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COBRE E ZINCO NO SOLO: CRESCIMENTO E PARÂMETROS FISIOLÓGICOS EM VIDEIRAS JOVENS E AVEIA PRETA

TESE DE DOUTORADO

Tadeu Luis Tiecher

Santa Maria, RS, Brasil

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COBRE E ZINCO NO SOLO: CRESCIMENTO E PARÂMETROS FISIOLÓGICOS EM VIDEIRAS JOVENS E AVEIA PRETA

por

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Orientador: Prof. Dr. Gustavo Brunetto
Co-orientador: Carlos Alberto Ceretta

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como requisito parcial para a obtenção do grau de
Doutor em Ciência do Solo

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“Eu acredito demais na sorte.
E tenho constatado que,
quanto mais duro eu trabalho,
mais sorte eu tenho.”
(Thomas Jefferson)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciência do Solo
Universidade Federal de Santa Maria

COBRE E ZINCO NO SOLO: CRESCIMENTO E PARÂMETROS FISIOLÓGICOS EM VIDEIRAS JOVENS E AVEIA PRETA

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A Campanha Gaúcha do Rio Grande do Sul é uma das principais regiões vitivinícolas do Brasil que sofre com fatores climáticos propícios para o desenvolvimento de doenças fúngicas foliares da videira (*Vitis vinífera*). Normalmente o controle dessas doenças é realizado com sucessivas aplicações de fungicidas a base de cobre (Cu), tais como a calda bordalesa, o que gera acúmulo de Cu no solo até teores tóxicos às videiras e às plantas que coabitam os vinhedos, como a aveia preta (*Avena strigosa* Schreb.). Nos vinhedos da região da Campanha Gaúcha, a toxicidade do Cu às plantas é constatada em teores disponíveis no solo relativamente baixos, se comparados a outras regiões vitivinícolas do mundo, o que se deve às características dos solos da região que, geralmente são arenosos, ácidos e com baixo teor de matéria orgânica do solo (MOS). Para reduzir a quantidade de Cu aplicado nos vinhedos, tem-se utilizado fungicidas alternativos à calda bordalesa que possuem zinco (Zn) na sua formulação. Como o número de aplicações de fungicidas ainda é elevado, tem sido observado aumento do teor de Zn em solos de vinhedo que já possuem alto teor de Cu. Em razão disso, o objetivo dessa Tese foi avaliar o efeito da adição combinada de teores elevados de Cu e Zn em solo arenoso da Campanha Gaúcha sobre o crescimento, o estado fisiológico e as alterações morfológicas do sistema radicular de plantas de videiras jovens e aveia preta. Para isso, foram realizados três estudos em casa de vegetação utilizando Argissolo Vermelho, com 5,4% de argila e 0,9% de MOS. Os estudos I e II foram realizados em vasos contendo 4,0 kg de solo com plantas de aveia preta (30 dias de cultivo) e videiras jovens (60 dias de cultivo), respectivamente, e os tratamentos consistiram de 0, 30 e 60 mg Cu kg^{-1} combinados com a adição de 0, 15, 30, 60, 120 e 180 mg Zn kg^{-1}. O estudo III foi realizado em recipientes do tipo *Rizobox*, contendo 2,4 kg de solo, com plantas de videiras jovens (60 dias de cultivo) e os tratamentos foram 0 e 120 mg Cu kg^{-1} combinados com a adição de 0, 120 e 240 mg Zn kg^{-1}. Nos estudos I e II, determinou-se o acúmulo de matéria seca de raízes e da parte aérea, os teores de Cu e Zn nas raízes e na parte aérea e a translocação destes metais das raízes para a parte aérea, a fluorescência da clorofila *a*, os pigmentos fotossintéticos e a atividade das enzimas antioxidantes. No estudo III, além das referidas análises, foram avaliados a morfologia do sistema radicular e as trocas gasosas realizadas pelas plantas. Em todos os estudos, as videiras jovens e a aveia preta apresentaram retenção de Cu e Zn nas suas raízes, diminuindo a translocação para a parte aérea. Contudo, nas maiores doses de Cu e Zn de todos os estudos foi observado diminuição do crescimento das plantas e eficiência fotoquímica associada ao decréscimo no teor de pigmentos fotossintéticos. Além disso, foram observadas alterações nas trocas gasosas e no sistema radicular das videiras jovens no estudo III. Em todos os estudos, a atividade das enzimas antioxidantes foi maior em doses intermediárias de Zn, indicando a ativação do sistema antioxidante. Porém, a condição de estresse em tratamentos com altos níveis de Cu e Zn não foi revertida. Desta forma, embora os teores de Zn atualmente observados em condições de campo na Campanha Gaúcha ainda estejam em níveis inferiores aos tóxicos às plantas, os solos arenosos de vinhedos dessa região que já estão contaminados com Cu, poderão ter seu efeito tóxico às plantas de videiras jovens e aveia preta potencializados à medida que o teor de Zn no solo aumentar. Para diminuir o potencial fitotóxico do Cu e Zn em solos arenosos de vinhedos da Campanha Gaúcha é imperativo utilizar práticas de manejo com objetivo de aumentar a capacidade de adsorção desses metais no solo e diminuir a adição de Cu e Zn. Isso só será possível utilizando manejo do solo que permita aumentar os teores de MOS e manter o pH do solo próximo ao ideal para a cultura da videira (6,0), além de utilizar estratégias de controle preventivo de doenças, de rotação de princípios ativos e, especialmente, de modos de ação de fungicidas.

Palavras-chave: Metais pesados, Vitivinicultura, Translocação, Estado fisiológico de plantas, Morfologia do sistema radicular, Estresse oxidativo.

ABSTRACT

Doctoral Thesis
Pos-Graduate Program in Soil Science
Universidade Federal de Santa Maria

COPPER AND ZINC IN THE SOIL: GROWTH AND PHYSIOLOGICAL PARAMETERS IN YOUNG GRAPEVINES AND BLACK OAT

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Date and Place of the defense: November, 10th, 2017, Santa Maria

The Campanha Gaúcha is one of the main wine regions of Brazil that suffers from climatic factors favorable to the development of fungal diseases on the leafs of the grapevine (*Vitis vinifera*). Generally, the control of these diseases is carried out with successive applications of copper-based (Cu) fungicides, such as the Bordeaux mixture, which generates Cu accumulation in the soil up to toxic levels for the vines and the plants that co-inhabit the vineyards, such as black oats (*Avena strigosa* Schreb.). In the vineyards of the Campanha Gaúcha, the Cu toxicity to plants is observed at relatively low Cu-levels in the soil, compared to other wine growing regions of the world, due to the characteristics of the soils from this region, that are generally sandy, acidic and have low soil organic matter (SOM) content. In order to reduce the amount of Cu applied in the vineyards, Zn-based fungicides have been used as an alternative to replace the Bordeaux mixture that have zinc (Zn) in its formulation. As the number of fungicide applications is still high, a quick increase in soil Zn content has been observed in vineyard soils that already have a high Cu content. Therefore, the objective of this Thesis was to evaluate the effect of the combination of Cu and Zn levels on a sandy soil from the Campanha Gaúcha on the growth, physiological status and the morphological changes in the root system of young grapevines and black oats. For that, three studies were carried out under greenhouse conditions using a Typic Hapludalf soil, with 5.4 % of clay and 0.9% of SOM. Studies I and II were carried out in pots containing 4.0 kg of soil, with black oats plants (30 days of cultivation) and young grapevines (60 days of cultivation), respectively, and treatments consisted of 0, 30 and 60 mg Cu kg^{-1} combined with the addition of 0, 15, 30, 60, 120 and 180 mg Zn kg^{-1}. Study III was carried out in Rizobox-type containers, containing 2.4 kg of soil, with young grapevines (60 days of cultivation) and the treatments were 0 and 120 mg Cu kg^{-1} combined with 0, 120 and 240 mg Zn kg^{-1}. In studies I and II, it was determined the accumulation of dry matter in the roots and shoots, Cu and Zn contents in the roots and shoots and the translocation of these metals from the roots to shoots, the chlorophyll *a* fluorescence, the photosynthetic pigments and the activity of antioxidant enzymes. In study III, in addition to these analyses, the root morphology and gas exchanges performed by plants were also evaluated. In all studies, young grapevines and black oats showed retention of Cu and Zn in their roots, reducing the translocation to the shoots. However, at the highest Cu and Zn doses of all the studies, it was observed decreased plant growth and photochemical efficiency was associated to the decrease of the content of photosynthetic pigments. In addition, changes in gas exchange and root system of young vines were observed in study III. In all the studies, activity of the antioxidant enzymes was higher in intermediate doses of Zn, indicating the activation of the antioxidant system. However, stressful conditions in with high levels of Cu and Zn was not reversed. This way, although the Zn levels currently observed under field conditions in the Campanha Gaúcha are still at levels lower than those that presented risk of toxicity to the plants, the sandy soils of vineyards from this region that are already contaminated with Cu may have their toxic effect on young grapevines and black oat plants potentiated as the soil Zn content increases. In order to decrease the phytotoxic potential of Cu and Zn in sandy soils from the Campanha Gaúcha vineyards, it is imperative to use management practices that aim to increase the adsorption capacity of these metals in the soil and decrease the addition of Cu and Zn. This can only be possible by using soil management that allows to increase the SOM content and to maintain soil pH close to the ideal for vines (6.0), besides using preventive strategies to control plant diseases, rotation of active principles and, especially, modes of action of fungicides.

Keywords: Heavy metals, Vitiviniculture, Translocation, Physiological state of plants, Root system morphology, Oxidative stress.

SUMÁRIO

1. INTRODUÇÃO

O acúmulo de metais pesados no solo tem sido tema de diversos estudos no mundo ao longo das últimas décadas. Na região Sul do Brasil um dos estudos pioneiros relacionados ao acúmulo de metais pesados como o cobre (Cu), zinco (Zn), chumbo (Pb), níquel (Ni) e manganês (Mn) no solo, foi o conduzido por Mattias (2006), em solos de Microbacias com histórico de aplicação de dejetos líquidos de suínos. Posteriormente, Girotto (2007) em sua Dissertação de Mestrado estudou especificamente o acúmulo de Cu e Zn em um solo Argissolo submetido a sucessivas aplicações de dejetos líquidos de suínos no Rio Grande do Sul (RS), onde foram encontrados expressivos aumentos nas concentrações de ambos os elementos. Este estudo contribuiu para o início de outros em solos de vinhedos contaminados. Os estudos foram realizados por participantes do grupo de pesquisas atualmente denominado Grupo de Estudos de Predição de Adubação e Potencial de Contaminação de Elementos em Solos - GEPACES, da Universidade Federal de Santa Maria (UFSM). A primeira obra oriunda dessa inserção do grupo de pesquisas foi a Tese de Doutorado de Girotto (2010), onde foram estudados os efeitos do Cu em solos contaminados com Cu oriundos da região da Campanha e da Serra Gaúcha sobre plantas de aveia preta em condições controladas. Além disso, foi estudado o efeito do incremento de Cu e Zn em solo com histórico de aplicação de dejetos líquidos de suínos, sobre aspectos bioquímicos e fisiológicos em plantas.

Em um segundo momento, Miotto (2012) em sua Tese de Doutorado estudou o acúmulo de Cu no solo e seu efeito em plantas de videiras adultas em produção e plantas que coabitam os vinhedos, tanto em condições de campo, como em condições controladas, especificamente com solos da região da Campanha Gaúcha do RS. Neste estudo, foi constatado que nos solos com longo histórico de cultivo de vinhedos estava ocorrendo além do acúmulo de Cu no solo, também o incremento dos teores de Zn. Isto motivou o início dos estudos com a combinação de doses (ou teores) de Cu e Zn em solos de vinhedos da região da Campanha Gaúcha. A primeira publicação do grupo de pesquisas sobre o tema foi derivada da Dissertação de Mestrado de Tadeu Luis Tiecher (2014). Neste estudo, foi coletado solo em um vinhedo contaminado com Cu na região da Campanha Gaúcha, onde foram adicionadas doses de Zn e estudados os efeitos da combinação de elevados teores de Cu e Zn sobre plantas de milho. Seguindo esta mesma linha de pesquisa foi elaborado o projeto da presente Tese de Doutorado, com o intuito de avaliar os efeitos da combinação de elevados teores de Cu e Zn em solos de vinhedos, sobre plantas de videiras jovens e plantas que coabitam os vinhedos

como a aveia preta. A relevância deste estudo está no seu viés ambiental, visto que os relatos de solos contaminados com metais pesados no mundo têm sido cada vez mais frequentes, bem como pela importância da atividade vitivinícola para o estado do RS.

A vitivinicultura é uma atividade que tem apresentado significativo crescimento nas últimas décadas, com grande destaque para a região da Campanha Gaúcha, no Estado do RS. O crescimento da atividade nesta região em muito se deve ao clima mais favorável ao crescimento e desenvolvimento da cultura da videira e ao relevo suavemente ondulado que facilita a mecanização da atividade. Outro fator relevante são as características do solo, que geralmente possui textura mais arenosa, o que possibilita aos produtores adicionar nutrientes em quantidade e momento mais adequado, propiciando vinhos de melhor qualidade. O conjunto destes fatores, que contribuem para a tecnificação e o incremento da qualidade da produção vitivinícola, tem atraído grandes empresas à região, além de pequenos produtores locais.

Embora o clima da região da Campanha seja mais favorável em relação a Serra Gaúcha, as precipitações pluviais ainda são consideradas elevadas para a cultura da videira. Isto obriga os viticultores a realizarem aplicações de fungicidas frequentemente, especialmente para o controle de míldio (*Plasmopara viticola*). Dentre os fungicidas, o que apresenta melhor custo benefício é a calda bordalesa, que contém grandes quantidades de cobre na sua composição, que, aliado às sucessivas aplicações, pode ocasionar o acúmulo deste elemento no solo e atingir teores tóxicos às plantas, o que já acontece em muitas áreas desta região.

Devido a este acúmulo, muitos viticultores tem reduzido a quantidade de fungicidas à base de Cu aplicada nos vinhedos, buscando o controle de doenças com produtos alternativos à calda bordalesa. Dentre estes produtos, os fungicidas do grupo dos ditiocarbamatos têm tido destaque, sendo observado um expressivo aumento do seu uso em vinhedos, especialmente nos últimos ciclos produtivos da cultura da videira. Contudo, estes produtos apresentam zinco na sua constituição. Como o número de aplicações dos fungicidas ainda é elevado e trata-se de produtos com ação de contato, tem sido observado também o acúmulo de Zn em solos de vinhedo.

No solo, o Cu e o Zn são retidos por ligações físico-químicas e sua labilidade depende do conteúdo de argilominerais, MOS, valor de pH do solo e capacidade de troca de cátions (CTC). Normalmente, a sorção do Cu e do Zn no solo ocorre primeiramente nos sítios de ligação mais ávidos e, em seguida, os íons remanescentes são redistribuídos em frações que são retidas com menor energia e, consequentemente, de maior disponibilidade e mobilidade.

Como os solos da região da Campanha possuem baixos teores de argila e de MOS, além de serem naturalmente ácidos, existem poucos sítios onde o Cu e o Zn podem se adsorver. Desta forma, as aplicações frequentes de fungicidas aumentam a quantidade de frações solúveis e trocáveis de Cu e Zn no solo, potencializando a toxidez às plantas, além de favorecer a transferência destes elementos para os ambientes aquáticos.

Os altos teores de Cu e Zn no solo podem levar a absorção excessiva destes elementos pelas plantas, o que pode causar estresse oxidativo devido ao desequilíbrio entre as respostas antioxidantes e o aumento da produção de espécies reativas de oxigênio (EROs). Pode, ainda, afetar negativamente a permeabilidade da membrana, a cadeia de transporte de elétrons e a absorção e translocação de nutrientes. Além disso, pode reduzir a taxa e a eficiência fotossintética, reduzir as trocas gasosas realizadas pelas plantas e causar clorose foliar, o que contribui para a inibição do crescimento das plantas.

Plantas que se desenvolvem em solos com altos teores de Cu e Zn podem apresentar alterações morfoanatômicas no desenvolvimento radicular. Pode ser observada redução da densidade de pelos radiculares, encurtamento e espessamento das raízes, o que resulta em redução da absorção de nutrientes, água e, consequentemente, reduz o crescimento das plantas. A soma de todos estes fatores dificulta a implantação das culturas de interesse econômico em solos de vinhedo sob estas condições, causando prejuízos econômicos e ambientais aos viticultores.

Atualmente, alguns estudos científicos tem explorado como o Cu e o Zn interferem isoladamente, ou mesmo a combinação entre os dois elementos, em uma variedade de processos fisiológicos e no sistema de defesa antioxidante das plantas. Contudo, tratam-se de estudos onde as plantas são cultivadas em solução nutritiva. Dentre os poucos relatos envolvendo a adição conjunta do Cu e Zn em solos, estes são em solos com incrementos nos teores destes dois elementos, juntamente com o aumento da MOS. No entanto, são escassos estudos sobre a combinação entre Cu e Zn em solos com acúmulo destes elementos em ambientes de vinhedos, conduzidos em solos ácidos, arenosos e com baixo teor de MOS, e seus efeitos às plantas.

Assim, torna-se necessário antever o que poderá acontecer num futuro próximo no campo, a partir das sucessivas aplicações de fungicidas que contém Zn na sua formulação e o consequente acúmulo deste elemento, em vinhedos que já possuem alto teor de Cu. De forma especial, devem-se investigar as implicações causadas pela adição destes metais pesados no solo ao estado fisiológico e à morfologia do sistema radicular de plantas de videiras jovens e de plantas que coabitam os vinhedos, como a aveia preta.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito da adição combinada de teores elevados de Cu e Zn em solo arenoso da Campanha Gaúcha sobre o crescimento, o estado fisiológico e as alterações morfológicas do sistema radicular de plantas de videiras jovens e aveia preta.

2.2 OBJETIVOS ESPECÍFICOS

i) Avaliar o estado fisiológico, através da atividade enzimática, da fluorescência da clorofila *a* e dos teores de pigmentos fotossintéticos, e a translocação de metais pesados do tecido das raízes para a parte aérea de plantas de aveia preta cultivadas em solo contendo concentrações elevadas de Cu e Zn, isoladamente ou combinados;

ii) Avaliar o estado fisiológico, através da atividade enzimática, da fluorescência da clorofila *a*, dos teores de pigmentos fotossintéticos, das trocas gasosas e da taxa fotossintética, e a translocação de metais pesados do tecido das raízes para a parte aérea de videiras jovens cultivadas em solo contendo concentrações elevadas de Cu e Zn, isoladamente ou combinados;

iii) Determinar aspectos morfológicos do sistema radicular, mensurando o comprimento e diâmetro de raízes, volume radicular e área superficial, de videiras jovens cultivadas em solo com a adição combinada de elevados teores de Cu e Zn.

3. HIPÓTESES

i) A adição inicial de doses de Zn em solo arenoso com alto teor de Cu pode amenizar a toxicidade do Cu às plantas de videiras jovens e aveia preta, por causa do efeito nutricional ocasionado nas plantas, uma vez que o Zn participa de processos fisiológicos importantes nas plantas e os Argissolos da região da Campanha Gaúcha naturalmente possuem baixos teores de Zn.

ii) A combinação de altos teores de Cu e Zn em solo arenoso potencializa a toxicidade às plantas de videiras jovens e aveia preta, alterando o seu estado fisiológico, devido à absorção e acúmulo de metais pesados em órgãos fotossinteticamente ativos das plantas, o que prejudica processos fisiológicos nas mesmas.

iii) A ocorrência de altos teores de Cu e Zn em solo arenoso altera a morfologia do sistema radicular de videiras jovens, provocando o aumento do diâmetro das raízes e a redução no comprimento do sistema radicular, como resposta à toxicidade causada por estes dois metais pesados.

4. REVISÃO BIBLIOGRÁFICA

4.1 ACÚMULO DE COBRE E ZINCO EM SOLOS DE VINHEDO

A viticultura brasileira, embora tenha apresentado retração da área cultivada nos últimos anos, é uma atividade de grande relevância econômica no país, especialmente no Estado do Rio Grande do Sul, onde atualmente são cultivados pouco mais de 49 mil hectares de videiras, representando aproximadamente 71% da área cultivada no país (IBGE 2017). Neste contexto, destacam-se as regiões da Serra Gaúcha, a maior e mais tradicional região produtora, e a Campanha Gaúcha, com crescente aumento de área cultivada, que começou a ser ocupada na década de 70 (MELLO, 2012) e teve grande expansão a partir dos anos 2000.

O crescimento da atividade vitivinícola na região da Campanha nas últimas décadas em muito se deve ao clima mais favorável ao crescimento e desenvolvimento da cultura da videira em relação à região da Serra Gaúcha. O clima da região da Campanha é subtropical, classificado como Cfa (classificação de Köppen) com pluviosidade média de 1388 mm ano^{-1}, distribuídas de forma igualitária em todos os meses do ano (IPA, 1989). O solo predominante da região é o Argissolo, derivado de rochas sedimentares (arenito com alto teor de silício), com predomínio de argilomineral do tipo 1:1, textura arenosa e baixo teor de matéria orgânica do solo (MOS), sendo o relevo suave a ondulado (STRECK et al., 2002), o que também contribuiu para o aumento da área vitícola, por facilitar a mecanização da atividade.

