the hypothesis of an advantage in the use of the agronomic practice intercropping in cases of Cu toxicity. Indeed, with respect to the development of the young grapevine plants, the negative impact caused by the Cu toxicity (specifically on leaves and stem biomass accumulation of vine plants) have been clearly mitigated by the co-presence of *Axonopus affinis* or *Paspalum plicatulum* plants (Figure 3) - with extraordinary effectiveness in the case of the soil treated with 40 mg Cu kg^{-1}. Differently, the efficacy of this recovery was only partial when the plants were grown in soil contaminated with 80 mg kg^{-1} of Cu. In fact, the leaf and stem biomass levels accumulated by these plants were less than half of those measured by the control vine plants (mono-cropped in a Cu-untreated soil). This result clearly indicates that in cases of severe soil Cu contamination an integrated approach of several strategies are necessary to guarantee an adequate development of new young plants, e.g. the use of cover crops in combination with soil amendments (Fuksova et al., 2010; Oustriere et al., 2016).

It is interesting to note that in stem and leaves the Cu contents, as well as those of Fe, were essentially unaffected by the different levels of Cu availability in the soil, indicating the operativity of some tolerance strategies. It is doubtless that the beneficial effect achievable with the intercropping approach. In fact, even if partial, this is most likely ascribable to some phenomena occurring at soil level, in particular, the space shared by the two plant species and where they can mutually interact. In this respect, it is interesting to note that the distribution of soluble Cu species during grapevine cultivation in intercropped approach with the grasses changed in favour of Cu-DOC and at the expenses of Cu^{2+} (Figure 2), particularly in the consociation grapevine-*Paspalum plicatulum* plants. Considering that the ionic Cu^{2+} is the form preferentially taken up by plants and microorganisms (McBride, 1994; Martínez & McBride, 1999), the consequences of this phenomenon on the extent of the Cu toxicity for the plants and on the onset of the related symptoms appear evident. In this regard, it should be highlighted that plants can modify the DOC composition through the root exudation of soluble organic compounds, such as organic acids and phenolic compounds, as described for malic acid in response to high levels of Cu (Nian et al., 2002), diminishing the phytotoxic potential of the contaminants (Kim et al., 2010; Dresler et al., 2014).

The metal complexing property of these compounds, their release in the rhizosphere has been also described as an adaptive attempt of plants to assure an equilibrate uptake in cases of nutrients shortage or to limit the availability of toxic elements (Jones, 1998; Pallarés et al., 2005; Malta et al., 2016). Specifically in the case of intercropped plant species, the phenomenon could mean that the inefficient plant species can take advantage of the adaptive response of the other one, as described for *Morus alba* L intercropped with *Pteris vittata* L in arsenic contaminated soil (Wan et al. 2017) or for citrus plants with covercrops species in Fe shortage (Cesco et al., 2006). Moreover, the Cu interaction with the functional groups of DOC was considered responsible of the drop of Cu^{2+} availability in soils treated with biochar and compost amendments (Oustriere et al., 2016). In addition, due to the chemical properties of these functional groups, a significant increase of the pH values, as detected in the intercropped system (Table 2) and ascribable, at least in part, to the preferential nitrate uptake of grasses in well-aerated soil (Marschner, 2011), could further favour this Cu-DOC interaction enhancing metal complexation phenomena (Pérez-Esteban et al., 2014). Moreover, it should also be emphasized that the pH values can directly affect the availability of soil elements/nutrients *via* mobilization/immobilization processes of the inorganic forms, especially in the rhizosphere (Hinsinger et al., 2009; Bravin et al., 2012). Therefore, the modifications of pH values together with the changes in DOC amounts achievable in intercropped conditions have higher potential for plant adaptation to Cu contaminated soils *via* governing the chemical Cu species in soil solution and, then, the levels of its availability for plants (Li et al., 2013, De Conti et al., 2016).

In the root systems it is interesting to note the higher accumulation of Cu in intercropped grapevine plants (Table 3). The augmented amount in the rhizosphere of exudates and their high affinity for the metal could be, at least in part, at the base of the phenomenon. In fact, it is reasonable to assume that under these conditions the metal complexation/immobilization processes are particularly pronounced at the root surface of both plant species, leading also to an enrichment of the root extraplasmatic space of these Cu forms (Cu-complexes). However, it is evident that the whole process determines a limitation of free Cu^{2+} fraction available for root acquisition, aspect particularly interesting and useful in Cu-contaminated soils for grapevine roots. In addition, the enhanced development of these roots observed in the intercropped system (Figure 4) could be part of a plant strategy to colonize preferentially less Cu-contaminated soil zones, such as the rhizosphere of roots of plant species resistant to toxic elements like *Axonopus affinis* and *Paspalum plicatulum* (Pallares et al., 2005). In this respect, it is interesting to note that a root growth directly towards less toxic patches of soil (Bochicchio et al., 2015) as well as the root intermingling between different plant species in the case of limited nutrient availability (Cesco et al., 2006) have been yet observed. It should however be highlighted that the coexistence of two species in the same soil volume, as in this work, can result in a series of competitive phenomena for nutrients and water between the roots of the two plant species. Obviously, its extent can be more relevant in chemically poor soils and in regions with low rainfall.

The morphological modifications of grapevine roots measured in this work could therefore be also ascribed, at least partly, to these circumstances. In fact, the limited P content in the plant tissues, especially evident in grapevine plants grown in the intercropped system, could be an example. On the contrary, the exceptionally high contents of Mn in tissues of both mono- and intercropped grapevine plants suggest the involvement of two plant species *Axonopus affinis* and *Paspalum plicatulum* to respond to the Cu toxicity. Also the limited Cu toxicity effect on grapevine plants co-coltivated with *Axonopus affinis* or *Paspalum plicatulum* plants can be partly attributed to the fact that the nutritional needs of two plant species rather than one has to be satisfied in the intercropped system, thus limiting the whole metal availability in the rhizosphere. Anyhow, regardless of these aspects, it should however be highlighted that the release of organic carbon by the two native grasses surely contributes to the maintenance/increase of soil organic matter content, which is of particular relevance in sandy soils (Brunetto et al., 2014). In this regard, it is interesting to highlight the positive effect of the use of native cover crops in the must quality of commercial vineyard under Mediterranean climatic conditions (Muscas et al., 2017), regardless of the problem of Cu toxicity.

5. Conclusions

The intercropping of *Paspalum plicatulum* and *Axonopus affinis* was efficient in promoting the growth of young grapevines at moderate and low levels of Cu-contaminated soils by reducing the Cu bioavailability due to an increase in pH and in the concentration of dissolved organic carbon in the soil solution. The phytotoxic effects of Cu on root morphology in young grapevines were reduced by intercropping in moderately Cu-contaminated soils (dose 40). This indicates that maintaining native grasses in young vineyards may be a strategy for phytoremediating Cu-contaminated soils and obtaining a grape production system with reduced interventions in the original environment. However, the excess of Mn uptake and the drop of P contents in grapevine tissues caused by Cu toxicity, even if attenuated in the intercropped systems, need to be taken into account for a balanced nutritional state of grapevines.

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Table 1. Physical and chemical characteristics of the 0.0-0.20 m layer in a Typic Hapludalf soil under natural grassland.

	Natural grassland
Clay (g kg^{-1})	54
Sand (g kg^{-1})	894
Silt (g kg^{-1})	52
Organic matter (g kg^{-1})	9.0
pH_{H2O} (1:1)	5.2
Exchangeable Al (mg kg^{-1})	0.4
Available Cu by EDTA (mg kg^{-1})	0.7
Available Zn by EDTA (mg kg^{-1})	0.9
Available K by Mehlich-1 (mg kg^{-1})	66.4
Available P by Mehlich-1 (mg kg^{-1})	3.6
Available Fe by EDTA (mg kg^{-1})	5.9
Available Mn by EDTA (mg kg^{-1})	15.4
Exchangeable Ca (mg kg^{-1})	0.5
Exchangeable Mg (mg kg^{-1})	0.2
CEC_{ef}^{*}, $cmol_c$ kg^{-1}	1.4
$CEC_{pH\ 7.0}^{**}$, $cmol_c$ kg^{-1}	3.2

$^{*}CEC_{ef}$ = Ability to effectively exchange cations; $^{**}CEC_{ph\ 7.0}$ = Cation exchange capacity the pH 7.0.

Table 2. Chemical characteristics of soil solution sampled throughout monocropping (Grapevine) and intercropping with *Paspalum plicatulum* (Grap.+*Paspalum*) and *Axonopus affinis* (Grap.+*Axonopus*) grown in soils with increasing Cu levels.

Solution chemical parameters	Cu doses	Cropping systems		
	(mg kg^{-1})	Grapevine	Grap.+*Paspalum*	Grap.+*Axonopus*
			1st Sampling	
pH	0	6.01aB^{1}	6.63 aA	6.07 aB
	40	5.33 bC	6.45 aA	5.85 aB
	80	5.30 bA	5.67 bA	5.53 bA
Dissolved organic carbon (mg L^{-1})	0	11.00 bB	17.00 aA	12.00 aB
	40	15.33 aB	18.67 aA	13.00 aB
	80	14.33 aB	16.33 aA	13.33 aB
Soluble Cu (mg L^{-1})	0	0.018 cA	0.021 cA	0.015 cA
	40	0.290 bB	0.422 bA	0.330 bB
	80	0.780 aA	0.757 aA	0.773 aA
			2rd Sampling	
pH	0	6.31 aC	7.30 aA	6.79 aB
	40	5.23 bB	6.68 bA	6.57 aA
	80	5.47 bB	6.23 cA	5.49 bB
Dissolved organic carbon (mg L^{-1})	0	17.67 aA	20.00 bA	19.00 aA
	40	12.67 bC	24.33 aA	18.67 aB
	80	9.67 bB	12.33 cB	15.33 bA
Soluble Cu (mg L^{-1})	0	0.019 cA	0.021 cA	0.024 cA
	40	0.338 bA	0.352 bA	0.353 bA
	80	1.039 aB	0.808 aC	1.512 aA

(1) Means followed by the same lowercase letter not differ between Cu doses in the same cropping system (column) and means followed by the same uppercase letter not differ between the cropping systems at the same Cu dose (row) by the Scott-Knott test ($p < 0.05$).