O clima subtropical da região faz com que tradicionalmente sejam feitas aplicações frequentes de fungicidas à base de Cu, como a calda bordalesa [$Ca(OH)_2$ + $CuSO_4$] e o oxicloreto de cobre [$CuCl_2.3Cu(OH)_2$], para o controle de doenças foliares, especialmente o míldio (*Plasmopara viticola*) (BRUNETTO et al., 2017). Em um ciclo da cultura da videira, o montante das aplicações de fungicidas pode chegar a adição de 30 kg Cu ha^{-1} (CASALI et al., 2008) o que ocasiona, muitas vezes, o aumento dos teores de Cu no solo até níveis tóxicos às plantas. Este incremento nos teores de Cu no solo pode ser visualizado na Figura 1. Por isso, alguns produtores passaram a utilizar fungicidas à base de Zn em detrimento dos fungicidas cúpricos. Como a calda bordalesa também contribui, em menor escala, para o aumento dos teores de outros metais pesados no solo, como o Zn (MIRLEAN et al., 2007), tem sido observado a ocorrência de altos teores de ambos os elementos, Cu e Zn, em solos de vinhedos, como já foi reportado ainda na década de 80 por Magalhães et al. (1985), sendo que na última década este fato tem tido maior notoriedade (BRUNETTO et al., 2014, 2017; CAMBROLLÉ

et al., 2015; FERNANDEZ-CALVIÑO et al., 2010, 2012; MACKIE et al., 2012; MIOTTO et al., 2014; GIROTTO et al., 2016; TIECHER et al., 2016a, 2016b, 2016c, 2017).

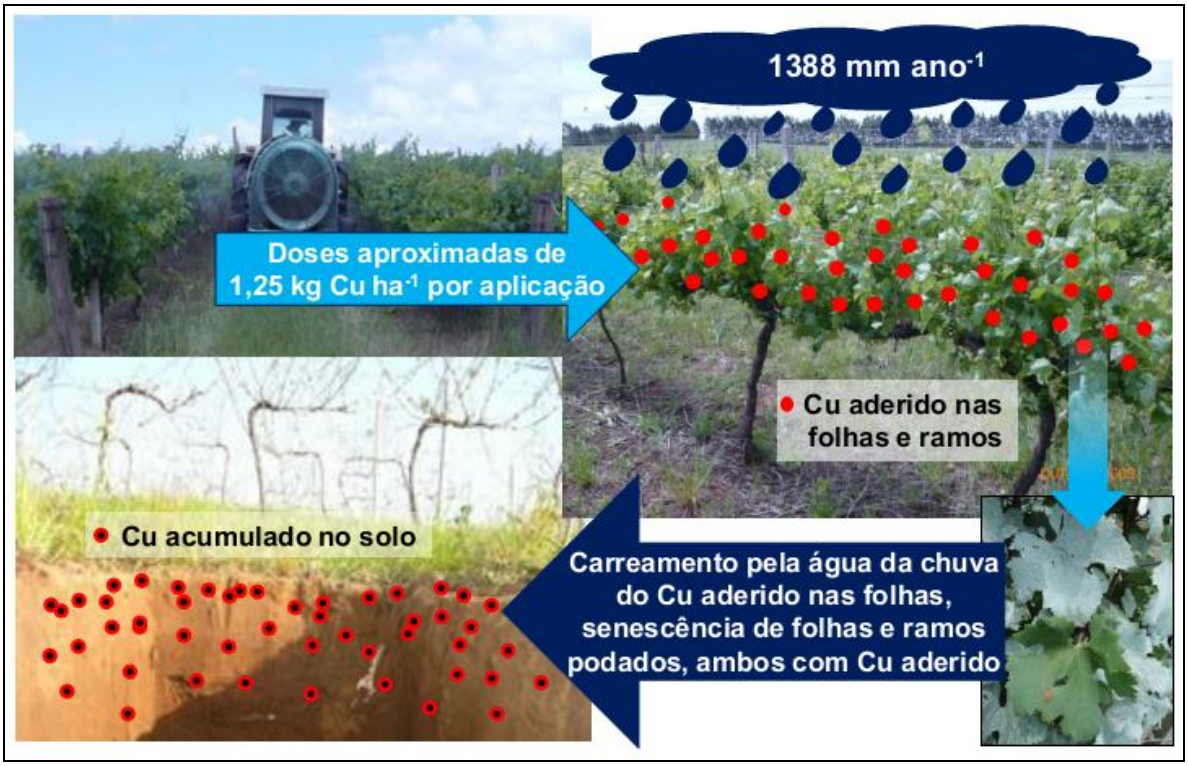

Figura 1: Fatores que contribuem para o incremento nos teores de Cu em solos de vinhedos do Sul do Brasil.

No Brasil, a viticultura tem pouco mais de um século e os estudos de acúmulo de Cu e Zn são escassos. Ainda assim, há uma grande amplitude de teores totais destes elementos documentados e tal fato é gerado por diferenças entre os solos e, principalmente, pela quantidade de Cu e Zn aplicadas. Este último fator é relacionado principalmente com a precipitação média da região (KOMÁREK et al., 2010; VAVOULIDOU et al., 2005). Como as regiões produtoras do Sul do Brasil estão situadas num clima subtropical, com elevadas precipitações anuais, as aplicações de fungicidas são frequentes e, consequentemente, os teores destes metais pesados acumulados no solo são muito maiores do que os encontrados em outras regiões produtoras de uvas no mundo (MIRLEAN et al., 2007), embora muitas vezes o tempo de cultivo seja o mesmo, ou até menor. Por isso, o tempo de cultivo com vinhedos nem sempre está bem correlacionado com os teores de Cu e Zn no solo (BRUN et al., 1998).

No solo, o Cu e o Zn são retidos por ligações físico-químicas e sua labilidade é dependente do ligante. O teor máximo de Cu e Zn de um solo está relacionado com a sua capacidade de sorção que depende de diversos componentes. Em geral, a capacidade de

sorção é maior quanto maior for o conteúdo de argilominerais, óxidos e hidróxidos de Fe, Al e Mn, carbonatos e MOS (BRADL, 2004; McBRIDE, 1994,). O pH do solo (BRADL, 2004; CHAIGNON et al. 2003), a Capacidade de Troca de Cátions (CTC) e a qualidade da MOS são importantes fatores que regulam a sorção e a biodisponibilidade de Cu e Zn no solo (ARIAS et al., 2005; BRUN et al., 2001). Somado a isso, os solos com valores de pH neutro a alcalino podem, em geral, suportar teores mais altos de Cu e Zn se comparado com solos ácidos, onde a biodisponibilidade destes elementos é maior (BRUN et al., 2001; CHAIGNON et al., 2003; PIETRZAK; Mc PHAIL, 2004).

Devido à interação entre estes fatores físico-químicos, o Cu e o Zn são encontrados em diferentes frações no solo, já que, naturalmente, eles são adsorvidos em diferentes graus de energia (BRUNETTO et al., 2014; CASALI et al., 2008; GIROTTO et al., 2014; TIECHER et al., 2013). Porém, o aumento dos teores de Cu e Zn no solo, por causa de aplicações frequentes de fungicidas, pode interferir na distribuição das suas frações no solo. Normalmente, a sorção do Cu e do Zn no solo ocorre primeiramente nos sítios de ligação mais ávidos e, em seguida, os íons remanescentes são redistribuídos em frações que são retidas com menor energia e, consequentemente, de maior disponibilidade e mobilidade (TIECHER et al., 2013). Há que se considerar, ainda, que o acúmulo de Cu e Zn no solo depende da interação competitiva dos mesmos pelos sítios de adsorção, bem como pelas demais espécies químicas presentes no meio (PÉREZ-NOVO et al., 2009, 2011). Por isso, a cinética de sorção desses elementos quando adicionados simultaneamente no solo é diferente do observado quando apenas um deles é depositado no solo (ARIAS et al., 2006), favorecendo o aumento dos teores de ambos os elementos em frações mais lábeis (TIECHER et al., 2016b), como pode ser observado na Figura 2.

Com as aplicações sucessivas de fungicidas e o consequente aumento da biodisponibilidade de Cu e Zn nos solos de vinhedos da região da Campanha Gaúcha, aumenta o risco de contaminação do ambiente, podendo, inclusive, ocorrer a contaminação de águas subterrâneas por metais pesados derivados de fungicidas (BRUNETTO et al., 2014, 2017). Isto foi constatado em um solo arenoso por Mirlean et al. (2007), que atribuiu tal fato à ocorrência simultânea de fatores como elevada precipitação, granulometria do solo, solo ácido e posição do aquífero a pequena profundidade. Além disso, o aumento dos teores de Cu e Zn em formas com maior biodisponibilidade pode ocasionar toxidez às plantas de videira e plantas que coabitam os vinhedos, como a aveia preta, especialmente nas condições de solo encontradas nos vinhedos da região da Campanha Gaúcha (GIROTTO et al., 2014, 2016; MIOTTO et al., 2014; TIECHER et al., 2016a, 2016b, 2016c, 2017).

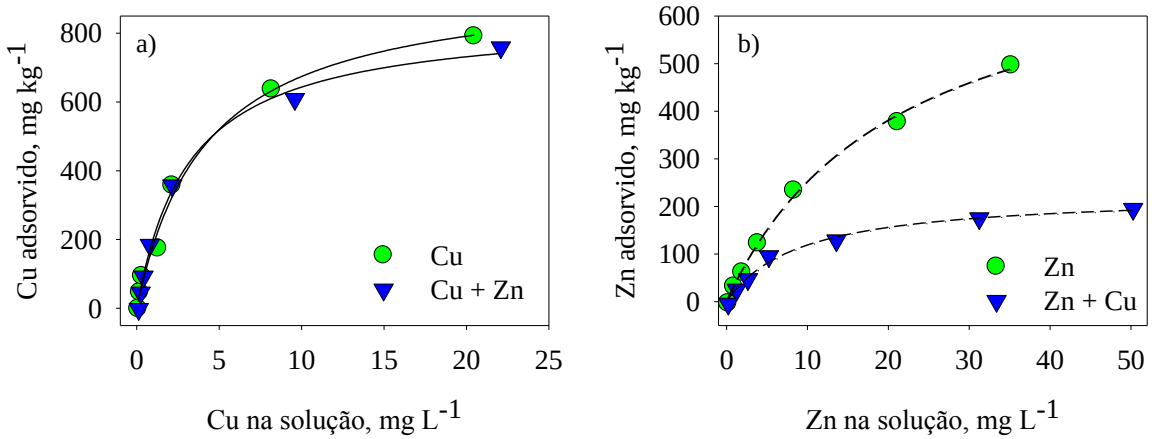

Figura 2: Isotermas de adsorção de Cu (a) e Zn (b) obtidas através da utilização de solução contendo apenas Cu, apenas Zn e solução mista com adição de Cu e Zn juntos, em um solo de vinhedo contaminado com Cu. Fonte: Adaptado de Tiecher et al. (2016b).

4.2 ALTERAÇÕES FISIOLÓGICAS E NUTRICIONAIS EM PLANTAS, OCASIONADAS PELOS ALTOS TEORES DE COBRE E ZINCO NO SOLO

Videiras jovens transplantadas em solos de vinhedos antigos erradicados, ou mesmo as gramíneas que coabitam os vinhedos, como a aveia preta, que é amplamente utilizada como planta de cobertura nas entrelinhas dos vinhedos, quando cultivadas em solos com altos teores de Cu e Zn, podem apresentar crescimento lento, clorose foliar e acúmulo destes metais pesados nos tecidos (AMBROSINI et al., 2015; GIROTTO et al., 2014, 2016; MIOTTO et al., 2014; TIECHER et al., 2016a, 2016c, 2017). O crescimento lento das videiras jovens gera um impacto econômico negativo aos viticultores, uma vez que retarda o início da produção dos vinhedos e, consequentemente, o retorno econômico da atividade. Por outro lado, o menor crescimento das plantas que coabitam os vinhedos afeta indiretamente a viticultura, especialmente nos aspectos relacionados à qualidade do solo. Como se tratam de solos arenosos e, portanto, mais frágeis, a menor produção de matéria seca das plantas de cobertura reduz a proteção do solo, deixando-o vulnerável à erosão, com a consequente perda de MOS e de nutrientes (LOURENZI et al., 2015), além de reduzir a ciclagem de nutrientes no solo.

O Cu e o Zn são micronutrientes e fazem parte da estrutura de compostos e ativadores enzimáticos (KABATA-PENDIAS, 2011; PILON et al., 2006). O Cu está envolvido em processos metabólicos, como fotossíntese, respiração, metabolismo de carboidratos e reprodução (KABATA-PENDIAS, 2011). O Zn está associado com o metabolismo de

carboidratos, regulação da expressão de genes, integridade estrutural do ribossomo e metabolismo de fosfato, além da síntese de enzimas como as desidrogenases, proteinases e peptidases (CHERIF et al., 2011; KABATA-PENDIAS, 2011). Todavia, plantas que crescem em solos com altos teores de Cu e Zn disponíveis podem acumular demasiadamente esses elementos em seus tecidos (KABATA-PENDIAS, 2011), resultando em respostas negativas em níveis bioquímicos, fisiológicos e nutricionais, que variam de acordo com o órgão da planta, a concentração dos elementos e a tolerância dos tecidos a altos níveis desses metais pesados (CAMBROLLÉ et al., 2012; MATEOS-NARANJO et al., 2013).

O Cu e o Zn geralmente apresentam interações antagônicas, onde a absorção de um elemento é competitivamente inibida pelo outro (KABATA-PENDIAS, 2011). Altas concentrações destes elementos nos tecidos das plantas induzem a mudanças nas propriedades da membrana, o que afeta a função de transportadores de membrana e canais iônicos (JANICKA-RUSSAK et al., 2008). Estes efeitos, ocasionam um aumento da permeabilidade da membrana não-específica, que pode ser responsável pelos desequilíbrios nas concentrações de nutrientes em plantas cultivadas em ambientes com altos teores de metais pesados (CAMBROLLÉ et al., 2013).

Plantas cultivadas em solo com alto teor de Cu podem apresentar redução na absorção de nutrientes, como Ca e Mg, devido a competição entre estes e o Cu. Isto ocorre porque estes elementos possuem a mesma valência, ou devido à adsorção de Cu na superfície das raízes, principalmente nos sítios com N das proteínas, com o qual possui forte interação, dificultando a passagem desses nutrientes para o interior da planta (KABATA-PENDIAS, 2011; KOPITTKE et al., 2011; LUO et al., 2008).

Já o Zn pode ser encontrado em grandes concentrações nos tecidos das plantas porque existe uma semelhança entre os raios iônicos dos cátions bivalentes, como o Mn, Fe e Cu. Assim, os íons de Zn podem substituir estes cátions bivalentes e ser absorvido pelas raízes (TEWARU et al., 2008). Uma vez no interior da planta, o Zn em excesso pode alterar o equilíbrio fisiológico pela competição com os demais cátions em diversos locais (TEWARU et al., 2008), como nos sítios de absorção primária ou nos locais de transporte de nutrientes das raízes (YANG et al., 2011). Assim, o Zn, em quantidade excessiva nas plantas, pode afetar a homeostase iônica normal, por interferir na absorção, transporte e regulação de íons essenciais (WANG et al., 2009).

Alguns distúrbios fisiológicos e bioquímicos em plantas são frequentemente associados ao aumento dos teores de Cu e Zn em solos e nas plantas (CAMBROLLÉ et al., 2012; MATEOS-NARANJO et al., 2013; TIECHER et al., 2016a, 2016c, 2017). Em espécies

sensíveis, concentrações nos tecidos entre 15-20 mg Cu kg^{-1} e 150-200 mg Zn kg^{-1} provocam redução do crescimento das plantas (KABATA-PENDIAS, 2011). Concentrações superiores a estas são frequentemente relatadas, tanto em condições de campo como em condições controladas de casa de vegetação (CAMBROLLÉ et al., 2012; GIROTTO et al., 2013; MIOTTO et al., 2014; TIECHER et al., 2016a, 2016c).

O aumento dos teores de metais pesados nos tecidos das plantas pode ocasionar distúrbios bioquímicos a nível celular, como o aumento da concentração de espécies reativas de oxigênio (EROs). Isto pode ocorrer porque o Cu pode ser encontrado em dois estados de oxidação nas plantas (YRUELA, 2005). Por meio da reação de Fenton, o ciclo redox entre Cu^{2+} e Cu^{+} catalisa a produção de radicais hidroxil (OH^{-}) provenientes de radicais superóxido ($O_2\bullet^{-}$) e de peróxido de hidrogênio (H_2O_2), aumentando a produção de EROs (BRIAT; LEBRUN, 1999). Por sua vez, o Zn pode gerar estresse oxidativo através da interferência com o sistema de defesa antioxidante das plantas (GRATÃO et al., 2005). Assim, a absorção excessiva de Cu e de Zn por plantas pode causar estresse oxidativo devido ao desequilíbrio entre as respostas antioxidantes e o aumento da produção de EROs (GIROTTO et al., 2013).

Embora as EROs formem-se naturalmente no interior das células, principalmente nos cloroplastos e mitocôndrias (FERREIRA et al., 2015), sua produção pode ser aumentada drasticamente em condições de altos teores de Cu e Zn no solo. Nestes casos, as EROs podem causar toxicidade através da sua reação com outras moléculas e podem causar mudanças significativas na permeabilidade seletiva de membranas (HERNANDEZ et al, 2001; DE GARA et al., 2003) e na atividade de enzimas ligadas à membrana (DEL RIO et al., 2006). Quando ocorre aumento na formação de EROs, a estratégia utilizada pelas plantas é a ativação do sistema antioxidante enzimático (GILL; TUTEJA, 2010), como pode ser observado na Figura 3, com o intuito de metabolizar as EROs e evitar a ocorrência de danos às membranas celulares, a peroxidação de proteínas e danos ao DNA. Segundo Andrade et al. (2010), plantas que aumentam a produção de enzimas antioxidantes exibem maior tolerância a metais pesados, como o Cu e o Zn.

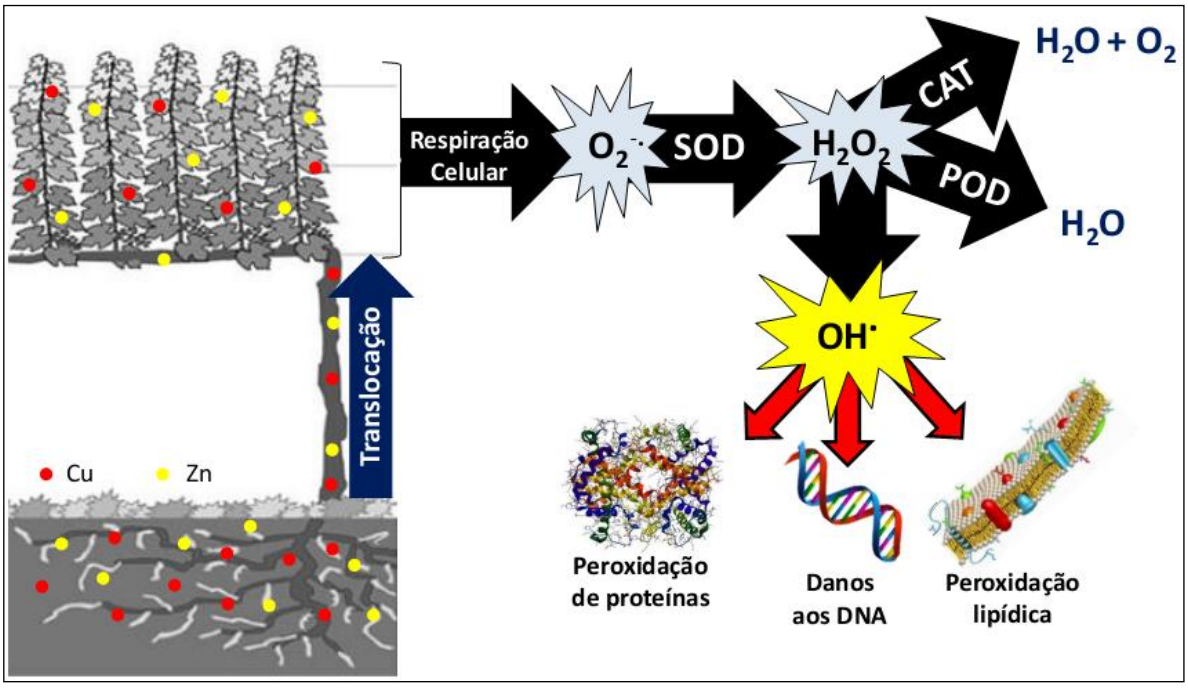

Figura 3: Formação de EROs em plantas de videira cultivadas em solo com altos teores de Cu e Zn, ação das enzimas antioxidantes e efeitos das EROs sobre proteínas, DNA e lipídios.

Dentre as enzimas antioxidantes, as enzimas da família superóxido dismutase (SOD) fazem parte do primeiro ajuste da tolerância das plantas ao estresse oxidativo. Estas enzimas dismutam o radical $O_2^{\cdot-}$, sendo o produto da sua atividade o H_2O_2 (ALSCHER et al., 2002). Embora o H_2O_2 seja menos tóxico que o $O_2^{\cdot-}$, seu excesso na célula também pode causar toxidez e, por isso, faz-se necessária a atuação de outras enzimas para que ocorra a degradação do excesso de H_2O_2 nas células. Sendo assim, as enzimas catalase (CAT) e as peroxidases (POD), podem ter as suas atividades aumentadas para que ocorra a eliminação do H_2O_2, obtendo-se como produtos da atividade destas enzimas a H_2O e o O_2. Baseado nestes mecanismos, diversos estudos de toxicidade ocasionada por Cu e Zn às plantas tem utilizado a atividade destas enzimas antioxidantes como biomarcadores (FERREIRA et al., 2015; GIROTTO et al., 2014, 2016; MIOTTO et al., 2014; SANTANA et al., 2015; TIECHER et al., 2016a, 2016b, 2016c, 2017). Isto porque são avaliações que possuem grande sensibilidade na detecção do estresse ocasionado pelos metais pesados, conferindo uma maior acurácia aos estudos de fitotoxidez.