Table 3. Macronutrient and micronutrient contents in shoot and root biomass of grapevine grown in monocropping and intercropping in soils with increasing Cu levels.

Nutrient	Cu doses	Cropping systems		
		Grapevine	Grapevine +*Paspalum*	Grapevine +*Axonopus*
	(mg kg^{-1})	Leaf		
P (g kg^{-1})	0	2.01aA[1]	1.40 aB	1.40 bB
	40	2.12 aA	1.56 aB	1.74 aB
	80	1.22 bA	1.24 aA	1.38 bA
Cu (mg kg^{-1})	0	9.07 cA	6.99 bB	8.43 cA
	40	14.07 aA	12.55 aB	14.83 aA
	80	10.95 bA	11.71 aA	12.55 bA
Fe (mg kg^{-1})	0	82.44 aA	45.60 aB	49.48 aB
	40	71.80 aA	50.88 aB	52.68 aB
	80	82.04 aA	53.16 aB	50.28 aB
Mn (mg kg^{-1})	0	106.83 cA	98.27 bA	88.23 bA
	40	244.99 bA	106.99 bB	112.83 bB
	80	580.75 aA	251.95 aC	454.43 aB
		Stem		
P (g kg^{-1})	0	1.19 aA	1.05 aA	1.08 aA
	40	1.04 bA	1.07 aA	1.19 aA
	80	0.55 cA	0.59 bA	0.65 bA
Cu (mg kg^{-1})	0	7.83 bA	5.19 bB	5.87 cB
	40	10.95 aA	8.35 aB	9.19 aB
	80	8.51 bA	7.39 aA	7.51 bA
Fe (mg kg^{-1})	0	35.83 aA	27.71 aA	34.23 aA
	40	33.99 aA	30.07 aA	31.55 aA
	80	34.35 aA	32.79 aA	30.11 aA
Mn (mg kg^{-1})	0	35.97 cA	54.05 bA	37.77 bA
	40	62.33 bA	63.65 bA	54.81 bA
	80	309.37 aA	87.29 aC	150.57 aB
		Root		
P (g kg^{-1})	0	1.06 aA	1.03 bA	1.07 aA
	40	0.79 aC	1.20 aA	1.09 aB
	80	0.69 aA	0.69 cA	0.76 bA
Cu (mg kg^{-1})	0	13.72 cA	16.24 cA	19.08 cA
	40	78.24 bB	120.32 bA	108.80 bA
	80	125.44 aC	165.64 aB	200.12 aA
Fe (mg kg^{-1})	0	417.96 aA	243.72 bB	259.24 aB
	40	152.32 bC	290.32 aA	209.96 bB
	80	114.64 bB	227.48 bA	201.40 bA
Mn (mg kg^{-1})	0	43.24 cA	48.60 cA	47.24 bA
	40	68.36 bA	73.88 bA	49.72 bB
	80	213.80 aA	106.64 aC	137.88 aB

(1) Means followed by the same lowercase letter do not differ between Cu doses within the same species (column) and means followed by the same capital letter do not differ between species within the same Cu dose (row) by the Scott-Knott test ($p < 0.05$).

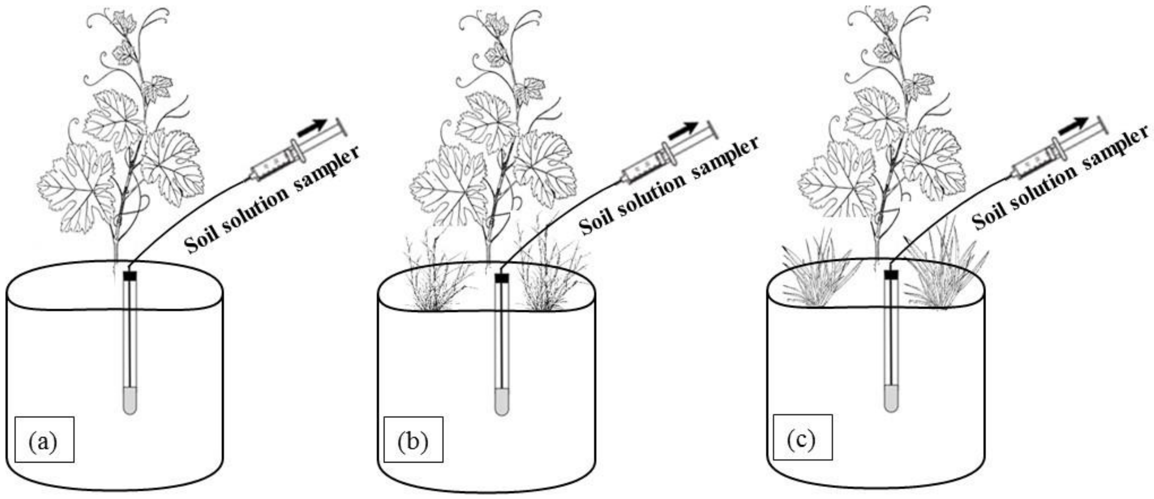

Figure 1. Schematic representation of the experimental units to experiment. Monocroppin of grapevine (a) intercropping with *Paspalum plicatulum* (b) and intercropping with *Axonopus affinis* (c). Rhizon samplers were arranged near the grapevine.

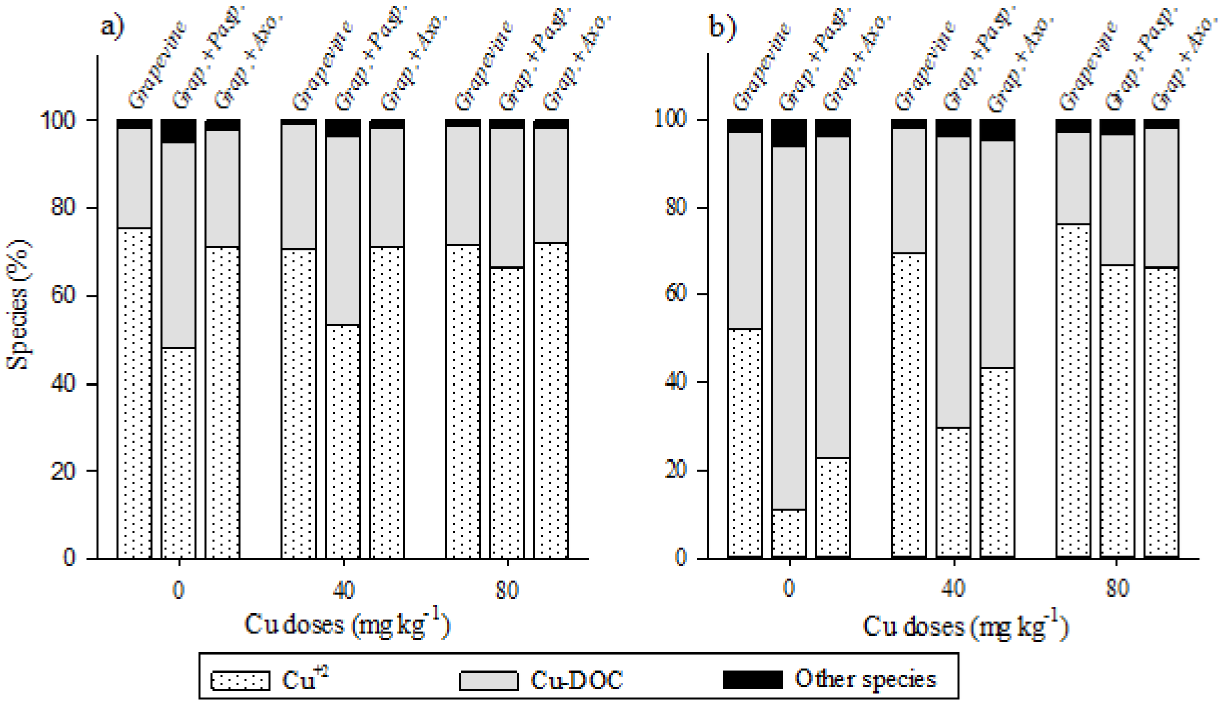

Figure 2. Distribution of the chemical species of Cu in the solution of the soil grown with monocropping (Grapevine), intercropping with *Paspalum plicatulum* (Grap.+*Paspalum*) and *Axonopus affinis* (Grap.+*Axonopus*) grown in soils with increasing Cu levels, in the 1st (a) and 2nd (b) sampling. Other species correspond to $CuOH^+$, $CuSO_{4(aq)}$, $CuNO_3^+$, $CuHPO_{4(aq)}$, $CuCl^+$ and $Cu(OH)_{2(aq)}$.