A alta concentração de Cu nos tecidos da planta pode ainda inibir o seu crescimento por interferência em processos celulares importantes, como a estrutura da cromatina, a síntese de proteínas e a atividade de enzimas nos processos de fotossíntese e respiração (GRATÃO et al., 2005; YRUELA, 2005). No caso do Zn, o aumento da sua concentração na planta pode

reduzir a eficiência fotossintética das plantas pela inibição da biossíntese de clorofila e diminuição da assimilação de carbono (CHEN et al., 2008; DHIR et al., 2008). Além disso, a toxicidade de Zn pode afetar negativamente a permeabilidade da membrana, a cadeia de transporte de elétrons (DE MAGALHÃES et al., 2004) e a absorção e translocação de nutrientes (JIANG; WANG, 2008; WANG et al., 2009). Isto resulta na ruptura dos processos metabólicos, como a transpiração e fotossíntese, responsáveis pela redução do crescimento das plantas (SAGARDOY et al., 2009).

Geralmente é observada redução do conteúdo de clorofila *a*, clorofila *b* e carotenoides em plantas cultivadas em solos com elevados teores de Cu e Zn (SANTANA et al., 2015; TIECHER et al., 2016a, 2016c, 2017). A diminuição destes pigmentos fotossintéticos pode ser atribuída ao efeito adverso do excesso destes elementos no transporte de elétrons na fotossíntese, causando diminuição da síntese de clorofila ou o incremento da sua degradação (CAMBROLLÉ et al., 2012; TIECHER et al., 2016a). Por outro lado, também pode ser observado aumento dos teores de pigmentos fotossintéticos nos tecidos foliares de plantas cultivadas em ambientes com elevadas concentrações de metais pesados (JAIN et al., 2010). Esta resposta provavelmente é causada pela menor produção de massa fresca das folhas e maiores quantidades de pigmentos por unidade de massa (HEWITT, 1983), ou seja, pela ocorrência de um efeito de concentração dos pigmentos fotossintéticos nas folhas. Apesar disso, quando os resultados são expressos em função da área foliar, observa-se redução nos teores de clorofilas e carotenóides por causa do aumento das doses de metais pesados no meio de crescimento das raízes (SAGARDOY et al., 2009).

No processo de fotossíntese das plantas, após os pigmentos fotossintéticos absorverem a energia dos fótons, existem três vias de dissipação desta energia, as quais podem ser visualizadas na Figura 4 e são descritas a seguir: i) dissipação fotoquímica, onde a energia luminosa é utilizada nos processos fotoquímicos da fotossíntese; ii) dissipação não fotoquímica, que é a produção de calor na forma de radiação infravermelha; e iii) fluorescência, que é a emissão da energia na região do visível (CAMPOSTRINI, 2001).

Plantas cultivadas em solos com altos teores de Cu e Zn geralmente apresentam aumento significativo na emissão da fluorescência pelas moléculas de clorofilas das antenas que foram excitadas, antes da transferência da energia para os centros de reação (KRAUSE; WEISS, 1991; TANYOLAÇ et al., 2007). Isso acontece porque o excesso de Cu e Zn nas plantas pode comprometer os centros de reação do PSII, ou a transferência da energia de excitação do complexo antena para os centros de reação pode estar prejudicado (BOLHAR-NORDENKAMPF et al., 1989). Geralmente, a emissão da fluorescência é alterada por

estresses do ambiente que causam alterações estruturais nos pigmentos fotossintéticos do PSII (CAMPOSTRINI, 2001), como a formação de complexos Cu-clorofila nos cloroplastos, os quais causam danos às funções fotossintéticas das plantas (ZVEZDANOVIC et al., 2007).

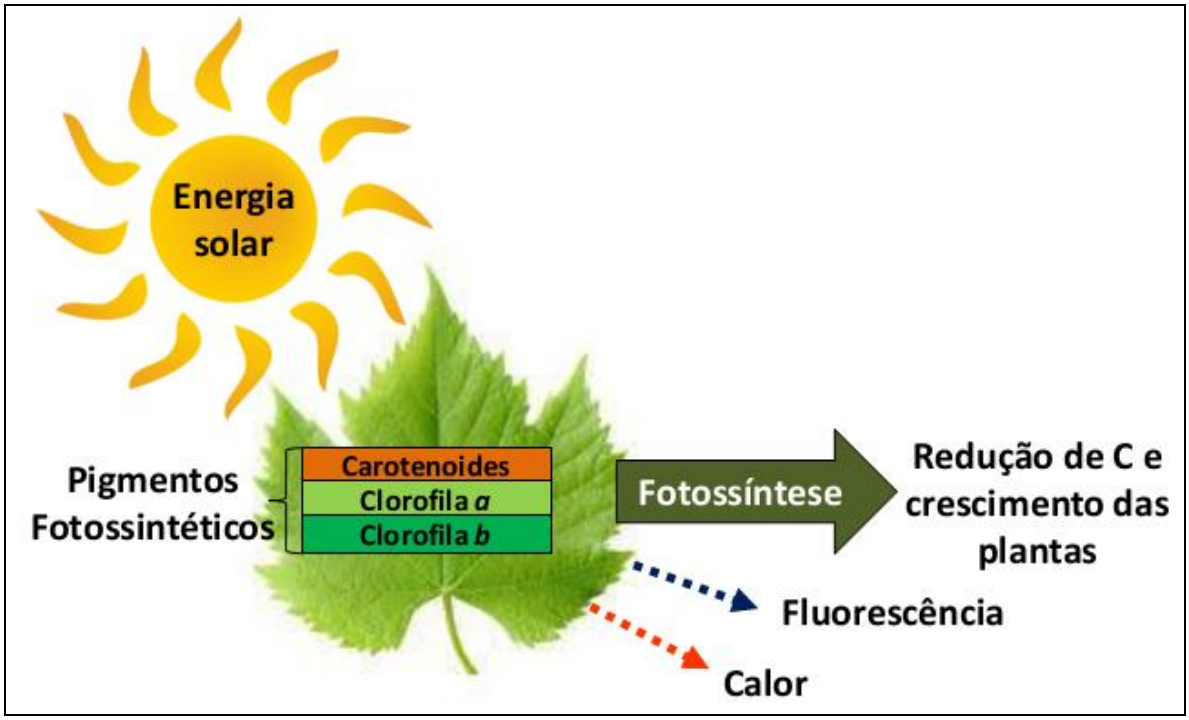

Figura 4: Vias de dissipação da energia solar captadas pelos pigmentos fotossintéticos.

Em condições de estresse, as plantas podem apresentar também redução acentuada na eficiência quântica máxima do PSII (Fv/Fm), o que caracteriza um estado de fotoinibição crônica (ALVES et al., 2002), a qual indica uma redução na proporção de centros de reação abertos (MAXWELL; JOHNSON, 2000). Nestas condições, geralmente ocorre o aumento da dissipação não fotoquímica (NPQ), o que indica que as plantas dissipam a luz na forma de calor, protegendo assim a folha de danos induzidos pela luminosidade (CAMBROLLÉ et al., 2012; MAXWELL; JOHNSON, 2000). Esta dissipação da energia de excitação das clorofilas pode prevenir a formação de moléculas altamente reativas, a partir de oxigênio (EROs), que podem danificar irreversivelmente proteínas, lipídeos e pigmentos de membranas fotossintéticas (HORTON; RUBAN, 2004).

O Cu e o Zn em altas concentrações nas plantas podem interferir negativamente nas trocas gasosas realizadas pelas plantas, reduzindo a sua taxa de fotossíntese líquida, resultando na redução do crescimento das plantas, devido à redução na assimilação de carbono fotossintético (CAMBROLLÉ et al., 2012, 2013, 2015). Geralmente altos teores de

Cu e Zn induzem a efeitos consideráveis sobre a taxa de fotossíntese líquida e condutância estomática. Contudo, não há relação direta entre os dois parâmetros, uma vez que não é observada redução na concentração de CO_2 intercelular (CAMBROLLÉ et al., 2013, 2015). Portanto, a redução da taxa de fotossíntese líquida pode ser atribuída a diferentes efeitos dos metais pesados sobre a integridade ou a função fotoquímica, bem como ao seu impacto sobre as concentrações de clorofila nas folhas (CAMBROLLÉ et al., 2015).

Outro fator que pode explicar a redução da taxa de fotossíntese é que o excesso de Cu e Zn pode diminuir a atividade de enzimas envolvidas na fixação carbono (MYSLIWA-KURDZIEL et al., 2004). Assim, o aumento da concentração de CO_2 intercelular encontrado em plantas expostas às concentrações elevadas de metais pesados pode ser explicada pelas alterações na atividade da enzima RuBisCO. A inibição da atividade desta enzima na presença de metais pesados pode ser devido a substituição de Mg^{2+} por íons metálicos, especialmente o Zn, no sítio ativo de subunidades de RuBisCO (SIEDLECKA; KRUPA, 2004), o que resulta em diminuição na atividade da RuBisCO carboxilase.

4.3 ALTERAÇÕES MORFOLÓGICAS NO SISTEMA RADICULAR DE PLANTAS, OCASIONADAS PELOS ALTOS TEORES DE COBRE E ZINCO NO SOLO

As plantas possuem mecanismos constitutivos e adaptativos para acumular ou tolerar altas concentrações de metais pesados na rizosfera (PAL; RAI, 2010). Uma estratégia comum de tolerância das plantas aos metais pesados é evitar a absorção excessiva e transporte de íons metálicos (KABATA-PENDIAS, 2011). Sabe-se que a exsudação de substâncias quelantes de metais é outra forma eficaz de reduzir a absorção e, assim, a toxicidade de metais pesados (PAL; RAI, 2010). Estas estratégias são de grande importância para as plantas cultivadas em solos com altos teores de Cu e Zn, pois uma vez no interior da planta, o Cu e o Zn em excesso, podem alterar o equilíbrio fisiológico em locais como os sítios de absorção primária ou nos locais de transporte de nutrientes das raízes (YANG et al., 2011).

As respostas das plantas em condições de estresse, como doses tóxicas de metais pesados, são muitas vezes semelhantes entre diferentes espécies (BOCHICCHIO et al., 2015). Isto porque a resposta morfogênica induzida pelo estresse é mediada por processos moleculares comuns, como o aumento de produção de EROs e alteração do equilíbrio de fitormônios (POTTERS et al., 2007). Dentre os hormônios, as auxinas apresentam grande relevância no desenvolvimento do sistema radicular (OVERVOORDE et al., 2010) e o aumento da disponibilidade de Zn no meio de crescimento das raízes pode estimular a sua

síntese nas plantas (HENRIQUES et al., 2012). Desta forma, um distúrbio da homeostase da auxina, provocado pelo excesso de Zn nas plantas, pode ter efeitos relevantes sobre o crescimento e o desenvolvimento das raízes, resultando em uma forte redução no número de raízes laterais (QUINT et al., 2009).

As altas concentrações de Cu ou Zn nas plantas são frequentemente apontadas como responsáveis por alterações morfológicas do seu sistema radicular. Contudo, quando as plantas se desenvolvem em ambientes com a combinação de altos teores de Cu e Zn, que resultam em elevadas concentrações de ambos os elementos nos seus tecidos, geralmente ocorre um efeito sinérgico, sendo observadas alterações mais significativas na morfologia das raízes das plantas que ali se desenvolvem (BOCHICCHIO et al., 2015; SOFO et al., 2013). O Cu e o Zn absorvidos pelas raízes são lentamente translocados para a parte aérea e, assim, acumulam-se nas raízes, onde podem exercer efeitos adversos às plantas (BOCHICCHIO et al., 2015; SOFO et al., 2013).

Dentre as alterações morfológicas, o aumento do diâmetro médio das raízes é frequentemente relatado em plantas cultivadas em solos com excesso de Cu e Zn (AMBROSINI et al., 2015; BOCHICCHIO et al., 2015). Isto pode ocorrer porque estes elementos se acumulam na endoderme e no periciclo da raiz, alterando a distribuição de auxina ou o equilíbrio hormonal (GIEHL et al., 2012), podendo ocasionar alterações no desenvolvimento da raiz, como a diferenciação prematura da endoderme e a lignificação dos tecidos corticais (ARDUINI et al., 1995). Esta diferenciação das células da endoderme pode resultar no espessamento das paredes celulares, o que pode ser uma estratégia de defesa das plantas, pois os metais pesados precisam atravessar a endoderme, via transportadores de membrana plasmática, para alcançar o xilema e então ser transportado para a parte aérea via apoplasto, o que contribui para a redução na translocação destes elementos.

Estas alterações podem se refletir em redução na absorção de nutrientes e água, provocando inibição do crescimento e menor incremento de biomassa de raízes e da parte aérea das plantas (KOPITTKE et al., 2009; TOSELLI et al., 2009). Os principais sintomas encontrados na anatomia de raízes de plantas cultivadas em solos com elevados teores de metais pesados são uma maior diferenciação das células, redução do tamanho das células meristemáticas e o encurtamento da região meristemática no ápice das raízes (AMBROSINI et al., 2015; GUIMARÃES et al., 2016). Isto pode estar relacionado às alterações no índice mitótico, reduzindo a frequência de divisão celular no ápice das raízes (JIANG et al., 2001).

Estas plantas também podem apresentar redução no tamanho da coifa das raízes, o que pode ser ocasionado pela redução na taxa de divisão celular na raiz, como foi observado por

Guimarães et al. (2016). Com a redução da coifa, todo o desenvolvimento da raiz pode ser comprometido, uma vez que as células desta região protegem o meristema apical e facilitam a penetração da raiz do solo, dentre outras funções (LYNCH et al., 2012). Além disso, a redução da multiplicação celular na região do meristema primário, no ápice radicular, é compensada pela maior multiplicação de células na região de alongamento distal, com rediferenciação do periciclo e consequente aumento no número de raízes laterais, na tentativa de encontrar um ambiente mais adequado para o desenvolvimento radicular (POTTERS et al., 2007).

Outros sintomas, como a deformação e a plasmólise de células do córtex das raízes e a desintegração das células da epiderme, também já foram relatados em trabalhos com exposição de plantas a altos teores de metais pesados (CHEN et al., 2013; JUANG et al., 2014). A plasmólise geralmente é constatada pela coloração marrom das raízes, que apresentam essa cor por causa da oxidação de fenóis liberados dos vacúolos da epiderme, como efeito dos metais pesados ao sistema radicular (AMBROSINI et al., 2015). O acúmulo de compostos fenólicos nas raízes das plantas inibe e elimina as EROs que são produzidas em condição de estresse (GRAEL et al., 2010), como a exposição a altas concentrações de metais pesados (YRUELA, 2009). Portanto, o maior acúmulo de compostos fenólicos nas raízes das plantas, geralmente, é atribuído a uma estratégia de defesa contra o estresse causado pelos metais pesados (CIPRIANI et al., 2013).

5. RESULTADOS

5.1 ESTUDO I

Physiological and nutritional status of black oat (*Avena strigosa* Schreb.) grown in soil with interaction of high doses of copper and zinc

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Physiological and nutritional status of black oat (*Avena strigosa* Schreb.) grown in soil with interaction of high doses of copper and zinc [1]

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Abstract

Vineyard sandy acid soils from South Brazil have experienced heavy metal contamination due to replacement of copper (Cu)-based by zinc (Zn)-based products to control foliar diseases. Thus, we evaluate physiological and nutritional status of black oat (*Avena strigosa* Schreb.), a common interrow crop in vineyards from this region. Soil was collected in a natural field from Santana do Livramento, in Rio Grande do Sul, the southernmost state of Brazil. Black oat was cultivated for 30 days in a greenhouse with application of 0, 30, and 60 mg Cu kg^{-1} combined with 0, 15, 30, 60, 120, and 180 mg Zn kg^{-1}. After the trial period, dry matter accumulation of roots and shoots, Cu and Zn contents in roots and shoots, chlorophyll *a* fluorescence, photosynthetic pigments and catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7) activity were determined. Cu and Zn toxicity was evidenced by the decrease in plant growth of black oat as well as by the decrease of photochemical efficiency associated with the decrease in photosynthetic pigment content, especially with the highest doses of Cu and Zn. Furthermore, the activity of antioxidant enzymes (CAT and POD) was increased in intermediate doses of Zn, indicating the activation of the antioxidant system, but the stress condition in treatments with high levels of Cu and Zn was not reversed.

Keywords: Acid soils. Cu- and Zn-based fungicides. Potentially toxic elements. Antioxidant enzymes. Photosynthetic pigments.

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1. Introduction

Viticulture is in full expansion in Brazil, especially in the state of Rio Grande do Sul, where there are currently about 50,000 hectares of vines, representing approximately 62% of the cultivated area in the country (IBGE, 2012). There has been significant growth of the Campanha Gaúcha region in the wine industry from the 2000s (Girotto et al., 2014). In viticulture in southern Brazil, frequent applications of Cu-based fungicides are traditional for the control of foliar diseases which often leads to increases in soil Cu content to levels which are toxic to plants (Miotto et al., 2014; Girotto et al., 2014; Cambrollé et al., 2015; Santana et al., 2015). As a result, producers began using Zn-based fungicides instead. As the Bordeaux mixture also contributes on a smaller scale to the increase in levels of other potentially toxic elements in the soil such as Zn (Mirlean et al., 2007), the occurrence of high levels of both Cu and Zn has been observed in vineyard soils. This fact was also reported in the 1980s by Magalhães et al. (1985) and it has had more notoriety in the last decade (Fernandez-Calviño et al., 2012; Brunetto et al., 2014; Couto et al., 2014; Tiecher et al., 2016a).

Cu and Zn in soil are retained by physical-chemical bonds and their lability is dependent on the ligand. In general, the sorptive capacity of a soil is higher, the higher the contents of clay minerals, Fe, Al and Mn oxides and hydroxides, carbonates and soil organic matter (SOM) (McBride, 1994 Bradl, 2004). The main soil in the Campanha Gaúcha region is Typic Hapludalf with the predominance of type 1:1 clay mineral, sandy texture and low SOM content (Streck et al., 2002). Thus, SOM content is one of the main factors that can be managed in the field to reduce the toxic effects of Cu and Zn to plants. It can be maintained or even increased by growing cover crops such as black oat (*Avena strigosa* Schreb.) in rows and in between rows of vineyards in winter (Girotto et al., 2014; Tiecher et al., 2016a).

Cu and Zn are micronutrient to plants, performing structural and enzymatic activation functions (Pilon et al., 2006; Kabata-Pendias, 2011). Cu is involved in metabolic processes such as photosynthesis, respiration, carbohydrate metabolism and reproduction (Kabata-Pendias, 2011), while Zn is associated with carbohydrate metabolism, regulation of gene expression, structural integrity of the ribosome and phosphate metabolism, in addition to synthesizing enzymes such as the dehydrogenases, proteinases and peptidases (Cherif et al., 2011; Kabata-Pendias, 2011).

However, plants grown in soils with high available Cu and Zn contents may excessively accumulate these elements in their tissues (Kabata-Pendias, 2011), resulting in changes in membrane properties, which in turn affects the function of membrane transporters and ion channels (Janicka-Russak et al., 2008). There may also be increased non-specific

membrane permeability, resulting in imbalances in concentrations of nutrients in plants grown in these adverse environments (Cambrollé et al., 2013). Negative responses in biochemical and physiological levels resulting from such disorders vary according to plant organ, element concentration and tissue tolerance to high levels of these elements (Mateos-Naranjo et al., 2013).

The absorption of excess Cu and Zn may cause oxidative stress due to imbalance between antioxidant responses and increased production of reactive oxygen species (ROS) (Girotto et al., 2013; Tiecher et al., 2016a). Through the Fenton reaction, the redox cycle between Cu^{2+} and Cu^{+} catalyzes the production of hydroxyl radicals (OH^{-}) from superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2) radicals, which enhances the harmful effects of these reactive species (Briat & Lebrun, 1999). High plant Cu content may also inhibit growth by interfering with important cellular processes in chromatin structure, in protein synthesis and enzyme activity in the processes of photosynthesis and respiration (Yruela, 2005; Gratão et al., 2005).

On the other hand, Zn may generate oxidative stress by interfering with the antioxidant defense system of plants (Gratão et al., 2005). The increase in plant Zn content may negatively affect photosynthetic efficiency by inhibiting chlorophyll biosynthesis and decreasing carbon assimilation (Chen et al., 2008; Dhir et al., 2008) and may reduce the rooting ability and even cause leaf chlorosis (Castiglione et al., 2007), which contributes to the inhibition of plant growth (Girotto et al., 2013). Furthermore, Zn toxicity may compromise the electron transport chain (De Magalhães et al., 2004).

Some studies have explored how Cu or Zn added to nutrient solution or sand interfere isolatedly in a variety of plant physiological processes (Jain et al., 2010; Cambrollé et al., 2012; Mateos-Naranjo et al., 2013; Cambrollé et al., 2015), while other studies on nutrient solution report the effects of the interaction between these two potentially toxic elements on the antioxidant defense system of plants (Upadhyay & Panda, 2010). However, there are few studies in the literature on the interaction between Cu and Zn in soil with high accumulation of these two elements in vineyard environments installed in sandy soils with low SOM content and its effects on cover crops such as black oat often found in vineyards. Therefore, this study was carried out aiming to evaluate physiological and nutritional status of black oat plants grown in soil with the interaction of high contents of Cu and Zn.

2. Material and methods

2.1. Description of the experiment

The soil used was a Typic Hapludalf (Soil Survey Staff, 2006) of an area of non-anthropic natural field (30º47'23.5"S and 55º22'7.0"W) with naturally low concentration of Cu and Zn, adjacent to a vineyard of the Campanha Gaúcha region in the municipality of Santana do Livramento (RS), located in Rio Grande do Sul, the southernmost state of Brazil. The soil was collected in the 0.00 to 0.15 m layer in November 2011. Subsequently, the soil was air-dried, ground, passed through a 2 mm mesh sieve and reserved. The physical and chemical characterization of the soil is shown in Table 1.

The soil was divided into 10 kg pots and then soil acidity was corrected with the addition of 670 and 830 mg kg^{-1} of $CaCO_3$ and MgO, respectively, to rise soil pH to 5.5. Correctives were added and mixed with the soil and then 100 ml kg^{-1} of distilled water was added to each pot. All content was immediately homogenized and incubated for a period of 60 days. Afterwards, the soil was dried, homogenized, and 40 mg P kg^{-1} and 100 mg K kg^{-1} were added in the form of triple superphosphate and KCl, respectively, and 100 ml kg $^{-1}$ of distilled water was added again. Finally, the soil was subjected to a 30-day incubation period. During the incubation period of correctives and fertilizers the soil remained in sealed plastic bags to prevent water evaporation.

Subsequently, the entire volume of soil was dried, homogenized and separated into 4 kg pots with six replications per treatment and randomized design. Afterwards, doses of 0, 30, and 60 mg Cu kg^{-1} were applied. The soil was then subdivided for each dose and 0, 15, 30, 60, 120, and 180 mg Zn kg^{-1} were added, totaling 18 treatments, totaling 108 pots (six replications per treatment). The statistical model used was a completely randomized bifactorial model. Cu and Zn doses were defined based on previous studies performed with maize (*Zea mays* L.) (Tiecher et al. 2016a), as well as from studies involving competitive adsorption of Cu and Zn (Tiecher et al. 2016b). The doses of Zn were added to the soil in the form of a solution containing $ZnSO_4.7H_2O$ (60%) and $ZnCl_2$ (40%), while the Cu doses were added in the form of $CuSO_4.5H_2O$. The solution was diluted with distilled water to obtain a final volume of 400 mL per pot and applied individually in the soil of each pot, which was then upturned so that homogenization occurred. The soil was incubated in a greenhouse for 180 days, with replacement of the evaporated water every 2 days to maintain humidity at 80% of the maximum water retention capacity (MWRC).

After incubation, black oat was sown with 25 seeds per pot, and at five days after emergence (DAE) 18 plants were kept per pot through 30 days of cultivation. Soil moisture was maintained at 80% of the MWRC with daily irrigation to replenish evaporated water and a 50 ml of a solution containing 50 mg N kg^{-1} was applied at 15 DAE.

2.2. Soil available Cu and Zn analyses

Contents of Cu and Zn available to plants were extracted with EDTA (Chaignon et al., 2009). Samples of 0.25 g of soil were added to 10 mL of solution containing 0.01 mol L^{-1} dissodic EDTA + 1.0 mol L^{-1} ammonium acetate, pH adjusted to 7.0, and placed into 15 mL centrifuge tubes, which were shaken for 2 h. After stirring, the suspension was centrifuged and filtered, and the concentrations of Cu and Zn were determined by flame atomic absorption spectrometry (AAS).

2.3. Dry matter production and content of Cu and Zn in roots and shoot of black oat plants

At 30 DAE, plants were uprooted manually, and roots washed in running water, immersed in EDTA (0.02 mol L^{-1}) for 1 min and washed with distilled water three times (Miotto et al., 2014). The shoots were cut close to the soil surface, and a leaf sample was immediately added to liquid N_2 and stored in an ultra-freezer at -80°C for biochemical analyses. The remaining leaf and root samples were dried in a forced-air oven (±65°C) until reaching constant weight.

An aliquot of the dried shoot and root samples were ground in a Wiley mill, and the total Cu and Zn concentrations were assessed after digesting the samples in a muffle furnace. The samples were calcined at 500–550 °C for 3 h, and the resulting ashes were diluted in a solution with 1 mol L^{-1} of ultrapure HNO_3. The total Cu and Zn concentrations were then analyzed by atomic absorption spectrophotometry (AAS; Varian SpectrAA-600, Australia).

2.4. Assessment of chlorophyll *a* fluorescence

The chlorophyll *a* fluorescence was analyzed at 30 DAE using a pulse amplitude modulated fluorometer JUNIOR-PAM (Walz, Effeltrich, Germany). The fluorescence was measured in the first fully expanded leaf in three plants per treatment during the period between 8:00 and 9:30 am (Souza et al., 2013). Prior to the measurements, the leaves were pre-adapted to the dark for 30 min to assess the initial fluorescence (Fo). Subsequently, the sample was subjected to a saturating light pulse (10,000 μmol m^{-2} s^{-1}) for 0.6 s to assess the

maximum fluorescence (Fm). The PSII maximum quantum yield (Fv/Fm), electron transport rate (ETRm), and PSII effective quantum yield (Y(II)) were assessed using the fluorescence induction curve.

2.5. Assessment of photosynthetic pigments

The leaves used for assessing the chlorophyll *a* fluorescence were collected for the analysis of the photosynthetic pigments when the plants were collected at 30 DAE. The concentrations of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) as well as the carotenoid levels were assessed according to the methodology described by Hendry and Price (1993) using 80% acetone with three samples per treatment. The pigment concentrations were calculated after reading the absorbances at 480, 645 and 663 nm according to the methodology by Lichtenthaler (1987).

2.6. Assessment of the enzymatic activity of catalase (CAT) and non-specific peroxidases (POD)

The crude enzymatic extract was prepared from leaf tissue (0.5 g) macerated using liquid N_2 and homogenized with 5.0 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 1.0 mM EDTA, 3.0 mM dithiothreitol (DTT) and 2% polyvinylpolypyrrolidone (PVPP) (m/v) (Azevedo et al., 1998). The homogenate was centrifuged at 14,000 g for 30 min at 4 °C, the supernatant was collected, and 0.5 mL aliquots were stored in a freezer at −80 °C until enzyme and total protein quantification. The total protein concentration of each sample was assessed in a spectrophotometer at 595 nm as described by Bradford (1976) using bovine serum albumin (BSA) as standard.

The activity of CAT (EC 1.11.1.6) was determined according to the spectrophotometric method described by Azevedo et al. (1998). The reaction mixture consisted of 1.9 mL of 100 mM potassium phosphate buffer (pH 7.5) and 20 mM H_2O_2 prepared immediately before use. The reaction was initiated by adding 100 µL of enzyme extract, and the activity was determined by the decomposition of H_2O_2 as the decrease in absorbance at 240 nm for 1 min (25 °C). The results were expressed as µmol min^{-1} mg^{-1} of protein, using the molar extinction coefficient of 40.0 M^{-1} cm^{-1} for the calculations.

The activity of non-specific peroxidases (POD, EC 1.11.1.7) present in the extract was assessed according to Zeraik et al. (2008) using guaiacol as the substrate and a molar extinction coefficient of 26.6 mM^{-1} cm^{-1} according to Chance and Maehley (1955). One unit

of POD was defined as the amount of enzyme that catalyzes the formation of 1mol of tetraguaiacol min^{-1} mL^{-1} extract at 470 nm.

2.7. Statistical analysis

First of all, experimental data were tested for normality and homogeneity of variance through Lilliefors and the Shapiro-Wilk tests. Subsequently, the data was submitted to analysis of variance, using the following completely randomized bifactorial statistical model:

$$Y_{ijk} = \mu + C_i + error\ a\ (i) + Z_j + error\ b\ (j) + CZ_{ij} + error\ c\ (i, j)$$

where μ = overall mean; C = Cu dose applied (i = 1, 2, 3); Z = Zn dose applied (j = 1, 2, 3, 4, 5, 6) and error = experimental error. When the interaction of the factors, doses of Cu and Zn was significant, the means were adjusted by polynomial regression.

3. Results

3.1. Soil available Cu and Zn

Soil available Cu and Zn showed no significant interaction between the Cu (p = 0.410) and Zn (p = 0.718) doses (Table 2). The higher the dose of Cu added to the soil, the higher the soil available Cu (p <0.0001, Figure 1a), without interference of Zn doses. Available Cu content was on average for all Zn doses (n = 36) 2.4±0.2, 22.6±3.1 and 41.9±2.1 mg kg^{-1} in the treatments with application of 0, 30 and 60 mg Cu kg^{-1}. In the same way, soil available Zn content showed a linear increase (p <0.0001, Figure 1b) with increasing Zn doses added to the soil, regardless the Cu dose, increasing from 18±0.3 mg kg^{-1} in the dose 0 mg Zn kg^{-1} to 141±5.2 mg kg^{-1} in the dose 180 mg Zn kg^{-1} (n = 18).

3.2. Plant growth and Cu and Zn concentration in roots and shoots

The interaction between Cu and Zn contributed to the reduction in dry matter production, especially in shoots (p <0.0001) of black oat plants (Figure 2a). The greatest reductions in dry matter production were observed with the highest Zn doses applied to the soil containing 60 mg Cu kg^{-1}. On the other hand, treatments without the addition of Cu presented the highest shoot dry matter production. For all the Cu doses, treatments with the addition of 15 mg Zn kg^{-1} had higher shoot dry matter production compared to treatments without the application of Zn.

Root dry matter production (Figure 2b) decreased, especially in treatments with the addition of 60 mg Cu kg^{-1} and 120 and 180 mg Zn kg^{-1}, respectively, compared to the other treatments. In treatments without the addition of Cu, a reduction in root dry matter production can be observed, especially in the treatment that received the dose of 180 mg Zn kg^{-1}.

Cu content in all treatments was higher in the tissue of leaves and roots (Figure 2c and 2d), the higher the Cu dose applied to the soil was, for the same amount of Zn applied to the soil. In treatments with the addition of 30 and 60 mg Cu kg^{-1} an increase in Cu concentration in shoot tissue was observed from the dose of 30 mg Zn kg^{-1} (Figure 2c), while in treatments without the addition of Cu an increase was observed only at the dose of 180 mg Zn kg^{-1}. Cu content in the root tissue (Figure 2d) in treatments with the addition of 30 and 60 mg Cu kg^{-1} was higher in treatments that received doses of 120 mg Zn kg^{-1} and 180 mg Zn kg^{-1}. Although soil available Cu content was only 42±2 mg kg^{-1} (Figure 1a) in the higher Cu dose (60 mg Cu kg^{-1}), Cu root content in this treatment ranged from 484±65 to 713±155 mg kg^{-1} (Figure 2d) whereas maximum Cu shoot content was 24±1 mg kg^{-1} (Figure 7c)

Zn content in the shoots showed a linear increase (Figure 2e) with increasing Zn doses added to the soil (p <0.0001), and the higher the dose of Cu added to the soil, the greater the Zn shoot content. This linear upward trend was also observed in Zn content in root tissue without Cu interference (p <0.0001, Figure 2f).

3.3. Translocation of Cu and Zn from root to shoot biomass

The ratio shoot/root for Cu and Zn concentration were affected by Cu doses (p <0.0001 and p <0.001, respectively, Table 2), with no interference of Zn doses. As a result, the proportion of total amount of Cu and Zn (concentration in tissue × dry matter) in shoot and root were affected only by Cu doses as well (p <0.0001 and p <0.05, respectively, Table 2). Ratio of shoot/root Cu concentration was 0.26±0.02, 0.06±0.01 and 0.04±0.01 (n = 36) for treatments with 0, 30 and 60 mg Cu kg^{-1} (Figure 3a). Ratio of shoot/root Zn concentration was 0.41±0.09, 0.67±0.05 and 0.98±0.16 (n = 36) for treatments with 0, 30 and 60 mg Cu kg^{-1} (Figure 3a). The amount of Cu in shoot biomass of black oat decreased from 47±4, 14±3 and 7±1% (Figure 3b), while Zn in shoot biomass increased from 58±5, 65±3 and 67±2% (Figure 3c) for treatments with 0, 30 and 60 mg Cu kg^{-1}.

3.4. Chlorophyll *a* fluorescence

The lowest values of the maximum quantum yield of PSII (Fv/Fm) were observed in treatments with the addition of 60 mg Cu kg^{-1} plus 120 and 180 mg Zn kg^{-1}, with reductions

of 21 and 14% when compared to the treatment without the addition of Zn, respectively (Figure 4a). The initial fluorescence (Fo) also showed reduced values in treatments with the addition of 60 mg Cu kg^{-1} plus 120 and 180 mg Zn kg^{-1} (Figure 4b), compared to treatments with the same Cu dose and lower Zn doses. As for the treatments without the addition of Cu, the lowest Fo values were found in treatments with the addition of 30, 60 and 120 mg kg^{-1} Zn.

The electron transport rate (ETRm) was lower with the addition of 60 mg Cu kg^{-1} plus 120 and 180 mg Zn kg^{-1}, with a reduction of 284 and 467% in comparison to the treatment without Zn addition, respectively (Figure 4c). Reduction in values of this variable was also observed in the treatment without the addition of Cu and with the addition of 180 mg Zn kg^{-1} in relation to other treatments without Cu addition. Among treatments with the addition of 30 mg Cu kg^{-1}, the highest ETRm value was found at a dose of 120 mg Zn kg^{-1}.

The highest effective quantum efficiency (Y(II)) was found in treatments without the addition of Cu and with the addition of 30, 60 and 120 mg Zn kg^{-1} (Figure 4d), causing a reduction in the value of treatment with the addition 180 mg Zn kg^{-1}. With the addition of 60 mg Cu kg^{-1}, Y(II) values remained stable in treatments with the addition of 0, 15, 30 and 60 mg Zn kg^{-1}, and the lowest value was observed in the treatment with the addition of 120 mg Zn kg^{-1}.

3.5. Photosynthetic pigments

The plants grown in soil with the addition of 30 mg Cu kg^{-1} showed a decrease in chl *a* content with increasing doses of Zn added to the soil (Figure 5a). With the addition of 60 mg Cu kg^{-1} plus 120 and 180 mg Zn kg^{-1}, the chl *a* reduced to 46 and 68%, respectively of the observed in the treatment without Cu. Contents of chl *b* and carotenoids showed similar trend in treatments with the addition of 60 mg Cu kg^{-1} (Figure 5b and 5c). Furthermore, a great reduction in the levels of these pigments was observed in plants with the addition of 120 mg Zn kg^{-1} compared to treatments with lower doses of Zn. As for the treatment with the addition of 60 mg Cu kg^{-1} + 180 mg Zn kg^{-1}, increased levels of chl *a* and *b* and carotenoids were observed in comparison to the treatment with the addition of 60 mg Cu kg^{-1} + 120 mg Zn kg^{-1}.

3.6. Activity of antioxidant enzymes

Catalase (CAT) activity showed an increase up to a dose of 120 mg Zn kg^{-1} in treatments without the addition of Cu (Figure 6a), but at the highest dose of Zn (180 mg Zn kg^{-1}) it decreased 112% in relation to the 120 mg Zn kg^{-1} treatment. Similarly, among

treatments with the addition of 30 mg Cu kg^{-1}, the treatment with the addition of 120 mg Zn kg^{-1} had the highest CAT activity with a reduction in this activity in the treatment with the addition of 180 mg Zn kg^{-1}. As for the treatments with the addition of 60 mg Cu kg^{-1} an increase in CAT activity was observed up to a dose of 30 mg Zn kg^{-1}, with subsequent reduction in treatments with the addition of 60, 120 and 180 mg Zn kg^{-1}.

Peroxidase (POD) showed similar activity with doses of 0, 15, 30 and 60 mg Zn kg^{-1} in soil without the addition of Cu (Figure 6b). The treatment with the addition of 120 mg Zn kg^{-1} presented the highest activity among treatments, but a reduction of 112% was observed at a dose of 180 mg Zn kg^{-1} compared to this treatment. Among treatments with application of 30 mg Cu kg^{-1} an increasing trend in POD activity was observed in line with the increase in the Zn dose applied to the soil. In treatments with the addition of 60 mg Cu kg^{-1} an increase in POD activity was observed in treatments with the addition of 15 and 30 mg Zn kg^{-1}, and the lowest activity among these treatments was observed in the treatment with the addition of 120 mg Zn kg^{-1}.

4. Discussion

The highest shoot dry matter production in treatments that received only the addition of Zn compared to the control treatment (without the addition of Cu and Zn) demonstrates the importance of Zn as a nutrient for plant growth. It is known that Zn concentration in plants acts in carbohydrate metabolism, regulation of gene expression, the structural integrity of the ribosome and phosphate metabolism, in addition to the synthesis and activation of enzymes (Cherif et al., 2011; Kabata-Pendias, 2011). However, the increase or maintenance of root and shoot dry matter production of black oat grown in soil with the addition of Cu (30 and 60 mg Cu kg^{-1}) in treatments with the addition of low doses of Zn (15 and 30 mg Zn kg^{-1}) may have occurred due to low free Zn concentrations in the soil solution of these treatments, because part of the element added to the soil may have been adsorbed to the functional groups of organic and mineral soil particles (Croué et al., 2003, Brunetto et al., 2014).

The sorption kinetics of these elements when added simultaneously in the soil is different from that observed when only one of them is deposited in the soil (Arias et al., 2006), which favors an increase in content of both elements in more labile fractions. However, in the present study, there was no interference in the soil available Zn content due to the addition of Cu doses, and in the same way, soil available Cu content was not affected by Zn doses. This might have occurred because the doses of both elements added to the soil were not high enough to saturate the adsorption sites for these elements in the soil. As showed

by Tiecher et al (2016b) in a competitive adsorption assay, at higher doses of Cu and Zn added to the soil there is a decrease in Zn adsorption as Cu addition increase, and consequently, increase Zn availability. It occurs because Cu has high reactivity with the functional groups of soil organic matter that contains S and N, in addition to carboxyl and phenolic groups (Croué et al., 2003), which results in high bonding energy and low desorption capacity (Brunetto et al., 2014).

Zn toxicity was less evident under low levels of Cu (0 and 30 mg Cu kg^{-1}). On the other hand, when there is interaction of high Cu and Zn doses, as in treatments with the addition of 60 mg Cu kg^{-1} plus 120 and 180 mg Zn kg^{-1}, the metal (Cu and Zn) toxicity to plants is evidenced by results of shoot and root dry matter, Fv/Fm, Fo, ETRm, Y(II), chlorophyll *a* and *b*, carotenoid and catalase. It demonstrate that the effect of the added copper was to increase the toxicity of the added zinc, as reported by Luo & Rimmer (1995). Previous studies in corn plants grown in soil contaminated with Cu and subjected to application of Zn doses showed similar effect (Tiecher et al., 2016a). Inhibition of cell elongation and division caused by the high concentration of Cu and Zn in the soil solution may help to explain the reduction in dry matter production (Hewitt, 1983; Arduini et al., 1994; Jain et al., 2010).

Cu and Zn contents in shoots of black oat plants increased in line with higher doses applied to the soil, and this has also been observed in other studies with different plant species (Jain et al., 2010; Cambrollé et al., 2012; Girotto et al., 2014; Tiecher et al., 2016a). It is known that in sensitive species leaf content between 15-20 mg Cu kg^{-1} and 150-200 mg Zn kg^{-1} causes reduction in plant growth (Kloke et al., 1984; Kabata-Pendias, 2011). In this study , plants grown in all treatments with the addition of 60 mg Cu kg^{-1} presented Cu leaf content above 15 mg kg^{-1}, and only the treatment with 0 mg Zn kg^{-1} + 60 mg Cu kg^{-1} showed a value below 20 mg Cu kg^{-1}, reducing, therefore, plant growth as we can see by results of shoot dry matter production (Figure 2a). Zn content in shoots was less than 200 mg kg^{-1} only in the treatments with 0 mg Zn kg^{-1} (in the three Cu doses), in treatments with the addition of 0 mg Cu kg^{-1} plus 15 and 30 mg Zn kg^{-1}, and 30 mg Cu kg^{-1} plus 15 mg Zn kg^{-1}. Unlike the literature Cu toxicity limits available in literature, results of dry matter production obtained in this study indicated that the limit for Zn in shoots maybe a little higher than 200 mg Zn kg^{-1} for black oat.

The high concentrations of Zn in shoots of black oat can also be attributed to the similarity of the ionic radii of bivalent cations, such as Cu itself, as well as manganese (Mn) and iron (Fe). Thus, Zn ions can replace any of these bivalent cations and be absorbed by the roots (Tewaru et al., 2008). Once inside the plant, excess Zn can alter the physiological

balance by competition with the other cations at various locations (Tewaru et al., 2008) such as the primary absorption sites or the nutrient transport sites of the roots (Yang et al., 2011).

Cu enhanced Zn uptake and translocation, while Cu in roots increase 10 to 20 fold but without translocation into shoots, Zn was taken up into both root and shoot at 10 fold the soil concentrations. Similar results are reported by Luo & Rimmer (1995) for barley (*Hordeum vulgare* L.), who observed a dominant effect of synergism, in which Cu additions increased Zn uptake, in spite of Zn-Cu interaction being often described as an antagonism (Kabata-Pendias, 2011). Studding another grass (wheat - *Triticum aestivum* L.), Tani & Barrington (2005) also report that increasing levels of Cu significantly increased Zn uptake by the plant shoots, grain and roots, while the higher level of Zn only slightly decreased Cu uptake.

Unlike for Zn, Cu accumulation occurred predominantly in the roots of black oat, without there being any proportional translocation to the shoots. These results are consistent with those found in black oat grown in vineyards soils contaminated with Cu (Girotto et al., 2014) and for other plant species grown in nutrient solution with high concentration of Cu (Cambrollé et al., 2013; Cambrollé et al., 2015). These results highlights the lower translocation of Cu compared to Zn in the plant system. The root system of black oat plants has mechanisms may prevent and/or reduce translocation of excess Cu to the shoots (Girotto et al., 2014). Moreover, the strong interaction of Cu with protein nitrogen also contributes to its lower translocation capability in the plant system (Kabata-Pendias, 2011).

The stress in black oat can also be observed by the reduction in the maximum quantum efficiency of PSII (Fv/Fm) and electron transport rate (ETRm), especially in treatments with the addition of 60 mg Cu kg^{-1} plus the addition of 120 and 180 mg Zn kg^{-1}, respectively, which characterizes a state of photoinhibition (Alves et al., 2002) and may be related to the reduction in chlorophyll content in plant leaves (Cambrollé et al., 2015). With the reduction Fv/Fm and ETRm, a smaller amount of energy taken up by the plant through the antenna complex is used to reduce carbon and produce dry matter, which helps to explain the low dry matter production in these treatments. Several authors relate the reduction in the Fv/Fm ratio to stress caused to plants of different species grown in an environment with an excess of potentially toxic elements (Cambrollé et al., 2012; Cambrollé et al., 2015; Santana et al., 2015; Tiecher et al., 2016a).