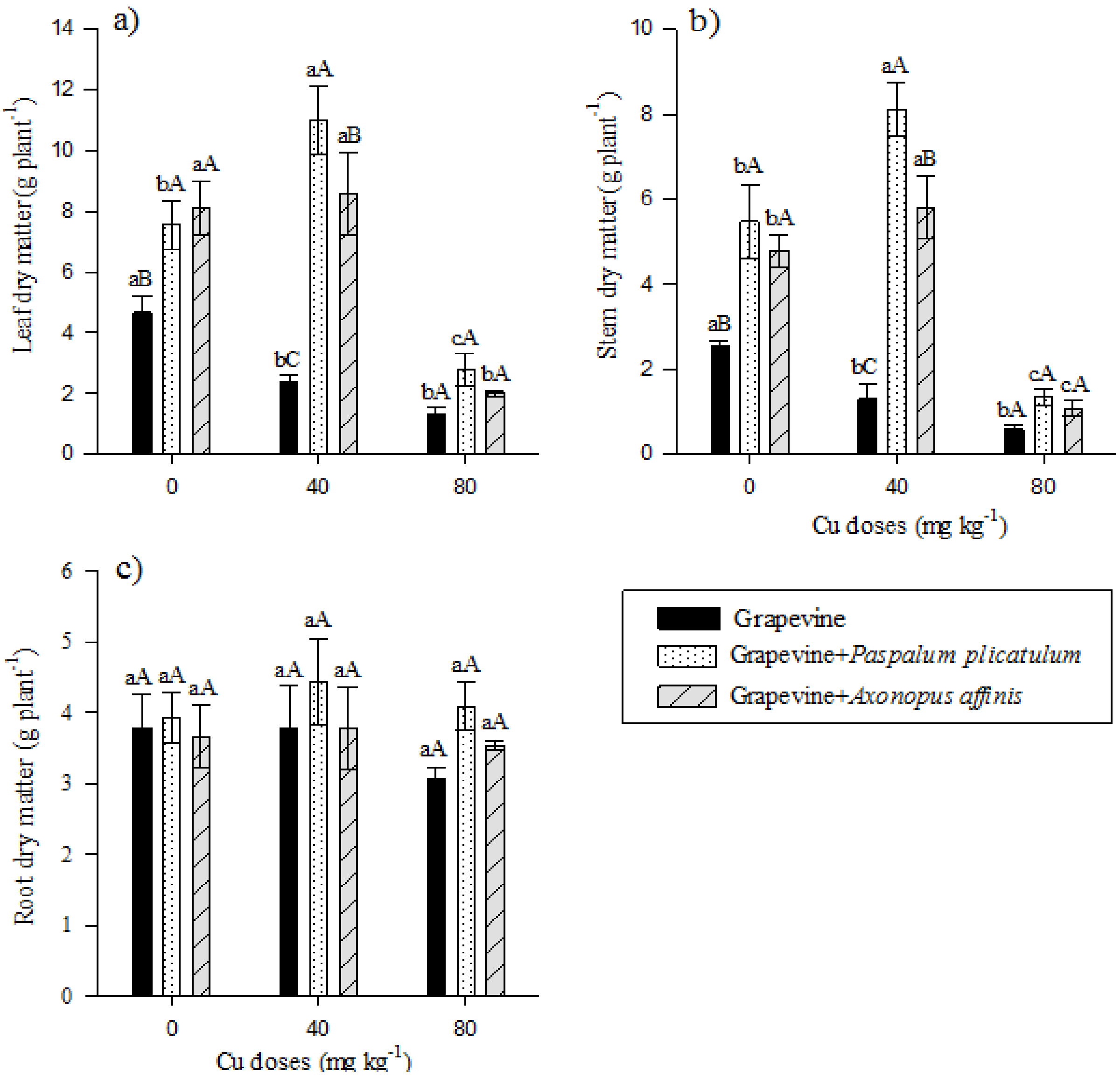

Figure 3. Dry matter yield of the leaves (a), stems (b) and roots (c) of monocropping (Grapevine) and intercropping with *Paspalum plicatulum* (Grapevine+*Paspalum plicatulum*) and *Axonopus affinis* (Grapevine+*Axonopus affinis*) grown in soils with increasing Cu levels. Different lowercase letters indicate differences between Cu doses in the same cropping system and different uppercase letters indicate differences between the cropping systems at the same Cu dose by Scott-Knott test ($p < 0.05$).

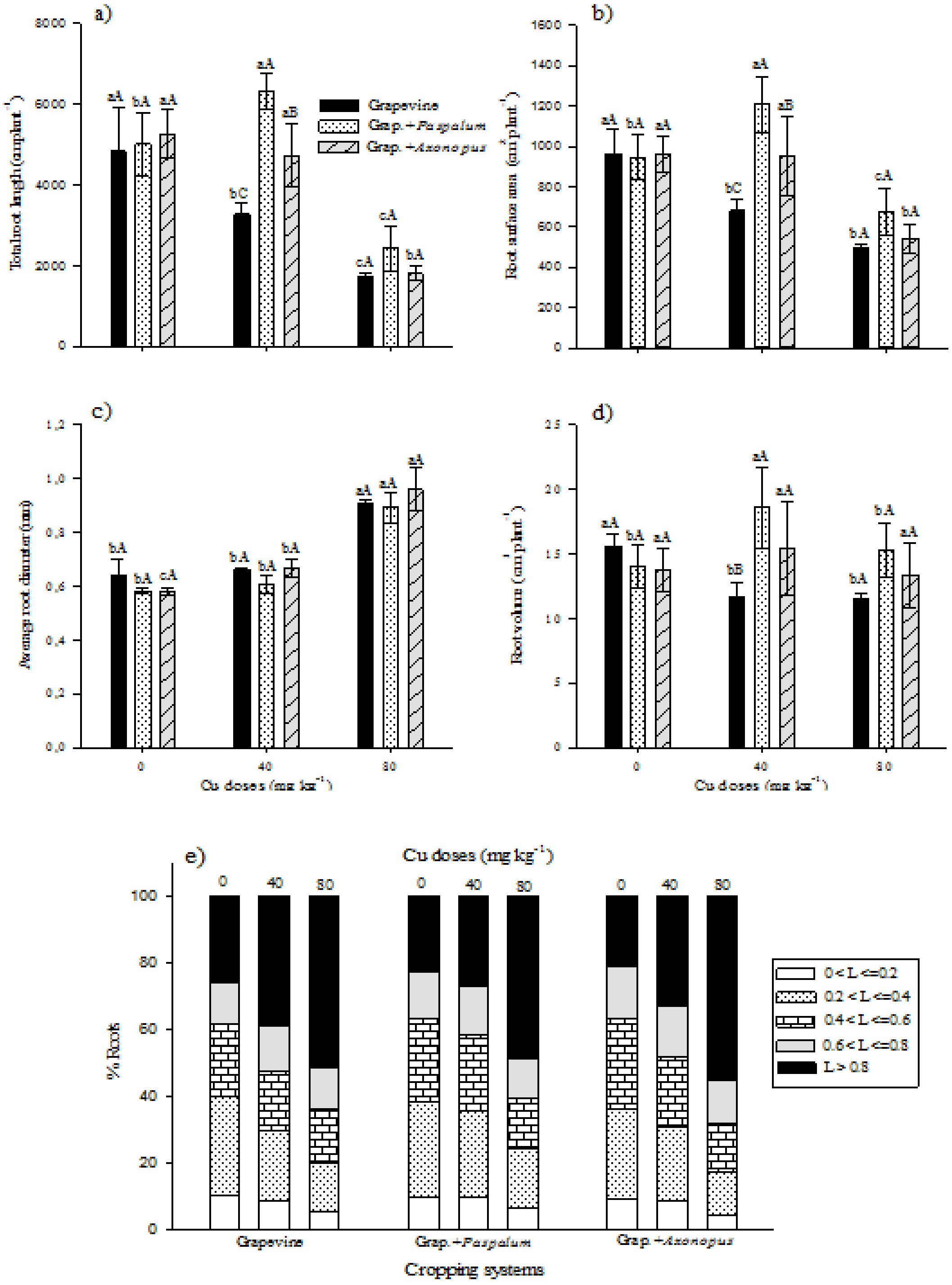

Figure 4. Root morphological parameters of grapevine grown in monocropping (Grapevine), intercropping with *Paspalum plicatulum* (Grap.+*Paspalum*) and *Axonopus affinis* (Grap.+*Axonopus*) in soils with increasing Cu content. Different lowercase letters indicate differences between Cu doses in the same cropping system and different uppercase letters indicate differences between the cropping systems at the same Cu dose by Scott-Knott test ($p < 0.05$).

5.5 ESTUDO V

Photosynthesis and growth of young grapevines intercropped with native grasses in soils contaminated with copper [5]

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Abstract

High copper (Cu) contents in vineyard soils due to long-term foliage-defense program based on Cu-containing fungicides may cause physiological and nutritional disorders in young grapevines, limiting plant growth and in some cases also compromising plant survival. This problem is particularly evident in viticultural areas of southern Brazil. Therefore, the study aimed to assess if the intercropping of grapevines with native grasses of southern Brazil can contribute to limit the soil Cu availability and thus the onset of toxicity symptoms (i.e. impairment of photosynthetic and growth parameters). In order to do this, we collected soil samples in natural grassland of the Pampa Biome (southern Brazil). The samples were air-dried; acidity, phosphorus and potassium levels were corrected and samples were consequently incubated. We used three Cu levels – control (i.e. no Cu addition), 40 mg Cu kg^{-1} and 80 mg Cu kg^{-1}. Such Cu levels are normally found in vineyard soils of the region. The experimental design was completely randomized with three replications. At each Cu level, we set up three combinations of monocropping (Grapevine), and intercropping with *Paspalum plicatulum* Michx. (Grapevine + *Paspalum plicatulum*) and *Axonopus affinis* Chase (Grapevine +

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Axonopus affinis). In the intercropped treatments, two grass seedlings were transplanted into each experimental unit, 35 days before transplanting the grapevines. The experiment was conducted for 70 days. At 41 days after transplanting the grapevines, gas exchange (IRGA) was measured. At 70 days, plant height and total dry matter yield were determined and the relative growth rate was quantified. The addition of Cu caused phytotoxicity in the single crop (Grapevine), reducing photosynthetic carbon assimilation and plant growth. The cultivation of young grapevines intercropped with native grasses, especially *Paspalum plicatulum Michx.*, promoted the growth of the grapevines in the control soil as well as in the moderately Cu-contaminated soils. This indicates that maintaining native vegetation in young vineyards can reduce Cu toxicity to transplanted grapevines.

***Keywords*:** cover crops, phytotoxicity, heavy metals, biome pampa, v*itis vinifera*.

INTRODUCTION

The application of cupric fungicides for the control of foliar diseases in grapevines may lead to an increase in copper (Cu) contents in vineyard soils (Miotto et al., 2014; Cambrollé et al., 2015; Brunetto et al., 2016). High levels of Cu can cause toxicity to young grapevines planted in soils of eradicated old grapevines and to the cover crops that co-inhabit these vineyards (Miotto et al., 2014).

Copper is an micronutrient to plants, but its excessive acquisition by roots severely compromises plant growth by impairing the functioning of the photosynthetic apparatus, reducing the carbon fixation (Kabata-Pendias, 2011; Cambrollé et al., 2015; Tiecher et al., 2016). The symptoms of phytotoxicity caused by excess Cu depend on the available fraction in the soil solution and on the plant species; in fact, excess Cu might trigger different tolerance mechanisms depending on the plant species (Leguizamo et al., 2017). Copper-tolerant plants either reduce the Cu availability in the rhizosphere by complexation reactions due to the release of ions and soluble organic compounds or by preventing Cu translocation to the shoots accumulating the metal in the root system (mainly in the apoplast) (De Conti et al., 2016; Brunetto et al., 2016). These tolerance mechanisms, can also favor the development of other plants grown in intercropping, due to the reduced bioavailability of the contaminant (Brunetto et al., 2016). Native species, such as the South American native grasslands, have mostly evolved in acidic and nutrient poor soils thanks to the ability of these plants to adapt to the conditions of low nutrient levels and the presence of toxic elements (Pallarés et al., 2005). The characteristics of these species, together with their natural occurrence in vineyards of southern Brazil, suggest great potential for the phytoremediation of these soils. Therefore, the study aimed at assessing if the intercropping of grapevines with native grasses of southern Brazil can contribute to limit the soil Cu availability and thus the onset of toxicity symptoms (i.e. impairment of photosynthetic and growth parameters).

MATERIAL AND METHODS

The soil used was a Typic Hapludalf (Soil Survey Staff, 2006) collected at 0-20 cm in an area of uncultivated grassland located in the Pampa Biome, southern region of Brazil (30°47'23.7''S and 55°22'7.3''W). The physical-chemical characterization of the soil is presented in Tiecher et al. (2016). After the collection, the soil was air dried, homogenized and passed through a 2 mm mesh sieve. Subsequently, soil acidity has been buffered adding a mixture of calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$) with a 2:1 ratio at a concentration of 0.57 g kg^{-1} soil and applied 40 mg P kg^{-1} and 100 mg K kg^{-1} of soil, in the form of triple superphosphate and potassium chloride, respectively. The treatments consisted of three Cu levels: control (i.e., no Cu addition), and the addition of 40 mg Cu kg^{-1} and 80 mg Cu kg^{-1}, which are levels usually found in vineyards where grapevines have been grown for approximately 15 and 30 years, respectively (Miotto et al., 2014). The addition of Cu occurred 50 days after the application of the corrective, by applying a solution of $CuSO_4$. $5H_2O$. Subsequently, the soil was incubated again for 115 days, with soil moisture kept at 80% of the maximum water holding capacity (MWHC).

The experimental design was completely randomized with three replications. The experimental units were pots of 8 L containing 7 kg of dry soil. At each Cu level, we set up three combinations of monocropping (Grapevine), as well as intercropping with *Paspalum plicatulum* Michx. (Grapevine + *Paspalum plicatulum*) and *Axonopus affinis* Chase (Grapevine + *Axonopus affinis*). In November 2015, we transplanted one grapevine (*Vitis vinifera* L. cv. Paulsen 1103) plant per pot, where it was grown for 70 days. In the intercropped treatments (Grapevine + *Paspalum plicatulum* and Grapevine + *Axonopus affinis*), two seedlings to native grasses were transplanted into each pot, 35 days before the transplanting of the grapevines. Soon after the transplanting, we determined the stem diameter at the ground level and the height of the grapevines. At 16 and 48 days after transplanting, we applied 20 and 10 mg N kg^{-1} of soil in the form of urea, respectively. In the intercropped treatments, the shoots of the cover crops were cut at 10 cm (height) and placed on the soil surface every 21 days, totaling three cuts along the cultivation of the grapevines. This management of the native grasses simulates the mowing typically used in managing cover crops in commercial vineyards. Soil moisture was maintained at 70% of the MWHC during cultivation, with daily weightings and the addition of distilled water when necessary.

At 41 days after transplanting the grapevines, gas exchange was measured in two fully expanded leaves (of the middle region), using an open system infrared gas analyzer (IRGA) (LI-6400XT LI-COR, Inc., Lincoln, NE, USA). Net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, transpiration rate, water use efficiency (WUE) and instantaneous carboxylation efficiency (CE) were determined at an ambient CO_2 concentration of 400 µmol mol^{-1} at 20–25 °C, 50 ± 5% relative humidity and a photon flux density of 1000 µmol m^{-2} s^{-1}. After 70 days of the transplanting, we determined the stem diameter at the ground level and the height of the grapevines in order to estimate the relative growth rate (RGR/day) using the following equation: $(\pi r^2 \times h)$/plant age (Sieverding, 1991), where r = 1/2 of the difference between the initial and final stem diameter; and h = the difference between the initial and final height of the grapevines. Afterwards, the grapevines were cut close to the soil surface and the roots were separated from the soil by hand, washed in running tap water to remove soil and then stored for the determination of dry matter (DM) in a forced air oven at ± 65°C, until reaching constant dry matter.

The results obtained were submitted to analysis of variance (ANOVA) using Sisvar software, version 5.6 (Ferreira, 2011), with the following completely randomized bifactorial statistical model: $Y_{ijk} = \mu + Ci + Z_j + CZ_{ij} + \text{error}\ (i, j)$. where μ= overall mean of the experiment; C = Cu levels (i= 1, 2, 3); Z= crops systems (j= 1, 2, 3) and error= experimental error. when the effects were significant, the means were grouped by the Scott-Knott test at 5% probability.

RESULTS AND DISCUSSION

The net photosynthetic rate decreased significantly with increasing Cu concentration added to the soil in grapevines (monocropping) and in grapevines intercropped with *Axonopus affinis* Chase (Figure 1). In grapevine intercropped with *Paspalum plicatulum* Michx., the reduction in the net photosynthetic rate occurred only at the highest Cu concentration used (80 mg kg^{-1}, Figure 1). This behavior was also observed in stomatal conductance and instantaneous carboxylation efficiency (Figure 1). Furthermore, the intercropped systems had a higher net photosynthetic rate compared to the monocropping system (grapevine): in control and 40 mg kg^{-1} Cu-treated plants we observed an increase of 38.9 and 97.8 % in grapevine + *Paspalum plicatulum* Michx., and 38.0 and 46.9% in grapevine + *Axonopus affinis* Chase, respectively (Figure 1). At these Cu levels, stomatal conductance, transpiration rate and instantaneous carboxylation efficiency were also higher in the intercropped systems (Figure 1). Intercellular CO_2 concentration and water use efficiency were slightly affected by Cu and did not differ between the cropping systems (Figure 1). These results show that the excess Cu compromises the functioning of the photosynthetic carbon assimilation pathway, reducing photosynthetic carbon assimilation. The excess Cu can further induce the reduction of the concentration of photosynthetic pigments and cause the degradation of the structure and the internal content of the chloroplast through the degradation of the membrane polar lipids (Maksymiec et al., 1995; Cambrollé et al., 2015).

The increase in the efficiency of the photosynthetic apparatus in the intercropped systems of the control and 40 mg kg^{-1} Cu treated plants is most likely due to the reduced Cu bioavailability and/or the increased bioavailability of other essential nutrients (Brunetto et al., 2016). Plant roots release in fact a myriad of compouds (i.e. root exudates) modifying the chemical characteristics of the rhizosphere. For instance, pH changes and the presence of organic ligands could increase the stable complexation of Cu^{+2} in the soil solution, thus reducing its bioavailability; such processes would favor plant growth, since the uptake of Cu by the roots occurs preferentially in the free form (McBride, 1994; De Conti et al., 2016).

Copper affected also plant growth both in terms of height and total dry matter, yet only in the monocropping systems and in the 80 mg kg^{-1} Cu treated intercropping systems (Figure 2). The intermediate Cu concentration (40 mg kg^{-1}) increased the plant height and total dry matter in the intercropping systems by 221 and 213% in grapevine + *Paspalum plicatulum* Michx., and 180 and 143% in grapevine + *Axonopus affinis* Chase, respectively. The relative growth rate was higher in the intercropped systems compared to the monocropping/grapevine in the control and at the intermediate Cu concentration (Figure 2). The reduction in grapevine growth at high Cu levels is probably related to the lower photosynthetic carbon assimilation (Figure 1). Furthermore, the excess Cu can affect the function of membrane transporters and ion channels, reducing their selective capacity; the resulting nutritional imbalances might also contribute to the reduction of plant growth (Cambrollé et al., 2015).

CONCLUSION

The addition of Cu caused phytotoxicity in the single crop (Grapevine), reducing photosynthetic carbon assimilation and plant growth. The cultivation of young grapevines intercropped with native grasses, especially *Paspalum plicatulum* Michx., promoted the growth of the grapevines in the control soil as well as in the moderately Cu-contaminated soils. This indicates that maintaining native vegetation in young vineyards can reduce Cu toxicity to transplanted grapevines.