A decrease of Fo values was observed in treatments with the addition of 60 mg Cu kg^{-1} plus the addition of 120 and 180 mg Zn kg^{-1}, representing the emission of fluorescence by chlorophyll molecules of the antennas that were excited before the transfer of energy to the reaction centers (Krause & Weiss, 1991; Tanyolaç et al., 2007). Generally, the Fo value is

changed by environmental stresses, such as high levels of potentially toxic elements in the soil, that cause structural changes in the photosynthetic pigments of PSII (Maxwell & Johnson, 2000). As complementary dissipation processes, when there is increased energy dissipation in the form of fluorescence, as a result reduced energy dissipation via photochemical process will occur, with reduction in the formation of ATP and NADPH and also in carbon assimilation (Krause & Weis, 1991). The effective quantum efficiency of PSII (Y(II)) was also reduced in plants grown in soil with the addition of the highest doses of Cu and Zn. This reduction may have occurred because of the increase of non-photochemical dissipation, which indicates that the plants dissipated light in the form of heat, thus protecting the leaf from damage induced by light (Maxwell & Johnson, 2000; Cambrollé et al., 2012) such as the increase in the ROS formation.

Plants grown in environments with high concentrations of potentially toxic elements generally have reduced chl *a*, chl *b* and carotenoid contents (Cambrollé et al., 2012; Cambrollé et al., 2015; Tiecher et al., 2016a). The decrease of photosynthetic pigments induced by high levels of Zn in the growth medium of plant roots may be attributed to the adverse effect of excess Zn in electron transfer in photosynthesis, causing decreased chlorophyll synthesis or increased degradation (Cambrollé et al., 2012). Coupled with this, excess Cu may cause degradation of the structure and internal contents of the chloroplast (Ciscato et al., 1997; Ouzounidou, 1996). Replacement of the central ion of magnesium (Mg) by Cu may also occur in the chlorophyll molecule, which can also damage chlorophyll synthesis (Küpper et al., 2002). In this study, the effect of pigment concentration may have also occurred due to reduced leaf expansion, especially in treatments with the addition of 60 mg Cu kg^{-1} and 180 mg Zn kg^{-1} compared to the treatment with the addition of 60 mg Cu kg^{-1} and 120 mg Zn kg^{-1}, as chlorophyll contents were higher in plants with lower dry matter production. This response is consistent with results observed by other authors who have studied the effects on plants of the addition of Cu and Zn to soil (Santana et al., 2015; Tiecher et al., 2016a).

The low chl *b* content shown by the treatment with the addition of 60 mg Cu kg^{-1} and 180 mg Zn kg^{-1}, and especially the treatment with the addition of 60 mg Cu kg^{-1} and 120 mg Zn kg^{-1}, may be related to the protection of PSII, since there is high chl *b* concentration in the antenna complex (Pearcy and Yang, 1998). An effective way to reduce the energy that reaches the PSII is through partial destruction of these pigments (Eckhardt et al., 2004), resulting in a lower energy capture and excited state of chlorophylls thereby preventing the formation of ROS (Ruban & Horton, 2004).

There was an unusual significant "recovery" in chlorophyll and carotenoid as zinc dose was increased from 120 to 180 mg kg^{-1} for the 60 mg Cu kg^{-1} (Figure 5). This maybe a result of concentration of these pigments due to the lower shoot dry matter production in the highest Zn doses (Figure 2a). On the other hand, it is that the central Mg can be chemically replaced by Zn resulting in the more stable [Zn]-chlorophylls with otherwise very similar properties (Rau et al., 2001; Takeuchi & Amao, 2005; Petrovic et al., 2006), and it may also contribute to the recovery in chlorophyll in the highest Zn doses. Further studies are needed to investigate this phenomenon at higher Zn doses.

The ROS, including H_2O_2 and radicals HO^- and O_2^- are formed naturally inside the cells, primarily in the chloroplasts and mitochondria (Ferreira et al., 2015). However, ROS production can be increased dramatically in conditions of high concentrations of Cu and Zn. Moreover, in this study the increased CAT and POD activity in plants indicates the activation of an enzyme response to prevent oxidative damage caused by toxicity of Zn (Jain et al., 2010) and Cu (Miotto et al., 2014). The lowest CAT and POD activity was observed with the lowest doses of Cu and Zn applied to the soil, which indicates the low production of ROS. However, while the increased activity of these enzymes suggests that H_2O_2 is being predominantly degraded in the cell, the decrease in their activities in treatments with the addition of 0 mg Cu kg^{-1} + 180 mg Zn kg^{-1}, 30 mg Cu kg^{-1} + 180 mg Zn kg^{-1} (only in CAT activity), 60 mg Cu kg^{-1} + 120 mg Zn kg^{-1} and 60 mg Cu kg^{-1} + 180 mg Zn kg^{-1} indicates that with higher levels of Zn the antioxidant enzymes cannot sufficiently protect black oat plants, which increases ROS in tissues and enhances oxidative damage. Symptoms of toxicity induced by high contents Cu and Zn in soil may be related to increase of Cu and Zn above levels considered normal in the leaves of black oat, causing oxidative stress. It may also be related to high levels of these elements in the roots, interfering with the absorption and plant nutrition.

5. Conclusions

High concentrations of Cu and Zn, especially in treatments with the addition of 60 mg Cu kg^{-1} and in treatments with the addition of the highest doses of Zn, 120 mg Zn kg^{-1} and 180 mg Zn kg^{-1}, cause toxicity to black oat plants and provide leaf contents above levels considered normal. The combination of high levels of Cu and Zn results in reduced production of plant dry matter of black oat due to impaired functioning of the photosynthetic apparatus and the change in plant enzyme activity. Cu enhanced Zn uptake and translocation, while Cu in roots increase 10 to 20 fold but without translocation into shoots, Zn was taken up

into both root and shoot at 10 fold the soil concentrations. An unusual recovery of chlorophyll was observed at the highest Zn and Cu doses, which may be result of concentration due to the lower shoot dry matter production, or due to the central Mg replacement by Zn ([Zn]-chlorophyll). Further studies are needed to investigate this phenomenon at higher Zn doses. Therefore, it is expected that the high Cu content observed in sandy soils with low SOM content of Campanha Gaúcha region may increase the toxic effect on plants as the Zn content is increased in the soil due to the use of Zn-based fungicides, replacing the traditional Cu-based fungicides.

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Table 1. Physical and chemical characteristics of the 0.0-0.15 m layer in a Typic Hapludalf soil under natural field (data from Tiecher et al. (2016b)). Values represents a mean of three replications.

Physical and chemical characteristics	Natural field
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 ($cmol_c$ 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})	132
Available P by Mehlich-1 (mg kg^{-1})	7.0
Available Fe by EDTA (mg kg^{-1})	21.9
Available Mn by EDTA (mg kg^{-1})	40.7
Exchangeable Ca ($cmol_c$ kg^{-1})	0.5
Exchangeable Mg ($cmol_c$ kg^{-1})	0.2
CEC_{ef} *, $cmol_c$ kg^{-1}	1.4
$CEC_{pH\ 7.0}$ **, $cmol_c$ kg^{-1}	3.2

*CEC_{ef} = Effective cation exchange capacity; **$CEC_{ph\ 7.0}$ = Cation exchange capacity at pH 7.0.

Table 2. Significance of the effects of the experimental factors and their interactions resulting from analysis of variance (ANOVA).

Variable	Effects		
	Cu doses	Zn doses	Cu × Zn
Soil parameters			
Soil available Cu	****	ns	ns
Soil available Zn	ns	****	ns
Production parameters			
Shoot dry matter	****	****	****
Root dry matter	**	****	****
Element concentration in shoot dry matter			
Cu	****	***	**
Zn	****	****	****
P	**	*	***
K	ns	ns	ns
Mg	****	ns	**
Ca	*	***	***
Fe	*	ns	ns
Element concentration in root dry matter			
Cu	****	**	*
Zn	ns	****	ns
P	ns	***	*
K	*	***	****
Mg	***	ns	****
Ca	****	*	*
Translocation parameters			
Cu content ratio in shoot/root	****	ns	ns
Zn content ratio in shoot/root	***	ns	ns
Cu distribution in shoot/root	****	ns	ns
Zn distribution in shoot/root	*	ns	ns
Chlorophyll a *fluorescence parameters*			
F_o	ns	**	***
Fm	****	****	****
Fv/Fm	ns	ns	*
Y (II)	ns	ns	*
ETR_{1500}	****	****	****
ETR_m	****	****	****
Photosynthetic pigments parameters			
Chl *a*	****	****	****
Chl *b*	***	***	*
Carotenoid	****	***	**
Chl total	***	***	*
Chl *a*/Chl *b*	***	***	*
Carot./Chl total	ns	*	ns
Activity of antioxidant enzymes			
POD	****	*	****
CAT	****	***	****

ns, not significant.
* Significant at $P<0.05$.
** Significant at $P<0.01$.
*** Significant at $P<0.001$.
**** Significant at $P<0.0001$.

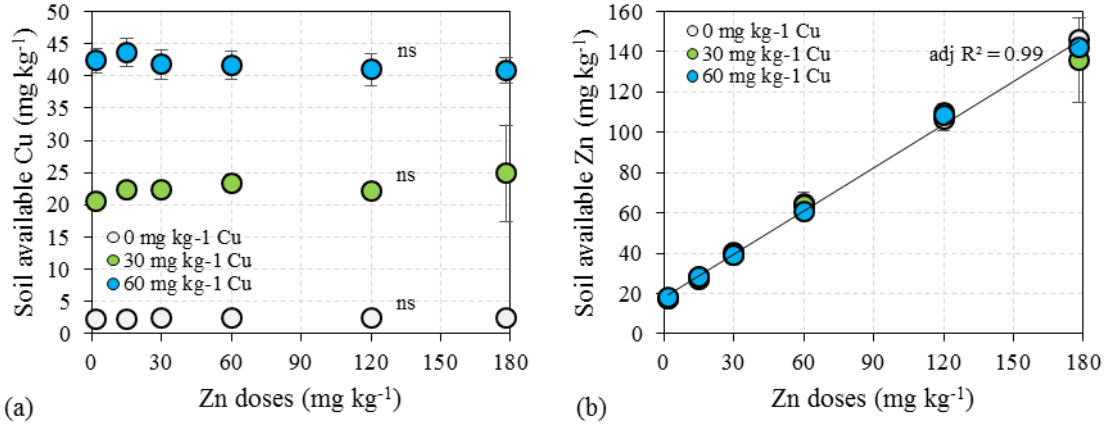

Fig. 1. Soil available Cu (a) and Zn (b) content in response to the addition of doses of Cu and Zn in the soil of natural field after cultivated of black oat for 30 days. Each value represents a mean of six replications. Continuous line in Figure 1b is the regression equation for the overall mean of three Cu doses following interpretation of ANOVA from Table 2, which shows no interaction between Cu and Zn doses.

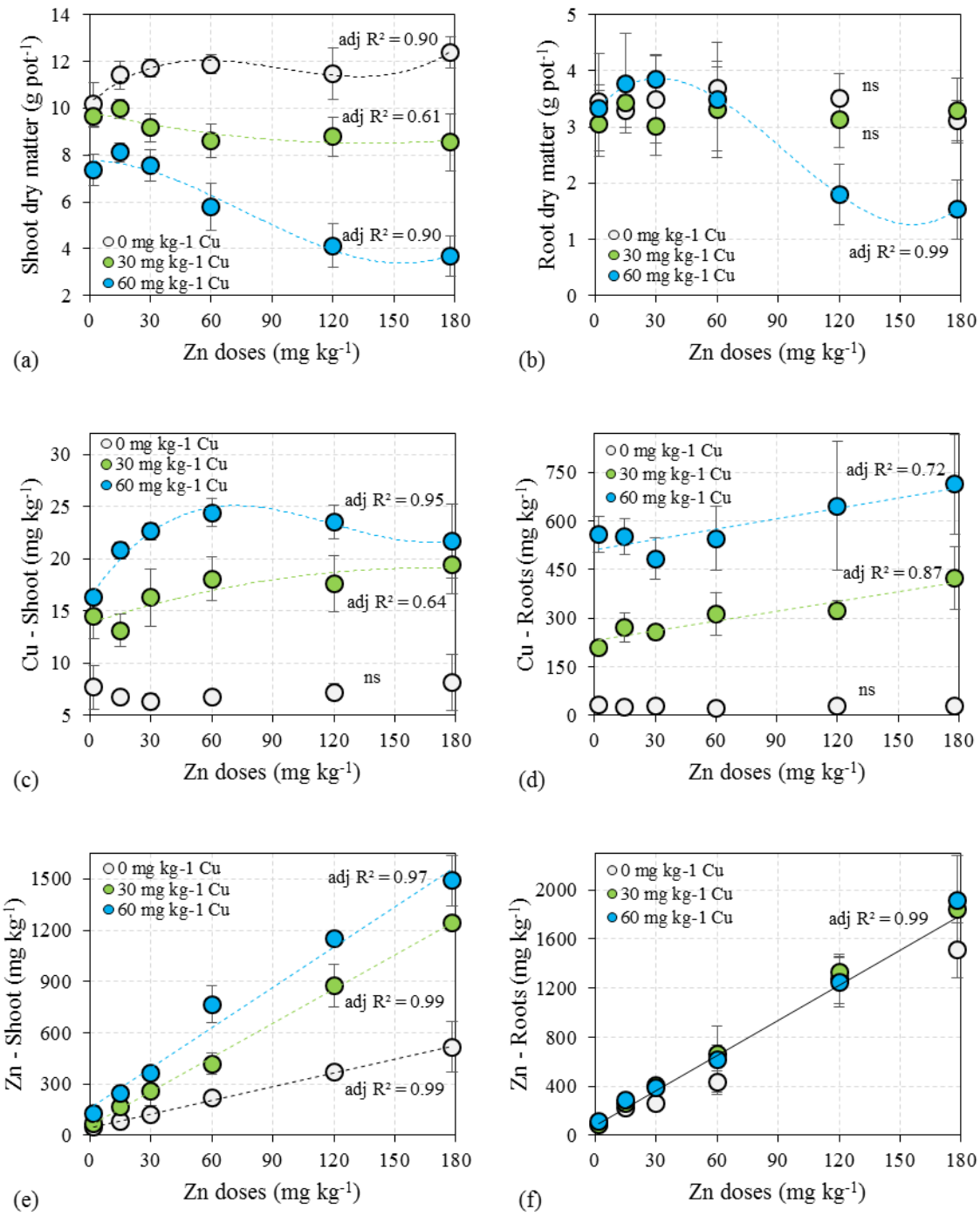

Fig. 2. Dry matter production of the shoots (a) and roots (b), total Cu concentration in the shoots (c) and roots (d), and total Zn concentration in the shoots (e) and roots (f) of black oat in response to the addition of doses of Cu and Zn in the soil of natural field cultivated for 30 days. Each value represents a mean of six replications. Continuous line in Figure 2f is the regression equation for the overall mean of three Cu doses following interpretation of ANOVA from Table 2, which shows no interaction between Cu and Zn doses.

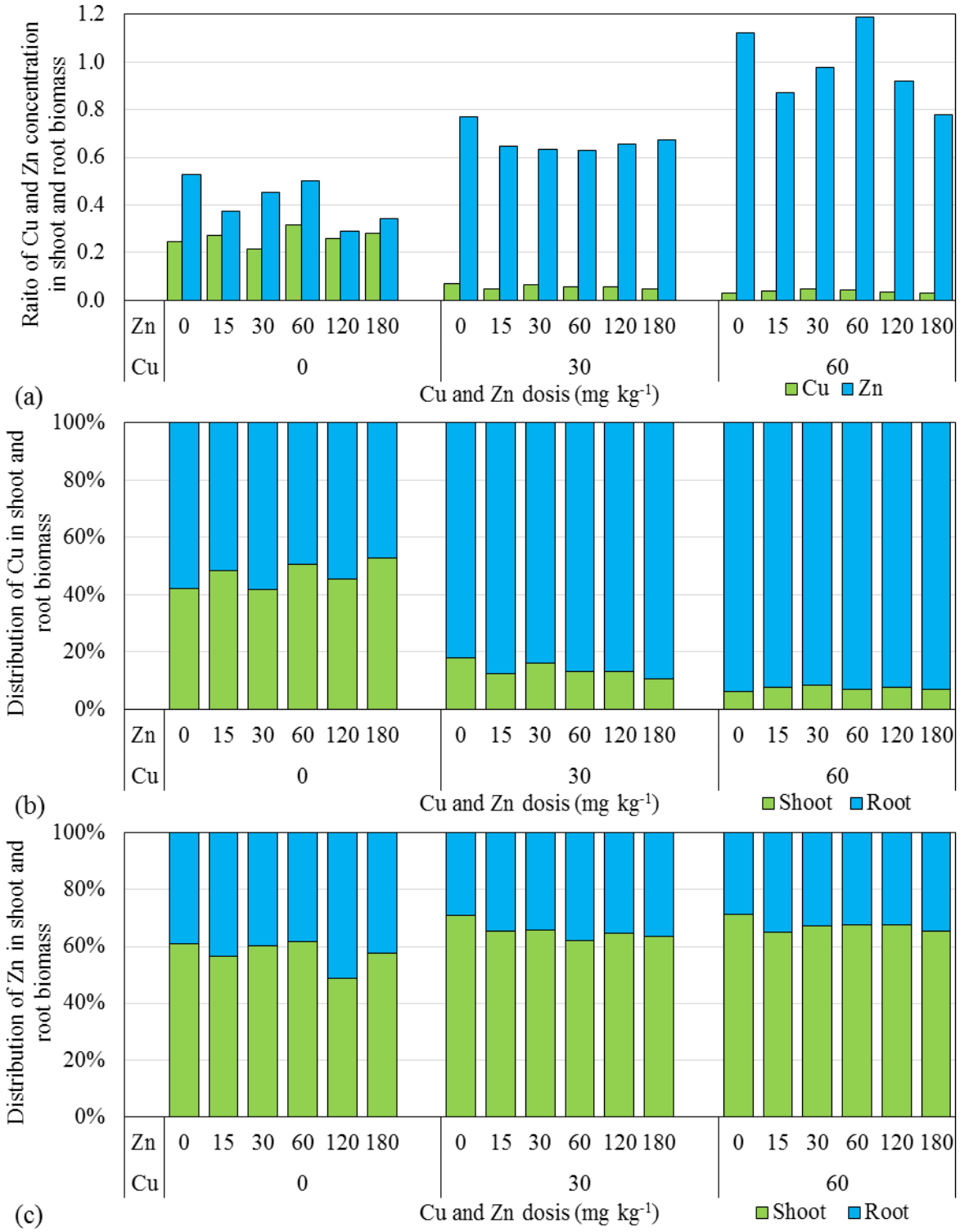

Fig. 3. Ratio of Cu and Zn concentration in shoot and root biomass (a) and distribution of Cu and Zn in root (b) and shoot (c) biomass (concentration in tissue × dry matter).

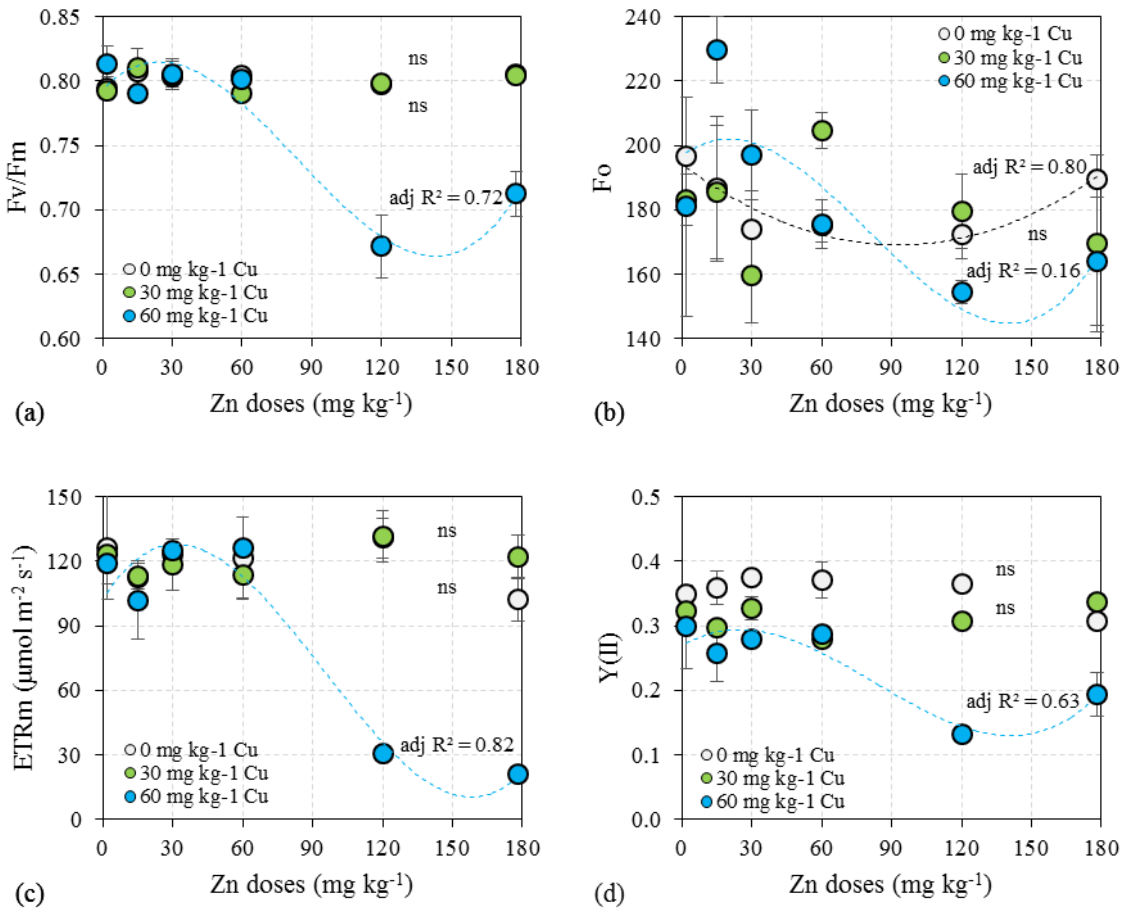

Fig. 4. Maximum quantum yield of PSII (Fv/Fm) (a), initial fluorescence (Fo) (b), electron transport rate (ETRm) and effective quantum efficiency of PSII (Y(II)) (d), in leaves of black oat in response to addition of doses of Cu and Zn in soil of natural field cultivated for 30 days. Each value represents a mean of six replications.

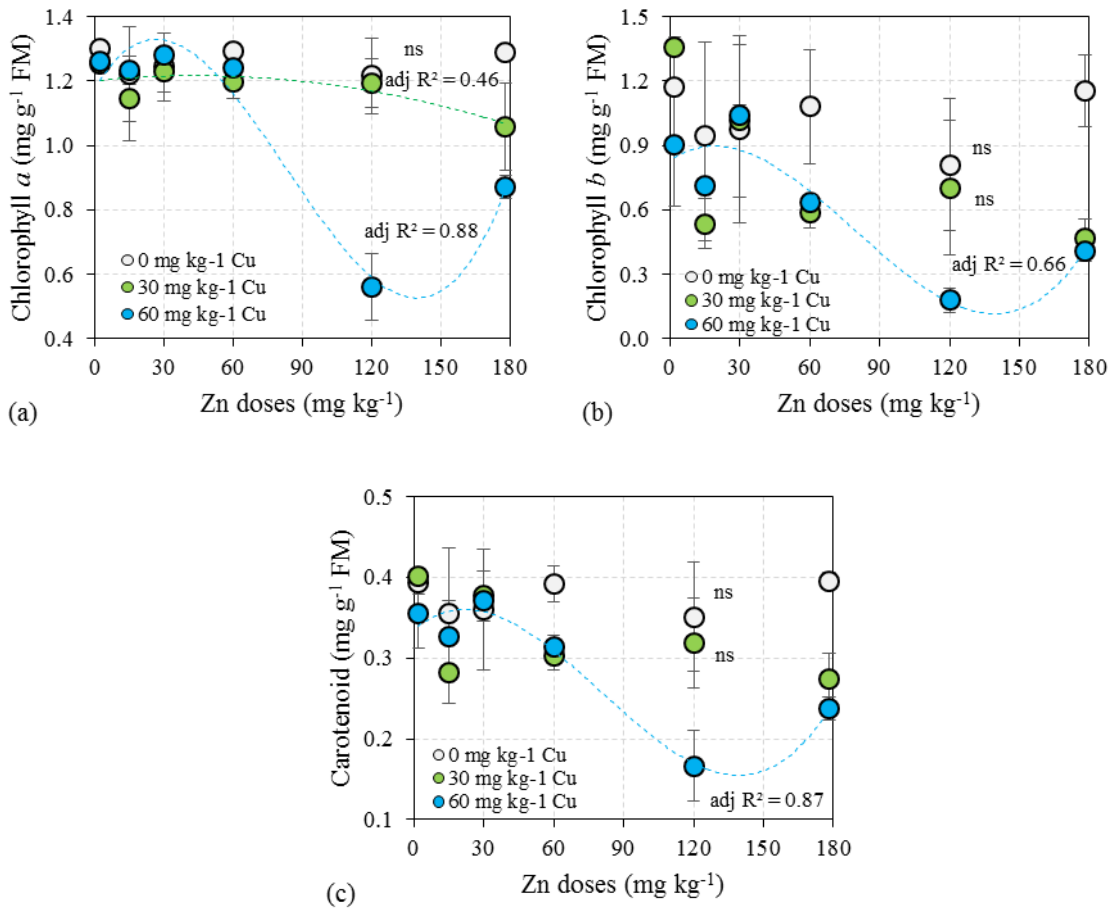

Fig. 5. Chlorophyll *a* (a), chlorophyll *b* (b) and carotenoid (c) content in leaves of black oat in response to addition of doses of Cu and Zn in soil of a natural field cultivated for 30 days. Each value represents a mean of six replications.

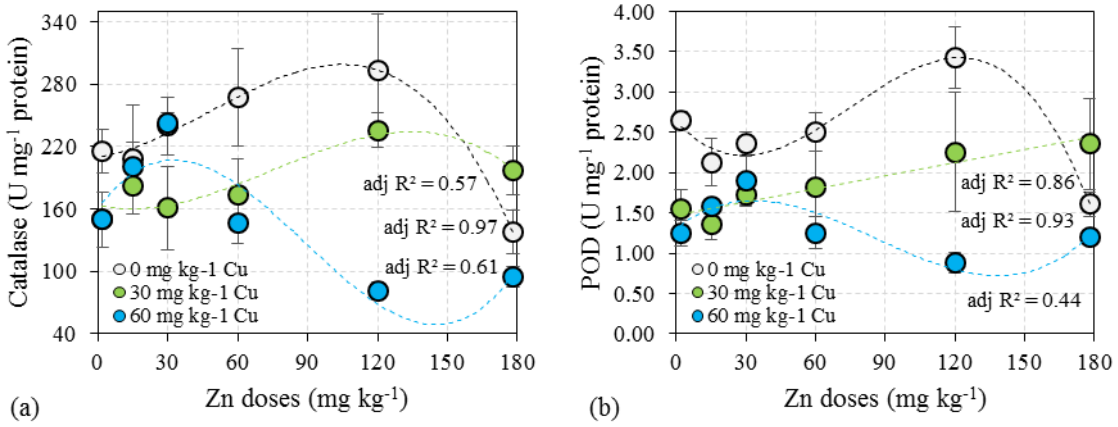

Fig. 6. Catalase (CAT) (a) and peroxidase (POD) (b) activity in leaves of black oat in response to addition of doses of Cu and Zn in soil of a natural field cultivated for 30 days. Each value represents a mean of six replications.

5.2 ESTUDO II

Tolerance and translocation of heavy metals in young grapevine (*Vitis vinifera*) grown in sandy acidic soil with interaction of high doses of copper and zinc

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Tolerance and translocation of heavy metals in young grapevine (*Vitis vinifera*) grown in sandy acidic soil with interaction of high doses of copper and zinc [1]

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Abstract
Vineyard soils may have high levels of copper due to frequent applications of Bordeaux mixture for the control of fungal diseases. Therefore, many winegrowers have used zinc-based fungicides instead of copper-based fungicides, resulting in high levels of Cu and Zn in vineyard soils. The aim of the study was to evaluate the tolerance of young grapevines grown in soil with interaction of high contents of Cu and Zn, and the translocation of these heavy metals from roots to shoots. We collected soil of a natural field of Rio Grande do Sul, the southernmost state of Brazil. Soil treatments were addition of 0, 30, and 60 mg Cu kg^{-1}, and addition of 0, 15, 30, 60, 120, and 180 mg Zn kg^{-1} for each copper dose. A seedling of grapevine per pot was grown for 60 days. After that, we measured root and shoot dry matter, Cu and Zn content in roots and shoots, chlorophyll *a* fluorescence, photosynthetic pigments, and peroxidase and superoxide dismutase activity. Young grapevines showed tolerance mechanisms to high concentrations of Cu and Zn by retaining these metals in the roots, decreasing translocation to the shoots. However, at the highest doses of Cu and Zn, we observed decrease in plant growth and in photochemical efficiency associated with the decrease in photosynthetic pigment content. Furthermore, the activity of antioxidant enzymes increased in intermediate doses of Zn, which indicates activation of the antioxidant system. However, the stress condition in treatments with high levels of Cu and Zn was not reversed.

Keywords: Vineyard soils. Phytotoxicity. Antioxidant enzymes. Chlorophyll fluorescence. Photosynthetic pigments.

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1. Introduction

Plants grown in soil with available Cu and Zn contents higher than those ideal may accumulate these heavy metals in tissue causing toxicity symptoms (Kabata-Pendias, 2011). Negative responses in biochemical and physiological levels resulting from such disorders vary according to species and plant organ, element concentration and tissue tolerance to high levels of Cu and Zn (Cambrollé et al., 2012; Mateos-Naranjo et al., 2013).

Excess Cu affects the function of membrane transporters and ion channels (Janicka-Russak et al., 2008) and there may be increased non-specific membrane permeability, resulting in nutritional imbalances (Cambrollé et al., 2013). In addition, it may cause oxidative stress due to imbalance between antioxidant responses and increased production of reactive oxygen species (ROS) by Fenton reaction (Girotto et al., 2013). Excess Zn may also generate oxidative stress by interfering with the antioxidant defense system of plants (Gratão et al., 2005) and negatively affect photosynthetic efficiency by inhibiting chlorophyll biosynthesis and decreased carbon assimilation (Chen et al., 2008; Dhir et al., 2008). Furthermore, it may reduce rooting capacity, cause leaf chlorosis and compromise the electron transport chain (De Magalhães et al., 2004; Castiglione et al., 2007), which contribute to the inhibition of plant growth (Girotto et al., 2013).

In viticulture, frequent applications of Cu-based fungicides are traditional for the control of foliar diseases, which often leads to increased Cu content in soil, even to toxic levels to plants (Miotto et al., 2014; Santana et al., 2015; Girotto et al., 2016). As a result, some viticulturists began using zinc-based instead of copper-based fungicides. As Bordeaux mixture also contributes (to a lesser extent) to increase levels of other heavy metals in soil, such as Zn (Mirlean et al., 2007), the occurrence of high levels of both Cu and Zn in vineyard soils of traditional wine-producing regions of the world has been observed. This fact had also been reported in the 80s by Magalhães et al. (1985), and it has had more notoriety in the last decade (Fernandez-Calviño et al., 2012; Brunetto et al., 2014; Cambrollé et al., 2015; Tiecher et al., 2016c). Grapevines may remain productive for decades in soils with high concentrations of heavy metals (Miotto et al., 2014). However, when there is a decline in production, with the subsequent eradication and implementation of young grapevine plants in contaminated soils, there may be decreased root and shoot growth, leaf chlorosis and accumulation of Cu and Zn in roots, resulting in economic losses.

Some studies have been conducted in order to assess the phytoremediation potential of some annual species, such as corn, which can be used over several cycles between rows of contaminated vineyard soils to promote phytoextraction of Cu and Zn, while also ensuring a

source of income to the farmer (Tiecher et al., 2016a; 2016b). The effects of high levels of Cu and Zn in black oat plants have also been studied. Black oats are traditionally used between rows of vineyards as cover crops for nutrient cycling and protection against soil erosion (Tiecher et al., 2016c). However, studies on the interaction between Cu and Zn in soil with high accumulation of these two heavy metals in vineyards installed in sandy soils with low organic matter content, and its effects on young grapevines (*Vitis vinifera*) are still scarce in international literature. For this reason, this study was conducted to assess the tolerance of young grapevines grown in soil with interaction of high contents of Cu and Zn, and the translocation of these heavy metals from roots to shoots.

2. Material and methods

2.1. Description of the experiment

The study was carried out using a Typic Hapludalf soil (Soil Survey Staff, 2006) of a natural field area (30º47’23.5’’S e 55º22’7.0’’W) with naturally low concentration of Cu and Zn. It is located adjacent to a vineyard of the Campanha Gaúcha region in city of Santana do Livramento (RS), Southern Brazil. The soil was collected in the 0.00–0.15 m layer in November 2011. Subsequently, the soil was air-dried, crushed, passed through a 2 mm mesh sieve and reserved. Physical and chemical characterization of the soil is shown in Table 1.

The soil was divided into pots containing 10 kg of soil each. We adjusted soil acidity with addition of 670 mg $CaCO_3$ and 830 mg MgO per kg^{-1} of soil. Correctives were added and mixed into the soil. Then we added 100 mL of distilled water per kg^{-1} of soil in each container with 10 kg of soil, where it was immediately homogenized and incubated for a period of 60 days. Afterwards, the soil was dried, homogenized and we added 40 mg P kg^{-1} and 100 mg K kg^{-1} in the form of triple superphosphate and KCl, respectively, in order to add 100 ml kg^{-1} of distilled water once more. Then the soil was subjected to another incubation period of 30 days. The soil remained in sealed plastic bags to prevent water evaporation during the incubation period of the correctives and fertilizers.

Subsequently, the entire volume of soil was dried, homogenized and separated into pots containing 4 kg of dry soil, with four replicates per treatment and randomized design. Afterwards, we applied Cu doses of 0, 30, and 60 mg kg^{-1}, and for each Cu dose applied, the soil was subdivided and we added 0, 15, 30, 60, 120, and 180 mg Zn kg^{-1}, totaling 18 treatments. Zn doses were added to the soil in the form of a solution containing $ZnSO_4.7H_2O$

(60%) and $ZnCl_2$ (40%), while Cu doses were added in the form of $CuSO_4.5H_2O$. The solution was diluted with distilled water to obtain a final volume of 400 mL per pot and it was applied individually in the soil of each pot, which was then revolved for homogenization to occur. The soil was incubated in a greenhouse for 180 days, with replacement of the evaporated water every 2 days to maintain humidity at 80% of the maximum water retention capacity (MWRC).

After grown black oat (*Avena strigosa* Schreb.) for 30 days (Tiecher et al. (2016c), we transplanted a young grapevine (*Vitis vinifera* cv. Paulsen 1103) plant per pot with 4 replicates per treatment and it was grown for 60 days from the date of the transplantation to pots containing treatment soils. Soil moisture was maintained at 80% of the MWRC with daily irrigation to replenish evaporated water. At 15 and 30 days after transplanting (DAT), 50 ml of a solution containing 50 mg N kg^{-1} and 50 mg K kg^{-1} was applied.

Contents of Cu and Zn available to plants were extracted with EDTA (Chaignon et al., 2009). Samples of 0.25 g of soil were added to 10 mL of solution containing 0.01 mol L^{-1} dissodic EDTA + 1.0 mol L^{-1} ammonium acetate, pH adjusted to 7.0, and placed into 15 mL centrifuge tubes, which were shaken for 2 h. After stirring, the suspension was centrifuged and filtered, and the concentrations of Cu and Zn were determined by flame atomic absorption spectrometry (AAS).

2.2. Dry matter production and total Cu and Zn content in the tissue

60 DAT, plants were uprooted manually, and roots washed in running water, immersed in EDTA (0.02 mol L^{-1}) for one minute and washed with distilled water three times (Miotto et al., 2014). Shoots were cut near to the soil surface; a leaf sample was immediately placed in liquid N_2 and stored in an ultrafreezer at –80°C until we carried out biochemical analyses. The remaining leaf and root samples were dried in a forced-air oven (±65 ºC) until reaching constant weight. Root and shoot dry matter was measured with a precision scale.

After drying, the samples were ground in a Wiley mill and total contents of Cu and Zn in the leaves and roots were measured after the digestion of 0.1g of tissue in 3.0 ml of HNO_3 plus 1 mL of $HClO_4$ (Embrapa, 2009). Sample digestion was performed in an open system with the use of a Velp Scientifica (Milano, Italy) block digester, which was heated at 130ºC for the period of 4 hours. Total contents of Cu and Zn were analyzed by atomic absorption spectrophotometer (AAS, VarianSpectrAA-600, Australia). Translocation of Cu and Zn was estimated by (*i*) Cu and Zn content ratio in shoot and root biomass and by (*ii*) Cu and Zn distribution in root and shoot biomass (content in tissue × dry matter).

2.3. Chlorophyll a fluorescence

We performed chlorophyll *a* fluorescence analysis using a pulse-amplitude modulated JUNIOR-PAM fluorometer (Walz, Germany) at 60 DAT. Fluorescence reading was performed in three replicates of each treatment, in the first fully expanded leaf of the plant, in the period between 8:00 and 10:00 a.m. (Tiecher et al., 2016a). Prior to measuring, the leaves were pre-adapted to the dark for a 30-minute period to determine initial fluorescence (Fo). Subsequently, the sample was subjected to a saturating light pulse (10,000 µmol m^{-2} s^{-1}) for 0.6 seconds, thereby determining maximal fluorescence (Fm). Through the induction curve we determined maximum quantum yield of PSII (Fv/Fm), basal quantum efficiency of PSII (Fv/Fo), electron transport rate (ETR_{1500}) and effective quantum efficiency of PSII (Y(II)).

2.4. Photosynthetic pigments

During plant collection, we collected leaf discs at 60 DAT to analyze photosynthetic pigments (using the same leaf used to determine chlorophyll *a* fluorescence) with three replicates per treatment. The discs were frozen in liquid N_2 and stored at –80°C. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid contents were determined according to the methodology described by Price and Hendry (1993). The absorbance of the supernatant was performed at 480, 645 and 663 nm to determine Chl *a*, Chl *b*, and carotenoids, respectively, using a spectrophotometer (model SF325NM - Bel Engineering, Italy). Pigment content was calculated according to the methodology proposed by Lichtenthaler (1987). Total chlorophyll content (Total Chl) was determined by adding the values of Chl *a* and Chl *b*, and the ratio between carotenoid and total Chl was determined by the ratio between the values of the variables.

2.5. Superoxide dismutase (SOD) and peroxidase (POD) activity

Samples for enzyme analyses in leaf tissue were collected at 60 DAT, using fully expanded leaves of three plants per treatment, which were immediately placed in liquid N_2 and stored at –80°C in an ultrafreezer until enzymatic extraction was carried out. The crude enzyme extract was obtained from 0.5 g of fresh leaf mass, which was macerated in liquid N_2 and homogenized with 5.0 mL of 100 mM potassium phosphate buffer (pH 7.5), containing 1.0 mM EDTA, 3.0 mM dithiothreitol (DTT) and 2% polyvinylpolypyrrolidone (PVPP) (m/v) (Azevedo et al., 1998). The homogenate was centrifuged at 14,000 rpm for 30 minutes at 4°C and the supernatant was collected. Aliquots of 0.5 mL were stored in a freezer at –80°C until

we quantified enzymes and total protein. The concentration of total protein of each sample was determined in a spectrophotometer at 595 nm as described by Bradford (1976) using BSA as standard. SOD activity (EC 1.15.1.1) was determined according to the spectrophotometric method described by Giannopolitis and Ries (1977), while peroxidase activity (POD, EC 1.11.1.7) present in the extract was determined in line with Zeraik et al. (2008).

2.6. Statistical analysis

Initially, experimental data were tested for normality and homogeneity of variance through Lilliefors and Shapiro-Wilk tests. Afterwards, data were submitted to analysis of variance, using the following completely randomized bifactorial statistical model:

$Y_{ijk} = \mu + C_i + Z_j + CZ_{ij} + error\ (i, j)$

where μ = overall mean of the experiment; C = Cu dose applied (i = 1, 2, 3); Z = Zn dose applied (j = 1, 2, 3, 4, 5) and error = experimental error. When the factors were significant, the means of Cu doses were compared by Tukey test at P <0.05 and the means of Zn doses were adjusted by polynomial regression.

3. Results

3.1. Soil available Cu and Zn

The content of soil available Cu increased with increasing Cu doses added to the soil (Table 2). Cu content available in soil also increased with increasing Zn levels added to the soil (Figure 1a). Likewise, soil available Zn content increased along with increasing Zn dose added to the soil (Figure 1b). There was an increase in available Zn content with increasing Cu dose added, especially for Zn doses higher than 120 mg kg^{-1} (Figure 1b).

3.2. Young grapevine growth and concentration of Cu and Zn in roots and shoots

There was no interaction between Cu and Zn in dry matter production of the roots and shoots of young grapevine plants (Table 2). However, there was a reduction in dry matter production of the shoots and roots upon addition of Zn doses applied to the soil for the three Cu doses (Figures 2a, b). Treatments without Cu addition showed the highest dry matter production of the shoots and roots (Table 2).

Cu content in shoots and roots were not affected by the addition of Zn, and there was not interaction between Cu and Zn treatments for these variables (Table 2 and Figures 2c, d). The lowest contents of Cu in shoots were observed in treatments without the addition of Cu.

However, there was no difference of Cu content in shoots with the addition of 30 and 60 mg Cu kg^{-1}. Cu content in roots was higher with increasing Cu doses applied to the soil (Table 2 and Figure 2d).

Zn content in shoots was altered only by doses of Zn added to the soil (Table 2). However, Zn content in roots increased upon addition of both Cu and Zn treatments added to the soil, as well as by the interaction between the doses of these metals (Table 2). Zn content in shoots increased linearly with increasing Zn doses added to the soil (Figure 2e).This linear upward trend was also observed in Zn content in the root tissue for the treatments with the addition of 60 mg Cu kg^{-1} (Figure 2f). On the other hand, treatments without Cu addition showed an increase in Zn content in roots up to 60 mg Zn kg^{-1}, and this increase was less evident upon addition of higher doses (120 and 180 mg Zn kg^{-1}) (Figure 2f).

3.3. Translocation of Cu and Zn from root to shoot biomass

The shoot/root ratio of Cu content, as well as Cu distribution between the shoots and roots was only altered by the Cu doses added to the soil (Table 2). The highest shoot/root ratio of Cu content was observed in treatments without Cu addition (1.0), while this ratio reduced significantly in treatments with the addition of 30 and 60 mg Cu kg^{-1} (0.2 and 0.1, respectively) (Figure 3a). A similar trend was observed in relation to Cu distribution between the shoots and roots. The percentage of Cu in shoots was 38 ± 10% in treatments without Cu addition, while it was 11 ± 1 and 7 ± 2% in treatments with the addition of 30 and 60 mg Cu kg^{-1}, respectively (Figure 3b).