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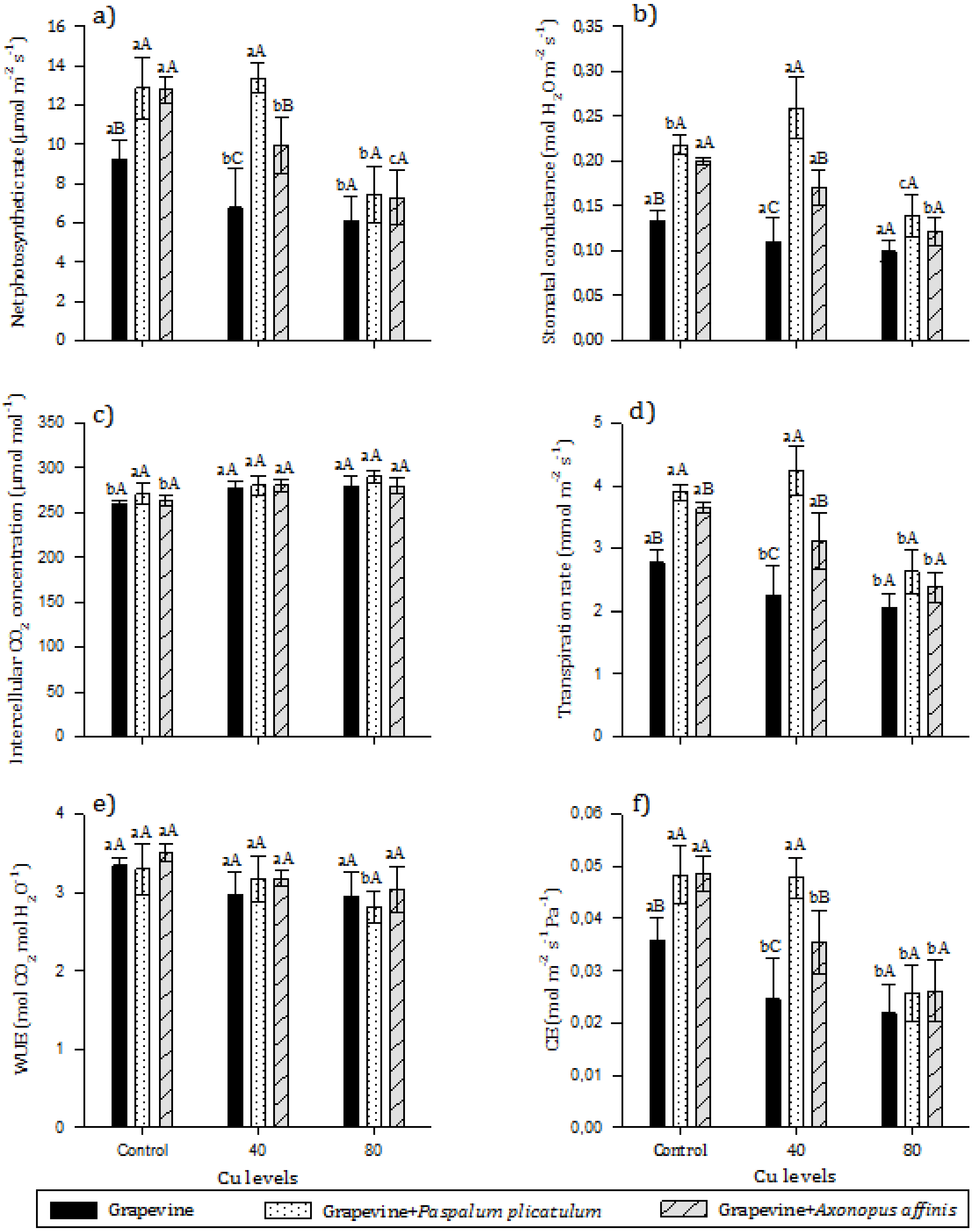

Figure 1. Net photosynthetic rate (a), stomatal conductance (b), intercellular CO_2 concentration (c), transpiration rate (d), water use efficiency (e) instantaneous carboxylation efficiency (f) of young grapevines. Means followed by the same lowercase letter do not differ between Cu levels in the same cropping system. Means followed by the same uppercase letter do not differ between cropping systems in same Cu level (Scott-Knott test, $p < 0.05$).

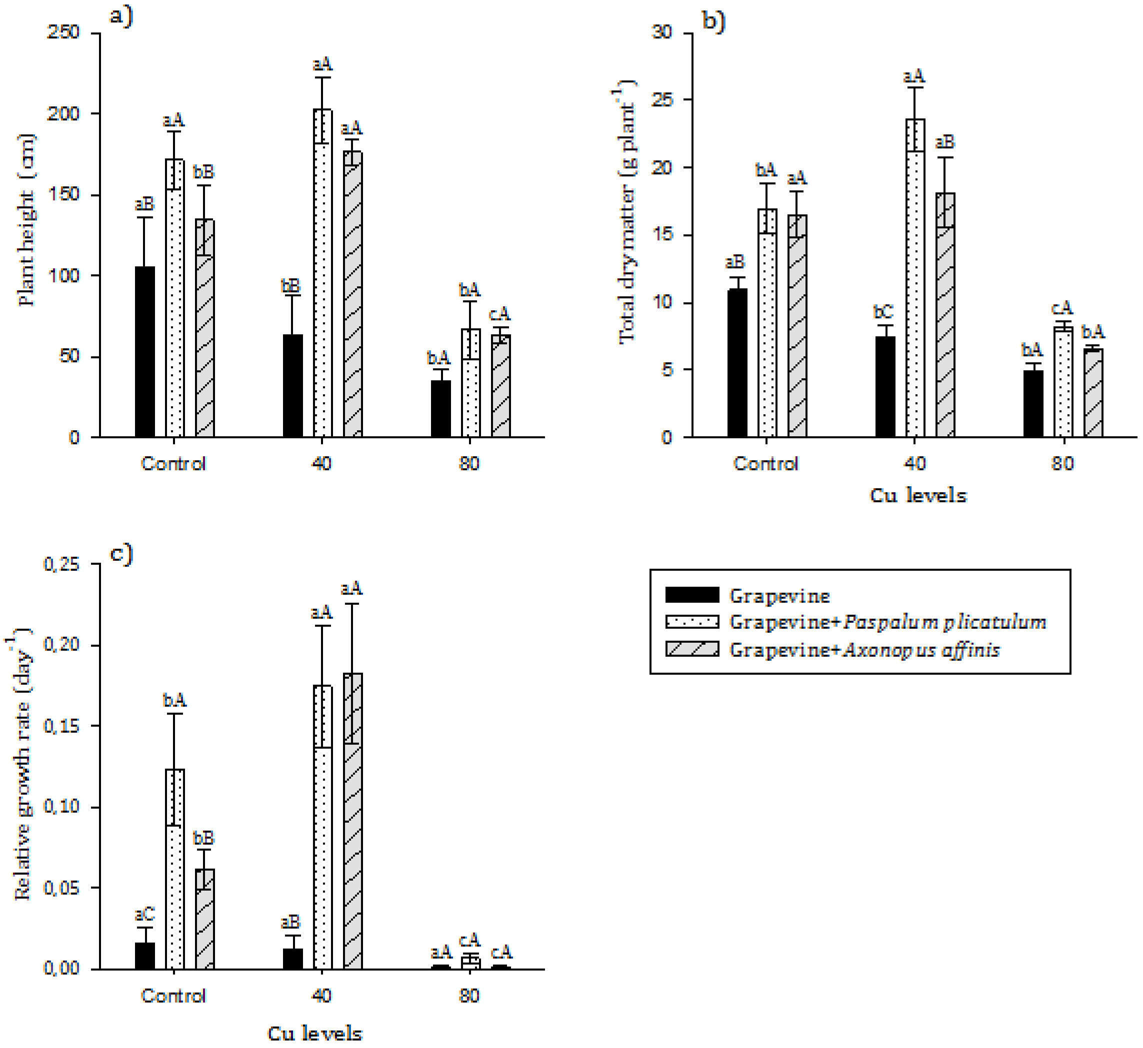

Figure 2. Plant height (a), dry matter yield (b) and relative growth rate (c) of young grapevines. Means followed by the same lowercase letter do not differ between Cu levels in the same cropping system. Means followed by the same uppercase letter do not differ between cropping systems at the same Cu level (Scott-Knott test, $p < 0.05$).

6. DISCUSSÃO GERAL

A contaminação dos solos de vinhedos com Cu é promovida principalemtne pela utilização intensiva de fungicidas cúpricos, utilizados no manejo das doenças foliares das videiras, podendo causar danos as plantas e organismos do solo que habitam nestes ambientes. Neste estudo foram simuladas condições de contaminação de Cu semelhantes às encontradas em vinhedos com 15 e 30 anos de cultivo, na região da Campanha Gaúcha (MIOTTO et al., 2014). Estes níveis de contaminação ocasionaram prejuízos ao crescimento das videiras jovens e plantas de cobertura de solo, especialmente, no maior nível de contaminação (Estudos II, III, IV e V - 80 mg kg^{-1} e estudo I - 50 μM L^{-1} de Cu), demonstrando o efeito tóxico do excesso do metal sobre as plantas nativas da região ou plantas exóticas, que podem ser introduzidas em áreas com histórico de cultivo de videiras.

A adição de Cu promoveu incremento na concentração de Cu presente na solução do solo, de forma mais expressiva na dose Cu 80 mg kg^{-1} (Artigos II, III e VI). Este incremento na concentração de Cu possivelmente ocorreu devido a saturação dos grupos funcionais, das partículas reativas de diversos constituintes da fase sólida do solo e, com isso, os íons remanescentes permaneceram na solução do solo, potencializando o risco de toxidez às plantas e também a transferência e contaminação dos recursos hídricos (BABCSÁNYI et al., 2016). Tais incrementos nas concentrações solúveis de Cu com o aumento dos anos de cultivo de videiras, já foram diagnosticadas em solos de vinhedos comerciais da região Sul do Brasil, como observado por BRUNETTO et al., 2014 e GIROTTO et al., 2014.

A baixa capacidade do solo em acumular Cu provavelmente está relacionada com as características físico-químicas dos solos sob vinhedos na região da Campanha Gaúcha, principalmente pelos baixos teores de argila e MOS, que são os principais sítios de sorção do metal no solo (McBRIDE, 1994; BRUNETTO et al., 2014). Esta hipótese é confirmada pelos resultados de Miotto et al. (2017), os quais observaram que aproximadamente 80% do Cu acumulado nos solos de vinhedos da Campanha Gaúcha é potencialmente disponível (extraível com EDTA). Isso demonstra que os teores de Cu podem atingir níveis tóxicos às plantas em poucos anos de cultivo, quando os vinhedos são implantados sobre solos arenosos e ácidos, pelo fato do Cu acumular no solo predominantemente em frações solúveis e trocáveis, formas consideras disponíveis para as plantas e microganismos. Estes resultados mostram claramente a necessidade de reduzir/suspender a entrada de Cu nestes sistemas, prevenindo o agravamento do problema da contaminação, bem como estabelecer estratégias para mitigação da toxidez nos solos que já possuem incrementos nos teores do metal.

A manutenção de plantas de cobertura do solo pode ser uma forma eficaz de amenizar a toxidez gerada pelo excesso de Cu, pois estas plantas podem expressaram mecanismos de tolerância ao excesso de Cu, tanto as espécies nativas do Bioma Pampa (*Axonopus affinis*, *Paspalum plicatulum* e *Paspalum notatum*), como as introduzidas (*Avena strigosa* e *Lolium perenne*). Durante a condução dos experimentos realizados nos estudos II e III foi observado alteração visual no padrão de respostas das plantas ao longo do cultivo, quando cultivadas nos níveis intermediários de contaminação, observando-se atenuação dos sintomas da toxidez com o passar do tempo. Este padrão de resposta também foi observado em outros experimentos com toxidez por metais pesados em plantas anuais, realizados pelo grupo de pesquisa GEPACES. Durante o cultivo das espécies nativas e da aveia preta, as plantas promoveram alterações nos atributos químicos da solução do solo, com destaque para o aumento do pH e concentração de carbono orgânico dissolvido (COD) (Estudos II, III). O aumento do pH e COD alterou a distribuição das espécies químicas de Cu na solução do solo, reduzindo a proporção da espécie livre (Cu^{+2}) e aumentando a proporção da espécie complexada com compostos orgânicos (Cu-COD), consequentemente, reduzindo a biodisponibilidade do contaminante, uma vez que a absorção ocorre preferencialmente na forma de Cu^{+2} (McBRIDE, 1994; KABATA-PENDIAS, 2011).

O aumento na complexação do contaminante é um mecanismo de tolerância desencadeado por algumas espécies de plantas e atua evitando e/ou reduzindo absorção excessiva e transporte de íons metálicos para o citoplasma celular (MEIER et al., 2012; MONTIEL-ROZAS et al., 2016). A magnitude da ocorrência deste mecanismo de tolerância é maior no apoplasto radicular e na rizosfera, como observado no estudo II, onde a porcentagem de Cu^{+2} foi menor na rizosfera da aveia preta em relação a solução do solo não rizosférico. A redução na biodisponibilidade de Cu, decorrente das alterações químicas promovida pelas plantas na solução do solo, foi mais intensa na úlima amostragem (3ª coleta), em relação às coletas do início dos cultivos (Estudos II, III e IV), o que pode explicar a redução dos sintomas visuais de fitotoxidez nos níveis intermediários de contaminação ao longo do cultivo.

Os principais ligantes orgânicos exsudados pelas raízes das plantas em condições de estresse são os ácidos orgânicos de baixo peso molecular (fenóis, ácidos orgânicos, amino ácidos e açúcares) (MONTIEL-ROZAS et al., 2016; ZAFARI et al., 2016). Estas substâncias, além de contribuirem para o aumento dos teores de COD, possuem elevada reatividade com o Cu, o que é conferida pela presença de grupos funcionais, especialmente o carboxila (—COOH) e hidroxila (OH), aumentando a proporção das espécies complexadas na solução do solo e reduzindo a biodisponibilidade dos metais (KIM et al., 2010; SESHADRI et al., 2015). No estudo I, o aumento dos níveis de Cu na solução de cultivo promoveu aumento na exsudação de compostos fenólicos totais e compostos quelantes pelo azevém perene. Estes resultados corroboram com o aumento no percentual de Cu-DOC verificado ao longo dos cultivos da aveia preta, espécies nativas e do cultivo consorciado das videiras com espécies nativas (Estudos II e III e IV).

Mecanismos de tolerância expressos no exterior das raízes, a exemplo da exsudação de substâncias quelantes, com capacidade de reduzir a biodisponibilidade do Cu, podem beneficiar outras espécies cultivadas em consórcio (WAN et al., 2017), como verificado nos estudos IV e V. Nestes estudos o cultivo consorciado das espécies nativas do Bioma Pampa, *Paspalum plicatulum* e *Axonopus affinis*, aumentaram o crescimento das videiras jovens, quando cultivadas em solos com teores naturais de Cu e em moderados níveis de contaminação por Cu (Cu 40 mg kg^{-1}). Além de aumentar o crescimento das plantas de videira, o cultivo consorciado nas condições descritas aumentou a eficiência do aparato fotossintético e contribuiu na manutenção do equilíbrio nutricional, aumentando a absorção de P e evitando a absorção excessiva de Mn (Estudo IV). Quando as videiras jovens foram cultivadas solteiras, a dose de Cu 40 mg kg^{-1} causou toxidez às plantas, que foi diagnosticado pela redução na taxa fotossintética e no crescimento das plantas. Isso demosntra que a manutenção da vegetação nativa do Bioma Pampa na reimplantação dos vinhedos, pode ser uma estratégia de fitorremediação, em solos moderadamente contaminados com Cu. Contudo, nos maiores níveis de contaminação (Cu 80 mg kg^{-1}) o cultivo consorciado não foi eficiente em reduzir os sintomas da fitotoxidez nas videiras jovens, indicando a necessidade associar outras técnicas de remediação, nestas condições de contaminação mais severas, como a adição de amenizantes e condicionadores de solo, como o calcário e composto orgânico.

A capacidade das plantas nativas do Bioma Pampa em reduzir a biodisponibilidade do Cu em solos contaminados com este metal (Estudos III, IV e V), possivelmente contribuem para a redução da fitotoxidez nas videiras adultas cultivadas na região da Campanha Gaúcha, onde estas plantas coabitam naturalmente os vinhedos e são manejadas como plantas de cobertura de solo. Esta influência do cultivo consorciado, possivelmente contribui para explicar a manutenção da produtividade e da ocorrência pontual e sutil dos sintomas da fitotoxidez de Cu em videras adultas, embora estas se desenvolvam em solos com altos teores de Cu disponível, diagnosticado por Miotto (2012) nos vinhedos com diferentes idades na região da Campanha Gaúcha. A manutenção da vegetação nativa coabitando os vinhedos tende a contribuir para o *terroir* dos produtos elaborados nesta região, imprimindo características específicas do local, que as diferenciam e aumentam a qualidade dos produtos gerados (MUSCAS et al., 2017). Manter a vegetação nativa em consorcio com as videiras reduz o impacto da atividade vitícula sobre a dinâmica ecológica e ambiental do local, além de ser uma excelente forma de fitorremediação *in situ* dos solos moderadamente contaminados, como demonstrado neste trabalho. O cultivo consorciado de plantas de interesse econômico e fitorremediadoras é uma forma de aumentar a eficiência no uso da terra, por possibilitar a remediação do ambiente e produção agrícola simultânea (WAN et al., 2017).

O consórcio de cultivo se caracteriza pela presença de duas ou mais espécies de plantas crescendo em um mesmo ambiente, o que eleva a demanda por água e nutrientes. Parte dos vinhedos na região da Campanha Gaúcha são irrigados, facilitando o controle da disponibilidade de água. Porém, cabe ressaltar a maior necessidade de adubação inicial dos pomares. O aporte extra de nutrientes visa atender a necessidade nutricional das diferentes espécies que crescem no ambiente, no entanto, como são plantas de cobertura de solo, não é realizada colheita das mesmas e, consequentemente, não há exportação de nutrientes. Assim, os nutrientes ciclam no sistema e com o passar do tempo reduz a necessidade de aporte externo de nutrientes, por aumentar a eficiência das adubações, somado a ocorrência de simbiose das plantas nativas com microrganismos, que promovem a fixação do N atmosférico e solubilizam nutrientes indisponíveis, disponibilizando-os para as videiras com a mineralização dos resíduos das roçadas (BRUNETTO et al., 2017).

O Cu absorvido pelas diferentes espécies de plantas de cobertura de solo e videiras jovens, acumulou predominantemente nas raízes, com baixa translocação para a parte aérea. O controle da translocação do Cu absorvido entre os órgãos das plantas consiste em um mecanismo de tolerância, expressado pela maioria das espécies vegetais quando cultivadas em ambientes com teores tóxicos do metal (KABATA-PENDIAS, 2011; CAMBROLLÉ et al., 2015). O aumento na exsudação de substâncias quelantes favorece a complexação e acúmulo do Cu no apoplasto, reduzindo a absorção excessiva e acesso do metal ao citoplasma das células radiculares (MEIER et al., 2012). Outro mecanismo de tolerância em nível radicular, porém intracelular, é a complexação e compartimentalização do metal em organelas inativas, como vacúolos, alterando a distribuição do metal via xilema na planta, reduzindo a translocação para a parte aérea, onde poderia causar maiores danos às plantas (DRESLER et al., 2014; KISA et al., 2016). No estudo I foi verificado aumento no conteúdo radicular de compostos fenólicos totais, com o aumento dos níveis de Cu na solução de cultivo do azevém perene, além de modificações nos conteúdos de ácidos orgânicos e aminoácidos. Provavelmente o aumento na concentração dos ácidos orgânicos de baixo peso molecular favoreça a complexação do Cu, contribuindo para o acúmulo do metal no sistema radicular, como verificado em todas as espécies avaliadas neste estudo. A complexação e compartimentalização do Cu nas raízes também pode ocorrer pela interação do Cu com o íon fosfato, no entanto, a formação deste complexo pode causar redução nos teores de P na parte aérea, onde o P participa de importante processos fisiológicos (MARSCHNER, 2011; BALDI et al., 2018). O aumento no conteúdo radicular e da exsudação de compostos fenólicos em *Lolium perenne*, também é uma forma que as gramíneas utilizam para aumentar a absorção de Fe em condições de deficiência do nutriente, conhecida como *estratégia II* para aquisição do Fe (MARSCHNER, 2011; PII et al., 2015). Isto provavelmente contribuiu para a manutenção dos teores de Fe na parte aérea do estudo I.