The shoot/root ratio of Zn content and distribution between the shoots and roots were affected only by Zn doses added to the soil (Table 2). There was a large reduction in the shoot/root ratio of Zn content in treatments with Zn addition (Figure 3a). The shoot/root ratio of Zn content was above 1.5 in treatments without the addition of Zn and less than 0.5 in all treatments with the addition of Zn. Consequently, the percentage of Zn in the shoots varied from 44-59% when Zn was not added and 13-24% when adding any of the Zn doses (Figure 3c).

3.4. Chlorophyll a fluorescence

There was no difference in the parameters of chlorophyll *a* fluorescence in young grapevine leaves due to the addition of Cu doses (Table 2). The parameters of initial fluorescence (Fo) and effective quantum efficiency (Y (II)) were not affected by the addition of Zn (Table 2). On the other hand, there was a reduction in the values of maximum quantum

yield of PSII (Fv/Fm), basal quantum yield of PSII (Fv/Fo) and electron transport rate (ETR_{1500}) upon addition of Zn (Figures 4a, b, c).

3.5. Photosynthetic pigments

Chl *a* and *b* contents were higher in treatments without Cu addition compared to those of 30 and 60 mg Cu kg^{-1} (Table 2). Carotenoid levels were lower in treatments with the addition of 30 mg Cu kg^{-1}, compared to the other Cu doses. On the other hand, the carotenoid/total Chl ratio was lower in the treatment without Cu addition, but it increased consistently with increasing Cu levels. Moreover, there was an increase in the carotenoid/total Chl ratio upon addition of Zn doses applied to the soil for all Cu doses (Figure 5d).

Chl *a* and *b* and carotenoid contents showed similar trends with the addition of Cu and Zn (Figures 5a, b, c). In treatments without Cu addition, there was an increase in concentration of these pigments up to Zn dose of 30 mg kg^{-1} and a decrease in treatments with 60 and 120 mg Zn kg^{-1}, with subsequent increase in treatment with 180 mg Zn kg^{-1}.

In treatments with the addition of 30 mg Cu kg^{-1}, the highest amounts of photosynthetic pigments were observed in the treatment without Zn, and there was a decrease in these levels due to the addition of Zn (Figures 5a, b, c). In treatments with the addition of 60 mg Cu kg^{-1}, there was a decrease in the pigment levels upon addition of Zn, levels exceeding 60 mg Zn kg^{-1} dose, and the lowest levels were observed in the treatment with the addition of the highest Zn dose (180 mg Zn kg^{-1}).

3.6. Antioxidant enzyme activity

Peroxidase (POD) activity was higher in treatments without the addition of Cu, compared to the treatments with the addition of 30 and 60 mg Cu kg^{-1} (Table 2). Among the treatments without Cu, there was an increase in POD activity up to the dose of 60 mg Zn kg^{-1}, but a decrease in POD activity in higher Zn doses (Figure 5e). In treatments with the addition of 30 and 60 mg Cu kg^{-1}, there was a decrease in POD activity starting at 60 mg Zn kg^{-1}.

Superoxide dismutase (SOD) showed the highest activity in treatments without the addition of Cu, whereas it decreased with increasing Cu doses added to the soil (Figure 5f). In treatments with the addition of 30 mg Cu kg^{-1}, there was an increase in SOD activity with the increasing amount of Zn added up to 60 mg Zn kg^{-1} (Figure 5f). However, at the highest Zn doses (120 and 180 mg kg^{-1}) there was a dramatic decrease in SOD activity. In treatments with the addition of 60 mg Cu kg^{-1}, there was stable SOD activity up to the dose of 15 mg Zn kg^{-1}, with subsequent reduction in activity along with increasing Zn doses.

4. Discussion

The availability of Cu and Zn was affected by the interaction between both metals in the soil, which demonstrates that the accumulation of both heavy metals in the soil depends on their competitive interaction for adsorption sites (Perez-Novo et al., 2009, 2011). It is known that Cu has a high affinity for the sorption sites of soil organic matter (Tiecher et al., 2013), while Zn remains mainly adsorbed in the functional groups of the mineral fraction, such as Fe oxides and the edges of phyllosilicate clay mineral (Perez-Novo et al., 2011). However, at high concentrations of both elements, competition for the same sorption sites may occur with a consequent increase in the availability of Cu and Zn, which becomes evident when we look at the treatments with the addition of the highest doses of Cu and Zn in soil (Tiecher et al., 2016b).

The highest availability of Cu and Zn contributed to a reduction in young grapevine root and shoot growth. Plants that grow in environments with high concentrations of available metals, such as treatments with the addition of 60 mg Cu kg^{-1} or with doses of 120 and 180 mg Zn kg^{-1}, generally have toxicity symptoms. The reduction in dry matter production may be the result of the inhibition of cell elongation and division caused by the high concentrations of Cu and Zn in the soil solution (Hewit, 1983; Arduini et al., 1994; Jain et al., 2010). Furthermore, the toxic effects may be attributed to the accumulation of Zn in the leaves (Cherif et al., 2011), which affects normal ion homeostasis by interfering with the absorption, transport and regulation of essential ions (Wang et al., 2009), resulting in breakdown of the metabolic processes, such as transpiration and photosynthesis, resulting in reduced growth (Sagardoy et al., 2009).

It is important to note that the behavior of adult grapevine plants may be quite different from that observed in young grapevines, such as those used in our study. The gradual increase of Cu and Zn in the soil over the years may allow adaptation of grapevine plants to high levels of the heavy metals. Such behavior was observed by Ambrosini et al. (2015), who found alterations in the root system of young grapevines after 70 days of exposure in soil with excess of Cu. Root thickening was observed, which may be a defense strategy of the plant in response to abiotic stress, increasing the area of the apoplast and, consequently, the retention of metals in the roots. Although Cu and Zn show some mobility in sandy soils, there is generally a gradient of concentration that is higher in the topsoil and decreases with depth (Brunetto et al., 2014). Therefore, adult grapevine plants with a well-developed root system can reach depths where Cu and Zn contents have not yet reached toxic

levels as in the superficial layer, thus reducing their toxic effects. Moreover, the exudation of low molecular weight organic compounds may be involved in external and internal tolerance mechanisms, which may contribute to the immobilization and decrease of bioavailability of heavy metals in the soil (Brunetto et al., 2016).

Cu and Zn contents in shoots of young grapevine plants increased along with the doses applied to the soil, as observed in other studies with different plant species (Jain et al., 2010; Cambrollé et al., 2012; Girotto et al., 2016; Tiecher et al., 2016a; 2016c). In plant species sensitive to high levels of heavy metals, leaf content between 15-20 mg Cu kg^{-1} and 150-200 mg Zn kg^{-1} causes severe decrease in plant growth (Kloke et al., 1984; Kabata-Pendias, 2011). In this study, plants grown in treatments with the addition of 30 and 60 mg Cu kg^{-1} had shoot Cu content above or close to 15 mg kg^{-1}, while shoot Zn content was higher than 200 mg kg^{-1} in all treatments with the addition of 60, 120 and 180 mg Zn kg^{-1}.

These high Zn levels in shoots of young grapevine plants can be attributed to the high amount of Zn present in the soil solution. This essential element has a similar ionic radius of bivalent cations, such as Cu, manganese (Mn) and iron (Fe) and, therefore, can be absorbed by the same transporters (Tewaru et al., 2008) and subsequently be translocated to the shoots. Once inside the plant, excess Zn may alter the physiological homeostasis by competition with other cations at various locations, such as the primary absorption sites or nutrient transport sites of the roots (Yang et al., 2011).

Cu accumulation occurred predominantly in the young grapevine roots, with low translocation to the shoots (Figure 3a). These results are consistent with those found in black oats grown in soils contaminated with Cu (Tiecher et al, 2016c.) and in grapevines cultivated in nutrient solution with high Cu concentration (Cambrollé et al., 2013; Cambrollé et al., 2015). This indicates that the root system of young grapevine plants has mechanisms that can prevent and/or reduce excess Cu translocation to the shoot. The lowest Cu content compared to Zn content in shoots may be associated with low Cu mobility in plants, since it has strong interaction with protein nitrogen (N) (Kabata-Pendias, 2011). In addition, Cu has a strong interaction with the sulfhydryl groups of enzymes and proteins in the apoplast of root cells, and can inhibit the activity of enzymes or cause changes in structure and replacement of key elements, resulting in a deficiency of other nutrients (Yruela, 2005; Kabala et al., 2008).

Young grapevine plants also showed mechanisms for Zn retention in the roots, because a small amount of the element translocated to shoots, probably due to Zn binding with proteins and amino acids in root tissues (Kabata-Pendias, 2011). This higher retention of Cu and Zn in the roots of young grapevines may be a survival strategy, since plants maintain a

lower concentration of metals in the most sensitive photosynthetic organs, such as the shoot and store most of the excess metal in less sensitive organs, such as the roots (Yang et al., 2011; Ambrosini et al., 2015).

The simultaneous increase in Cu and Zn levels in roots, particularly in the treatment with the highest doses of Cu and Zn, may indicate that both elements may use a similar absorption mechanisms and transport sites (Kabata-Pendias, 2011). High concentrations of these elements in plant tissues induce changes in membrane properties, which affect the function of membrane transporters and ion channels (Janicka-Russak et al., 2008). These effects cause increased non-specific membrane permeability, which may be responsible for the imbalance in nutrient concentration in plants grown in environments with high levels of heavy metals (Cambrollé et al., 2013). On the other hand, the lack of interference in Cu content in the roots and shoots in terms of Zn doses applied to the soil may be explained by Cu-Zn interaction, which is often described as antagonistic (Kabata-Pendias, 2011).

The stress on young grapevine plants may also be observed by the reduction of maximum quantum efficiency of PSII (Fv/Fm), basal quantum efficiency of PSII (Fv/Fo) and electron transport rate (ETR_{1500}) due to Zn addition to the soil. Our results are in agreement with authors that relate reduction in parameters of chlorophyll *a* fluorescence to the stress caused to plants of different species grown in environments with excess Zn (Cambrollé et al., 2012; Tiecher et al., 2016a, 2016c). The reduction of Fv/Fm, Fv/Fo and ETR_{1500} characterizes a state of photoinhibition in plants (Alves et al., 2002), and because of this a smaller amount of energy taken up by the plant through the antenna complex is used to reduce carbon and produce dry matter. Dissipation processes are complementary in plants and when there is increased energy dissipation in the form of fluorescence, reduced energy dissipation via photochemical process will consequently happen, with reduced formation of ATP and NADPH as well as carbon assimilation (Krause and Weiss, 1991). This reduction may also have occurred because of increased non-photochemical dissipation, which indicates that the plants dissipated light in the form of heat, thus protecting the leaf from damage induced by light (Maxwell and Johnson, 2000; Cambrollé et al., 2012), as in the case of increased formation of ROS.

Plants grown in environments with high concentrations of heavy metals generally have reduced contents of Chl *a*, Chl *b* and carotenoids (Cambrollé et al., 2012; Cambrollé et al., 2015; Tiecher et al., 2016a; 2016c). The decrease in photosynthetic pigments, induced by high levels of Zn in the growth medium of plant roots may be attributed to the adverse effect of Zn on electron transfer in photosynthesis, causing decreased chlorophyll synthesis or increased

degradation (Cambrollé et al., 2012). Furthermore, excess Cu may cause degradation of the structure and the internal contents of the chloroplast (Ouzounidou, 1996; Ciscato et al, 1997). The substitution of the central Mg ion for Cu in the chlorophyll molecule may also happen, impairing chlorophyll synthesis (Küpper et al., 2002). In this study, the effect of pigment concentration due to the reduction in leaf expansion may have also occurred, especially in treatments with the addition of 0 mg Cu kg^{-1} and 180 mg Zn kg^{-1} compared to the treatment of 0 mg Cu kg^{-1} and 120 mg Zn kg^{-1}, since chlorophyll levels were higher in plants with lower dry matter production. This response is consistent with the results observed by authors who have studied the effects of Cu and Zn addition on plants (Santana et al., 2015; Tiecher et al., 2016c).

The Carotenoid/total Chl ratio may be used as a potential indicator of photooxidative damage caused by high irradiation (Hendry and Grime, 1993). Chlorophyll tends to be photooxidized at high light radiation and carotenoids may prevent this photooxidation. Due to this capacity, the increase in Carotenoid/total Chl indicates the presence of stressed plants, a phenomenon observed due to increasing doses of Cu and Zn applied to the soil, highlighting the adverse effect of increasing concentrations of heavy metals in soil to plants.

ROS, including H_2O_2 and HO^- and O_2^- radicals, are formed naturally in the cells, especially in chloroplasts and mitochondria (Ferreira et al, 2015), due to electron transfer and cellular respiration. However, ROS production may be increased dramatically in conditions of high concentrations of Cu and Zn. In these cases, ROS may cause toxicity by their reaction with other molecules and may cause significant changes on selective membrane permeability (Hernandez et al, 2001; De Gara et al., 2003) by lipid peroxidation and activity of enzymes bound to the membrane (Del Rio et al., 2006). When there is an increase in the formation of ROS, the strategy used by plants is the activation of the antioxidant enzyme system (Gill and Tuteja, 2010), and according to Andrade et al. (2010), plants that enhance the production of antioxidant enzymes show increased tolerance to heavy metals such as Cu and Zn.

In this study, increased POD activity observed in plants grown in treatments without the addition of Cu and with the addition of low doses of Zn (up to 60 mg Zn kg^{-1}) and in SOD activity in treatments with the addition of 30 mg Cu kg^{-1} combined with the addition of low doses of Zn (up to 60 mg Zn kg^{-1}) indicate the activation of an enzyme response to prevent oxidative damage caused by Zn and Cu toxicity. In these treatments, we observed the lowest POD and SOD activity at the lowest doses of Cu and Zn applied to the soil, which indicates low production of ROS. This increased POD and SOD activity suggests that H_2O_2 is being predominantly degraded in the cell. However, decreased activity observed especially in

treatments with the addition of the highest dose of Cu (60 mg Cu kg^{-1}) combined with the addition of Zn indicates that antioxidant enzymes cannot sufficiently protect young grapevine plants, with increased ROS in tissues, which enhances oxidative damage. Symptoms of toxicity induced by high concentrations of Cu and Zn may be related to increased levels (above those considered normal) of both Cu and Zn in leaves of young grapevine plants, causing oxidative stress, as well as high contents of these elements in the roots, interfering with absorption and plant nutrition.

5. Conclusions

Young grapevines showed tolerance mechanisms to high concentrations of Cu and Zn, especially through the retention of these metals in the roots, decreasing translocation to the shoots, where toxic effects could be more severe. Still, the highest doses of Cu and (especially) Zn in soil provide leaf Zn and Cu contents above those considered normal, resulting in toxicity to young grapevine plants. In the soil assessed in this study, Cu and Zn contents available in the soil above 25 mg kg^{-1}, obtained through the application of Cu and Zn doses higher than 30 mg kg^{-1}, caused reduction in dry matter production of young grapevine plants due to impaired functioning of the photosynthetic apparatus and change in plant antioxidant enzyme activity. Thus, the high Cu levels observed in acidic sandy soils with low organic matter content grown with young grapevine may have their toxic effect enhanced as Zn content is increased in soil, due to the use of zinc-based fungicides in place of traditional copper-based fungicides.

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Table 1. Physical and chemical characteristics of the 0.0-0.15 m layer in a Typic Hapludalf soil under natural field (data from Tiecher et al. (2016b)). Values represents a mean of three replications.

Physical and chemical characteristics	Natural field
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 ($cmol_c$ 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})	132
Available P by Mehlich-1 (mg kg^{-1})	7.0
Available Fe by EDTA (mg kg^{-1})	21.9
Available Mn by EDTA (mg kg^{-1})	40.7
Exchangeable Ca ($cmol_c$ kg^{-1})	0.5
Exchangeable Mg ($cmol_c$ kg^{-1})	0.2
CEC_{ef} *, $cmol_c$ kg^{-1}	1.4
$CEC_{pH\ 7.0}$ **, $cmol_c$ kg^{-1}	3.2

*CEC_{ef} = Effective cation exchange capacity; **$CEC_{ph\ 7.0}$ = Cation exchange capacity at pH 7.0.

Table 2. Significance of the effects of the experimental factors and their interactions resulting from analysis of variance (ANOVA) and the effects of the Cu doses.

Variable	Effects of ANOVA			Cu doses (mg kg^{-1})[a]		
	Cu doses	Zn doses	Cu × Zn	0	30	60
Production parameters						
Shoot dry matter	**	****	ns	4.2 ±1.6^{A}	3.1 ±1.5^{B}	3.3 ±1.8^{B}
Root dry matter	*	****	ns	6.0 ±2.7^{A}	5.1 ±2.2^{AB}	4.6 ±2.6^{B}
Soil parameters						
Available Cu content	****	****	*	5 ±1^{C}	30 ±2^{B}	56 ±3^{A}
Available Zn content	****	****	****	54 ±52^{B}	57 ±57^{B}	62 ±62^{A}
Element concentration						
Cu in shoot dry matter	****	ns	ns	5 ±0.7^{B}	15 ±2.5^{A}	15 ±1.8^{A}
Cu in root dry matter	****	ns	ns	7±4^{C}	79±20^{B}	192±88^{A}
Zn in shoot dry matter	ns	****	ns	259 ±181	245 ±152	262 ±205
Zn in root dry matter	**	****	****	923±684^{B}	886±692^{B}	1252±1306^{A}
Translocation parameters						
Cu content ratio in shoot/root	****	ns	ns	1.0±0.38^{A}	0.2±0.03^{B}	0.1±0.04^{B}
Zn content ratio in shoot/root	ns	****	ns	0.6±0.9	0.5±0.6	0.5±0.6
Cu distribution in shoot/root	****	ns	*	38 ±10^{A}	11±1^{B}	7±2^{B}
Zn distribution in shoot/root	ns	****	ns	24 ±17	21 ±12	22 ±13
Chlorophyll a *fluorescence parameters*						
F_o	ns	ns	ns	146 ±21	144 ±31	156 ±39
Fm	ns	ns	ns	663 ±78	671 ±78	702 ±132
Fv/Fm	ns	**	ns	0.78 ±0.03	0.79 ±0.03	0.78 ±0.04
Fv/F_o	ns	***	ns	3.6 ±0.5	3.7 ±0.5	3.6 ±0.7
Y (II)	ns	ns	ns	0.28 ±0.06	0.30 ±0.05	0.27 ±0.05
ETR_{1500}	ns	***	ns	60 ±17	66 ±19	60 ±19
Photosynthetic pigment parameters						
Chl *a*	****	**	****	3.01 ±0.87^{A}	1.93 ±0.54^{B}	2.22 ±0.78^{B}
Chl *b*	****	**	****	1.23 ±0.35^{A}	0.84 ± 0.24^{B}	0.99 ±0.31^{B}
Carotenoid	****	ns	****	0.76 ±0.23^{A}	0.57 ±0.14^{B}	0.72 ±0.17^{A}
Total Chl	****	**	****	4.25 ±1.21^{A}	2.77 ±0.77^{B}	3.20 ±1.08^{B}
Chl *a*/Chl *b*	*	ns	ns	2.45 ±0.17^{A}	2.30 ±0.20^{AB}	2.24 ±0.28^{B}
Carotenoid/Total Chl	**	**	ns	0.18 ±0.04^{B}	0.21 ±0.04^{AB}	0.24 ±0.07^{A}
Activity of antioxidant enzymes						
POD	***	**	*	11.8 ±5.1^{A}	7.7 ±4.8^{B}	5.8 ±3.7^{B}
SOD	****	****	***	10.2 ±4.3^{A}	6.3 ±4.2^{B}	3.5 ±2.3^{C}

[a] Cu doses followed by the same letter are not significantly different according to Tukey test at P <0.05.
ns, not significant.
* Significant at P<0.05.
** Significant at P<0.01.
*** Significant at P<0.001.
**** Significant at P<0.0001.

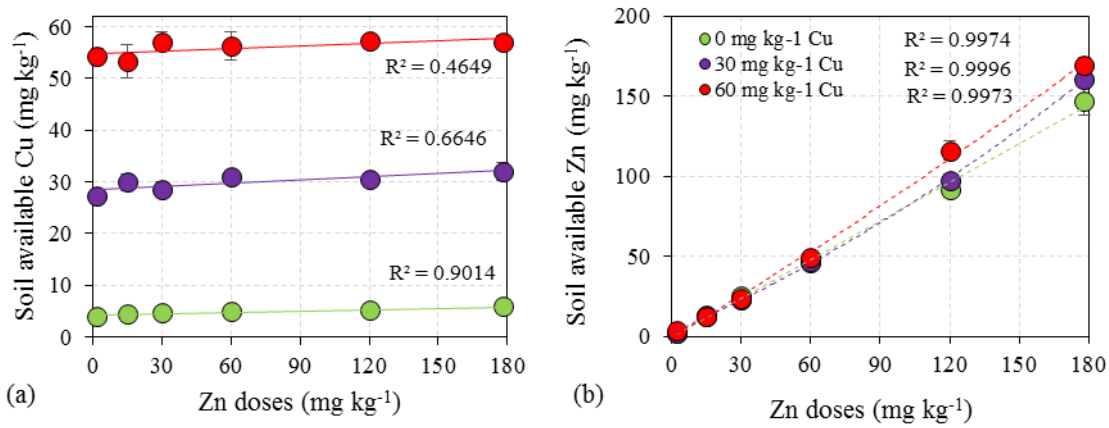

Fig. 1. Available Cu contents with the addition to soil doses of Cu (a) and the addition of increasing doses of Zn (b) to natural field soil.