A absorção excessiva de Cu promoveu alterações na morfologia do sistema radicular em todas as espécies de plantas avaliadas neste trabalho. As alterações morfológicas radiculares comuns entre as espécies foram a redução do comprimento, área superficial e volume, com excessão da videira (Estudos I, II, III e IV). Estas modificações morfológicas podem estar relacionadas às alterações da expansão celular e no índice mitótico, reduzindo a frequência de divisão celular na região do meristema primário (JIANG et al., 2001). Normalmente a redução no alongamento das raízes é acompanhada pelo espessamento, lignificação dos tecidos corticais e aumento do número de raízes laterais, possivelmente como forma de buscar zonas no solo mais adequadas para o desenvolvimento radicular (POTTERS et al., 2007; BOCHICCHIO et al., 2015). As alterações morfológicas, promovidas pelo aumento na concentração de Cu na solução de cultivo podem ser observadas na Figura 2, onde a videira, *Paspalum plicatulum* e *Paspalum notatum* foram cultivadas em solução nutritiva completa (HOAGLAND & ARNON, 1950), em três concentrações de Cu (0,5; 20 e 40 μM de Cu L^{-1}) por 10 dias.

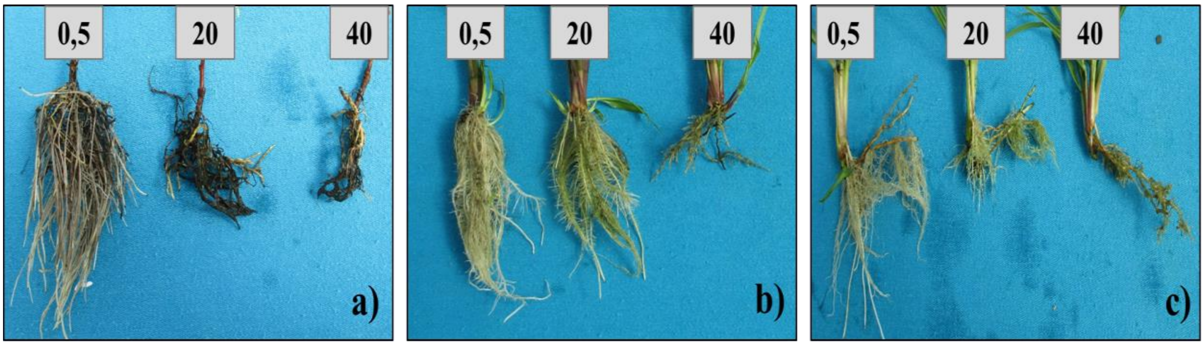

Figura 2: Sistema radicular de videira (a), *Paspalum Plicatulum* (b) e *Paspalum notatum* (c), cultivados em solução nutritiva completa (Hoagland & Arnon, 1950), nas concentrações de 0,5; 20 e 40 μM de Cu L^{-1}, durante 10 dias.

As alterações observadas na morfologia radicular reduzem o volume de solo explorado pelas plantas, podendo refletir em redução na absorção de água e nutrientes, contribuindo para ocorrência de deficiências e desbalanços nutricionais, especialmente de nutrientes que possuem a difusão como principal mecanismo de suprimento, com destaque para o P (TOSELLI et al., 2009; BALD et al., 2018). Aliado a isso, a toxidez por Cu induz mudanças nas propriedades da membrana, função de transportadores e canais iônico, ocasionado pelo aumento da permeabilidade da membrana não-específica, que pode ocasionar deficiência ou absorção excessiva de outros nutrientes (YRUELA, 2005; KABATA-PENDIAS, 2011). Estas alterações possivelmente explicam os elevados teores de Mn no tecido vegetal, diagnosticados nos estudos II, III e IV, quando as plantas foram cultivadas em condições de toxidez por Cu. Pode-se inferir que a redução no crescimento das plantas cultivadas nos solos contaminados, não é decorrente apenas da absorção excessiva do Cu, mas também de efeitos fisiológicos e nutricionais desencadeados pelo excesso do metal.

Desta forma, a adoção de práticas agronômicas que reduzem a necessidade da aplicação de fungicidas à base de Cu para o manejo das doenças nas videiras é fundamental, visando prevenir a contaminação dos solos de vinhedos, ou o agravamento do problema nos solos já contaminados com Cu. Entre as práticas a serem adotadas, cabe destacar o manejo integrado de doenças, o qual estabelece a utilização de fungicidas (controle químico); como a última ferramenta de manejo das doenças a ser adotada, e quando necessária, deve-se priorizar produtos com baixa concentração de Cu em sua composição, além de rotacionar os princípios ativos para evitar a resistência dos fungos. Com a adoção destas práticas agronômicas o aporte de Cu nos vinhedos será menor, contribuindo para manter a fertilidade e qualidade do solo que é a base do sistema de produção, viabilizando a manutenção da atividade a longo prazo nas regiões com vocação para viticultura.

7. CONCLUSÕES GERAIS

Níveis elevados de Cu no ambiente de cultivo reduziram o crescimento das videiras jovens e plantas de cobertura do solo, tanto as nativas do Bioma Pampa como as introduzidas nos vinhedos durante o período de inverno. A absorção excessiva de Cu comprometeu a eficiência da fotossíntese, reduzindo a assimilação de carbono, além de desencadear alterações morfológicas no sistema radicular, que culminam na redução do volume de solo explorado pelas plantas, diminuindo a absorção de água e nutrientes, contribuindo para os desequilíbrios nutricionais observados.

As plantas cultivadas em condições de toxidez pelo Cu expressaram mecanismos de tolerância ao excesso do metal, que consistiram no aumento do pH da solução do solo e da exsudação de ligantes orgânicos, elevando a complexação do Cu^{+2} na solução do solo. As plantas cultivadas em ambientes contaminados acumularam o Cu absorvido predominantemente no sistema radicular, diminuindo a translocação via xilema para parte aérea, onde poderia causar distúrbios mais severos em importantes processos fisiológicos da planta.

O cultivo consorciado de *Axonopus affinis* e, especialmente de *Paspalum plicatulum* foi eficiente em reduzir os sintomas de fitotoxidez nas videiras jovens cultivadas em solos com baixos e moderados níveis de contaminação por Cu. Isso demosntra que as plantas nativas do Bioma Pampa apresentam grande potencial de utilização na fitorremediação dos solos de vinhedos contaminado com Cu, devido a sua ocorrência espontânia e adaptação as condições locais, em que os vinhedos são implantados. Além da proteção do solo e ciclagem de nutrientes, a manutenção da vegetação nativa coabitando os vinhedos na região da Campanha Gaúcha, reduz o impacto da atividade sobre a dinâmica ecológica e ambiental do local, podendo também contribuir com a diferenciação (*terroir*) dos produtos elaborados nesta região, imprimindo nos mesmos, especialmetne nos vinhos, características específicas do local, que os diferenciam e elevam a qualidade e o seu valor comercial.

8. PERSPECTIVAS DE ESTUDOS FUTUROS

I) Avaliar os efeitos do aumento nos teores de Cu no solo sobre a diversidade botânica das espécies de plantas nativas do Bioma Pampa, que naturalmente coabitam os vinhedos comerciais da região da Campanha Gaúcha.

II) Determinar a influência da vegetação que coabita os vinhedos sobre a nutrição da videira, disponibilidade e ciclagem de nutrientes, considerando a grande diversidade de espécies presentes nos vinhedos e das possíveis associações destas plantas com microrganismos, que promovem a fixação de N atmosférico e solubilização de nutrientes indisponíveis.

III) Avaliar a capacidade do cultivo consorciado de plantas de cobertura do solo em mitigar a fitotoxidez de Cu em videiras jovens, sob condições de campo, bem como, avaliar a contribuição das plantas que coabitam os vinhedos adultos, em reduzir os efeitos do excesso do metal nas videiras em produção.

IV) Selecionar os porta-enxertos de videiras que apresentam maior tolerância aos teores elevados de Cu no solo, direcionando estes para reimplantação de vinhedos antigos erradicados.

V) Analisar se o Cu está sendo introduzindo na cadeia alimentar, através de avaliações dos teores de Cu nas uvas e derivados. Caso estiver ingressando, identificar se a fonte do metal é a absorção do solo e translocação para o fruto ou decorrente do contato direto do metal devido às aplicações dos fungicidas cúpricos nas plantas.

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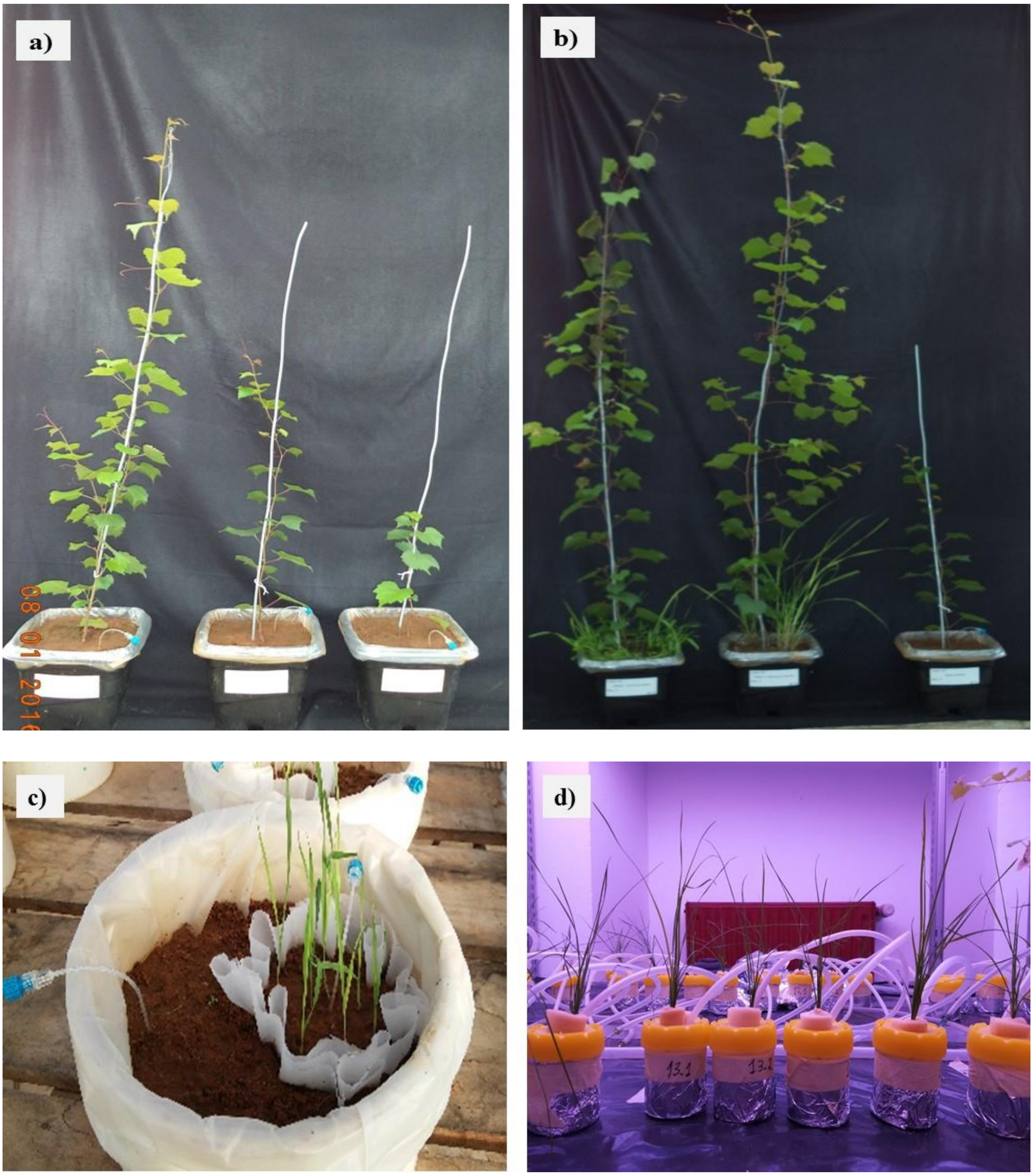
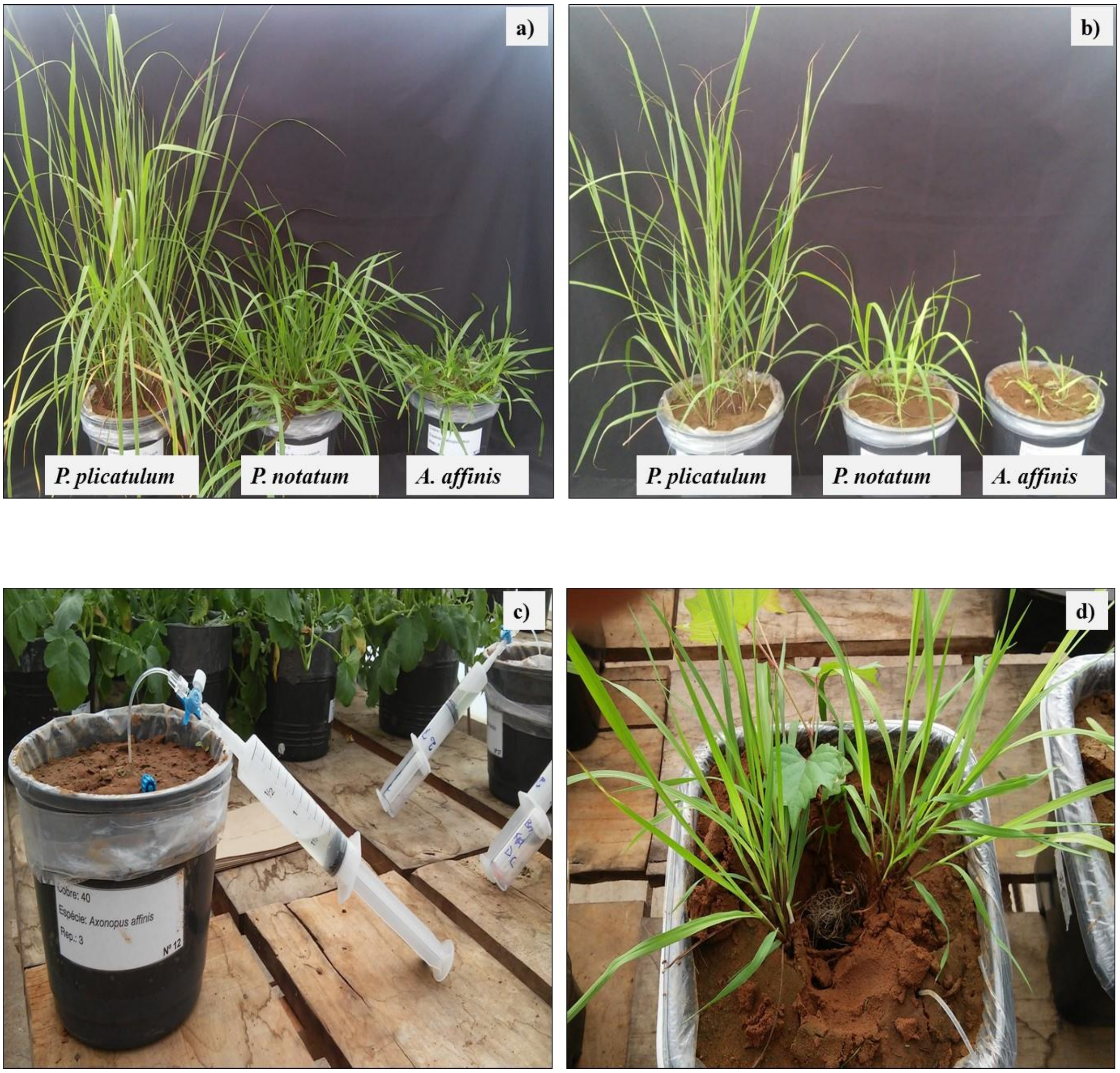
APÊNDICES

APÊNDICE 1: Espécies nativas do Bioma Pampa e espécies hibernais de cobertura de solo, coabitando os vinhedos no final do inverno (a), aplicação de cálda bordalesa (b), nos vinhedos da região da Campanha Gáucha.



APÊNDICE 2: Efeito do aumento nos teores de Cu sobre o crescimento das videiras (a), mitigação da toxidez por Cu na videira, com o cultivo consorciado de *Axonopus affinis* e *Paspalum plicatulum*, na dose 40 mg Cu kg^{-1} (b), unidade experimental do estudo II, com delimitação do ambiente rizosférico (c) e coleta dos exsudatos radiculares no azevém perene (d).



APÊNDICE 3: Crescimento do *Paspalum plicatulum* (*P. plicatulum*), *Paspalum notatum* (*P. notatum*) e *Axonopus affinis* (*A. affinis*) no solo com teor natural de Cu (a) e no solo com adição de 80 mg Cu kg^{-1}(b). Extração da solução do solo antecedendo a implantação do experimento (c) e plantio das mudas de videira dos estudos IV e V, no sistema consorciado (d).

APÊNDICE 4: Polenta Italiana.

Caracterização:

A Polenta é um alimento típico da culinária italiana, com origem na região do Vêneto, Norte da Itália. Sua receita é simples, composta apenas por farinha de milho, água e sal. Era considerado o “alimento dos pobres” pelo baixo custo e simplicidade no preparo, porém era o alimento que matava a fome dos camponeses desta região da Itália.

Ao chegar no Brasil os imigrantes italianos enfrentaram inúmeras dificuldades, incluindo a escasses de alimentos, permanecendo a polenta como base da alimentação das famílias numerosas da época. Além de alimento, a polenta é tema de uma das músicas símbolo da imigração italiana no Sul do Brasil - *la bella polenta*. Por ser um alimento rico e com sabor neutro, atualmente a receita ganhou novos ingredientes e acompanha diversos pratos.

Soluções:

- 2 litros de água;
- 400 g de farinha de milho fina;
- sal a gosto;

Procedimento de análise:

a) Adicione a água em uma panela (preferencialmente uma polenteira de ferro fundido), leve ao fogo e acrescente o sal;

b) Quando iniciar a fervura, acrescente a farinha de milho aos poucos, mexendo sempre para não embolotar;

c) Cozinhar por 30-40 minutos em fogo baixo, mexendo constantemente.

d) Despejar a polenta sobre uma tábua de madeira e está pronta para ser servida;

Determinação:

A polenta acompanha muito bem carnes ao molho e queijos. Um bom vinho também harmoniza muito bem com o prato;

Observação: Por mais longe que vamos, nunca esquecer de onde viemos!!!

VITAE

Lessandro De Conti, filho de Alberto Antônio De Conti e Lourdes Menusi De Conti, nasceu dia 10 de fevereiro de 1988, em Tucunduva, Rio Grande do Sul (RS). Filho de agricultores familiares, desde jovem participou das atividades agropecuárias na propriedade da família. Aos 7 anos de idade inicou sua alfabetização na Escola Municipal de Ensino Básico São Fancisco de Sales, localizada na zona rural de Tucunduva – RS. Em 2003 ingressou no colégio - Sociedade Educacional Três de Maio, cursando o Ensino Médio concomitante com o curso Técnico em Agropecuária , obtendo o título em 2006.

Em 2007 ingressou no Curso de Agronomia da Universidade Federal de Santa Maria (UFSM), trabalhou como bolsista de Iniciação Científica (CNPq) no Laboratório de Química e Fertilidade dos Solos, de 2008 até a conclusão do curso, sob orientação do professor Carlos Aberto Ceretta. Realizou o estágio do curso de Agronomia pelo Programa Novos Talentos, das empresas Fundação MT e Tropical Melhoramento e Genética (TMG), obtendo o título de Engenheiro Agrônomo em 2012. Realizou o curso de Mestrado em Ciência do Solo (agosto de 2012 a julho de 2014) no Programa de Pós-Graduação em Ciência do Solo (PPGCS) da UFSM sob orientação do professor Carlos Alberto Ceretta (título da dissertação: *Teores no solo e espécies químicas na solução de P, Cu e Zn com adições sucessivas de dejetos líquidos de suínos*).

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