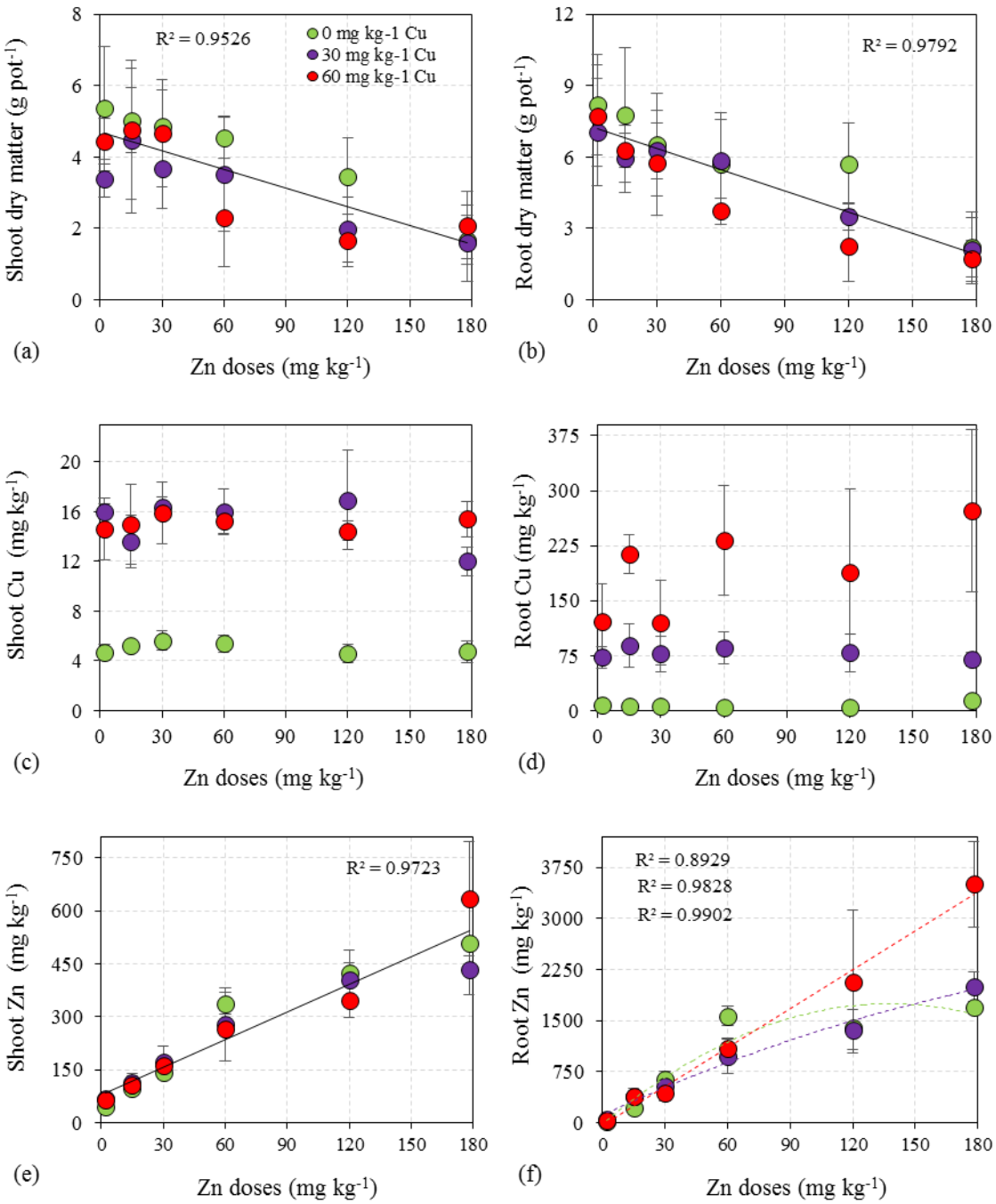

Fig. 2. Dry matter production of the shoots (a) and roots (b), total Cu content in the shoots (c) and roots (d), and total Zn content in the shoots (e) and roots (f) of young grapevine in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Continuous line in Fig. 2a, 2b, and 2f is the regression equation for the overall mean of three Cu doses following interpretation of ANOVA from Table 2, which shows no interaction between Cu and Zn doses.

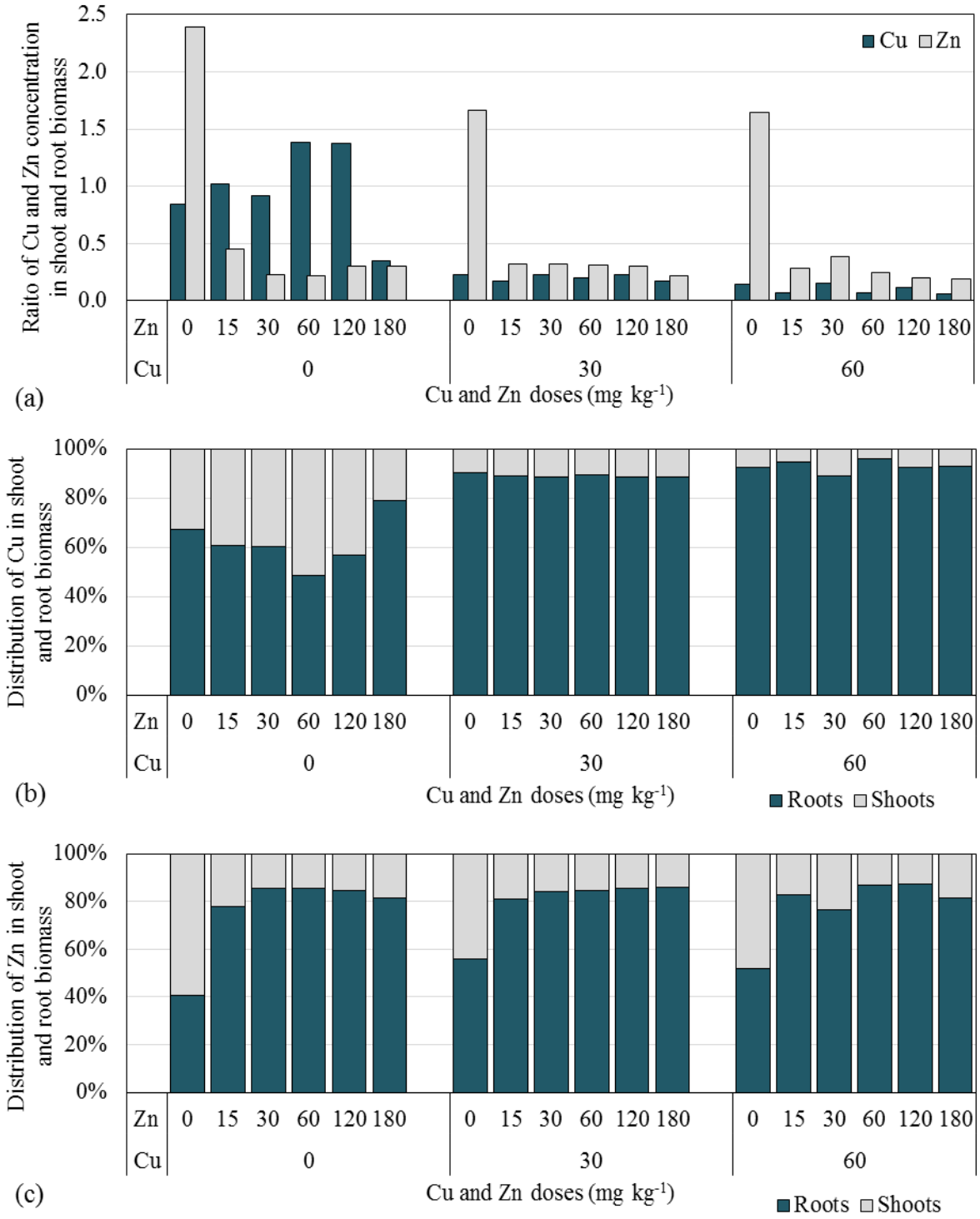

Fig. 3. Cu and Zn content ratio in shoot and root biomass (a) and Cu (b) and Zn (c) distribution in root and shoot biomass of young grapevines grown in sandy acidic soil with interaction of high doses of copper and zinc (content in tissue × dry matter) (n = 3).

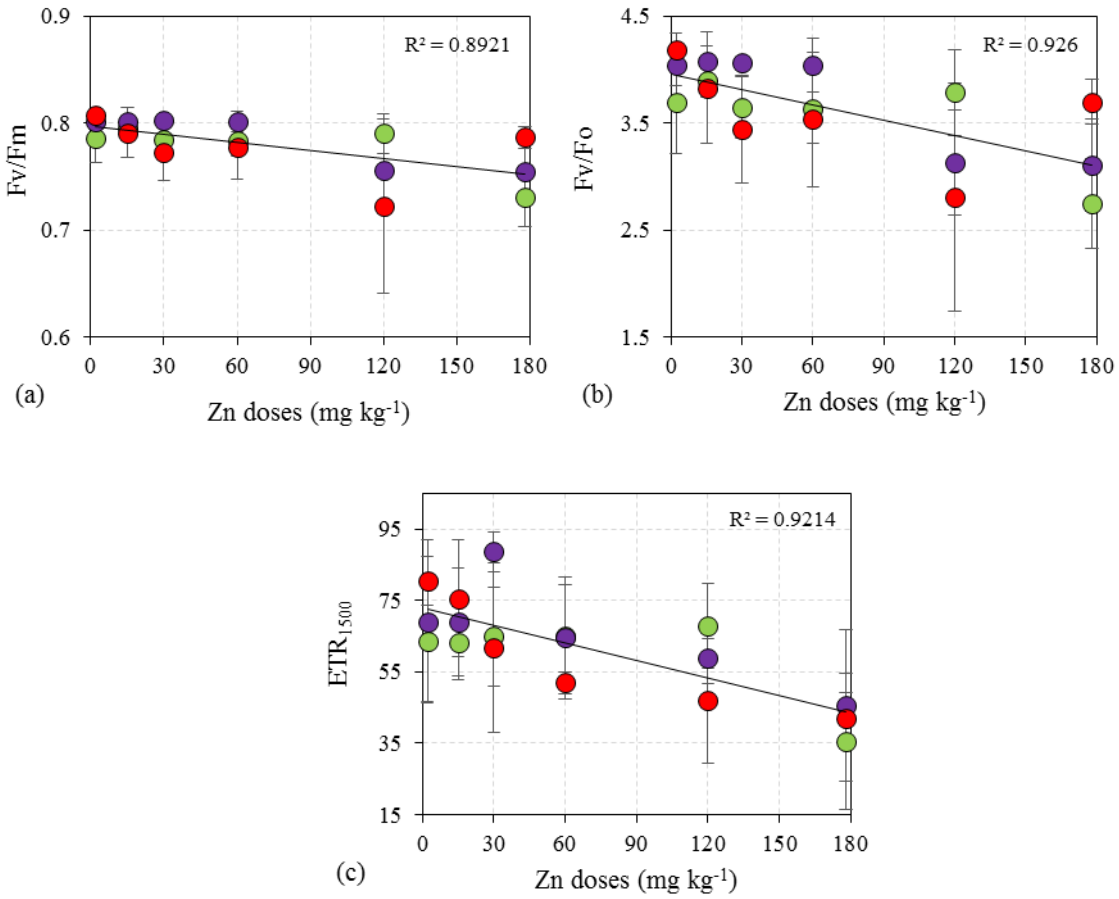

Fig. 4. Maximum quantum yield of PSII (Fv/Fm) (a), basal quantum yield of PSII (Fv/Fo) (b), and electron transport rate (ETR_{1500}) (c) in young grapevine leaves in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Continuous line in Fig. 4a, 4b, and 4c is the regression equation for the overall mean of three Cu doses following interpretation of ANOVA from Table 2, which shows no interaction between Cu and Zn doses.

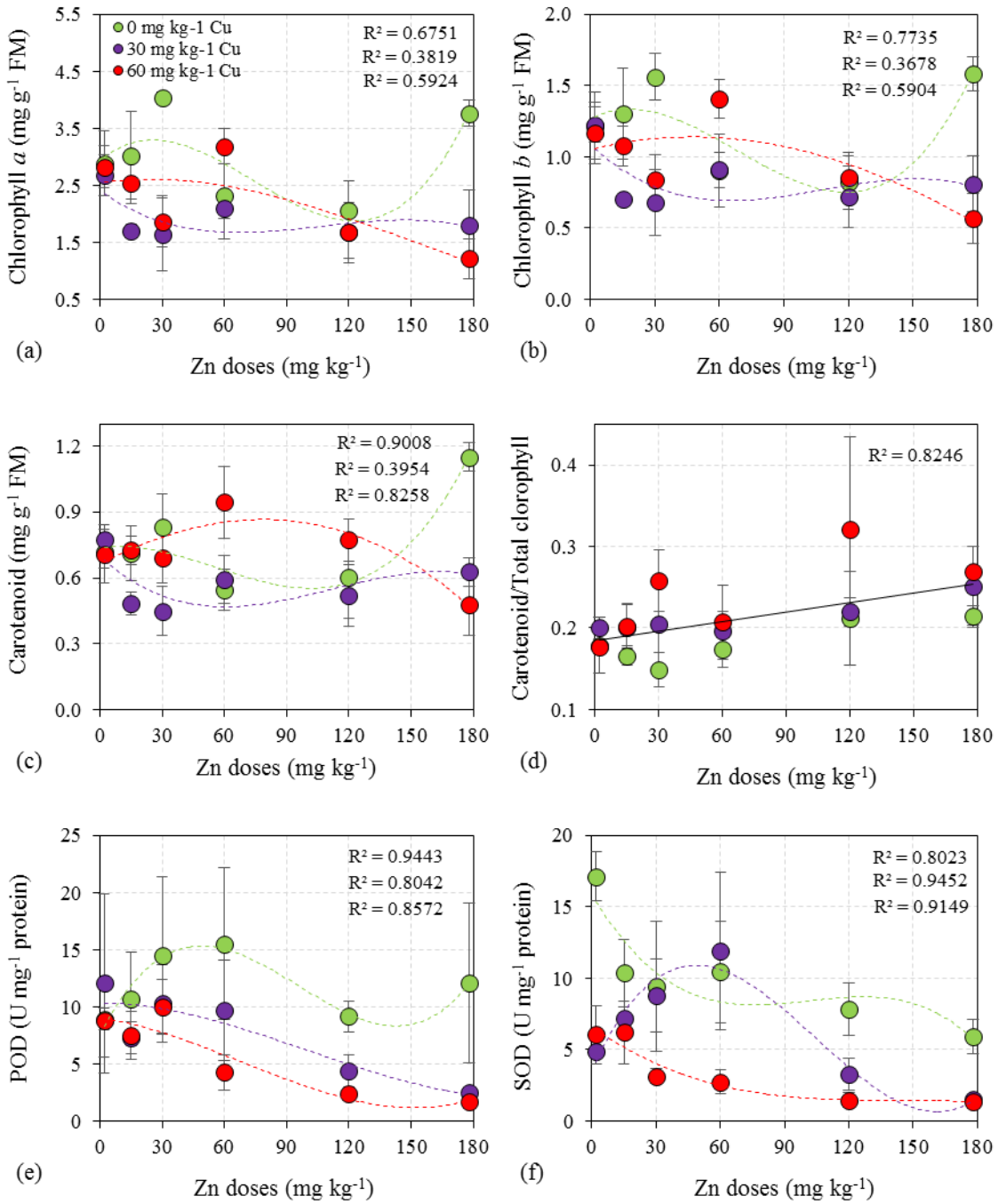

Fig. 5. Chlorophyll *a* (a), chlorophyll *b* (b), and carotenoid (c) content, carotenoid/chlorophyll ratio (d), peroxidase (POD) (e) and superoxide dismutase (SOD) (f) activity in young grapevine leaves in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Continuous line in Fig. 5d is the regression equation for the overall mean of three Cu doses following interpretation of ANOVA from Table 2, which shows no interaction between Cu and Zn doses.

5.3 ESTUDO III

The interaction of high copper and zinc doses in acid soil changes the physiological state and development of the root system in young grapevines (*Vitis vinifera*)

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The interaction of high copper and zinc doses in acid soil changes the physiological state and development of the root system in young grapevines (*Vitis vinifera*) [1]

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Abstract

Old vineyards may present high copper (Cu) content in the soil due to the frequent application of Bordeaux fungicide to control leaf fungal diseases. Thus, many wine makers replace copper fungicides by those made of zinc (Zn) and it leads to the accumulation of these two elements in vineyard soils, fact that may potentiate the occurrence of physiological disorders and morphological changes in the plant root system. The aim of the current study was to assess the effects of high Cu and Zn contents in a sandy acid soil on the physiological state and development of the root system in young grapevines. The soil was taken from a vineyard from Southern Brazil, and then it was sieved and had its acidity and P and K contents corrected. Next, the soil was subjected to the application of 0 and 120 mg Cu kg^{-1}; each one of these doses was added with 0, 120 and 240 mg Zn kg^{-1}, thus totaling six treatments. After the treatments were added to the soil samples, 2.4 kg of soil was stored in rizobox-type containers. One young grapevine plant was transplanted to each box. The transplanted plants were cultivated for 60 days in greenhouse. The accumulation of root and shoot dry matter was set after the experimental period, as well as the Cu and Zn contents in the roots and shoot, the root system morphology, the chlorophyll *a* fluorescence, the photosynthetic pigments, the gas exchanges and the superoxide dismutase enzyme activity (SOD). Young grapevines presented mechanisms to tolerate high Cu and Zn concentrations in the soil, mainly through the retention of such metals in their roots to diminish translocation to the shoot. However, the highest Cu and Zn doses led to grapevine plant growth decrease, to gas exchange alterations and to photochemical efficiency reduction associated with photosynthetic pigment decrease and to non-photochemical energy dissipation increase. Moreover, the SOD activity was greater in intermediate Zn doses, thus indicating antioxidant system activation. Thus, the combination between high Cu and Zn concentrations in vineyard soils will enable minimizing the toxic effects of these metals to young grapevines cultivated in these soils.

Keywords: Vineyard soils. Gas exchange. Chlorophyll fluorescence. Root system. Photosynthetic pigments. Heavy metals.

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1. Introduction

Wine regions can be located in areas presenting climatic conditions favorable for the occurrence of leaf fungal diseases, mainly mildew (*Plasmopara viticola*) (Brunetto et al., 2017). Thus, it is worth regularly using fungicides made of copper (Cu), such as the Bordeaux fungicide, to protect grapevines from fungi. Since successive leaf applications of these fungicides are conducted in the same area for many years, Cu accumulates in the soil, mainly in the upper soil layers (Brunetto et al., 2014). Annual applications can reach 30 kg Cu ha^{-1} (Casali et al., 2008), so, the Cu content in the soil can often reach toxic levels to plants (Miotto et al., 2014; Girotto et al., 2016; Guimarães et al., 2016). Accordingly, some winemakers start using fungicides made of zinc (Zn) to the detriment of copper fungicides. Therefore, one can observe the combination of high Cu and Zn contents in vineyard soils in traditional wine regions worldwide, fact that has been gaining researchers' attention in the last decades (Fernandez-Calviño et al., 2012; Brunetto et al., 2014; 2016; Cambrollé et al., 2015; Tiecher et al., 2016a; 2016c; Tiecher et al., 2017).

Plants grown in soils presenting Cu and Zn content availability higher than other soils may strongly accumulate these heavy metals in their tissue and develop toxicity symptoms (Kabata-Pendias, 2011). Overall, there are alterations, such as growth reduction, mean diameter increase, abnormal branching, root thickening and darkening, and root length reduction, in the root system morphology of plants subjected to high heavy metal concentrations (Ambrosini et al., 2015; Bochicchio et al., 2015; Guimarães et al., 2016). Moreover, there are negative biochemical and physiological responses due to such disturbances, which vary depending on the plant species and organ, on element concentration and on tissue tolerance to high Cu and Zn levels (Cambrollé et al., 2012; Mateos-Naranjo et al., 2013).

High Cu and Zn concentrations in plant tissues may negatively influence the gas exchanges performed by plants, since they often induce the occurrence of considerable effects on the net photosynthesis and stomatal conductance rates (Cambrollé et al., 2012; 2015). Yet, Cu excess may affect the membrane transport function and the ionic channels (Janicka-Russak et al., 2008). Such changes may lead to increase in the non-specific permeability of the membrane, which results in nutritional imbalance (Cambrollé et al., 2013). Moreover, it is possible observing oxidative stress caused by unbalance between antioxidant responses and the increased production of reactive oxygen species (ROS) through Fenton reaction (Girotto et al., 2013). The excess of Zn may also generate oxidative stress, because of its influence on the antioxidant defense system of plants (Gratão et al., 2005), as well as negatively affect the

photosynthetic efficiency by inhibiting chlorophyll biosynthesis. Such inhibition leads to leaf chlorosis and carbon assimilation reduction, since it compromises the electron transport chain (De Magalhães et al., 2004; Castiglione et al., 2007; Chen et al., 2008; Dhir et al., 2008), fact that helps inhibiting plant growth (Girotto et al., 2013).

Although adult grapevines are capable of remaining productive for decades in soils presenting high heavy metal concentrations (Miotto et al., 2014), the implantation of young grapevine plants in contaminated soils after production decline, or even the eradication of old vineyards, may lead to lower plant growth and seedling percentage, to leaf chlorosis, and to Cu and Zn accumulation in the tissues, all factors resulting in economic losses. Some studies have been performed in order to assess the phytoremediation potential of some annual species such as maize and black oats, which can be used between soil lines in vineyards contaminated with Cu and Zn (Tiecher et al., 2016a; 2016b; 2016c). However, studies about the interaction between Cu and Zn in soil presenting high accumulation of heavy metals, and their associated effects on young vineyards transplanted in sandy soils showing low organic matter content (OMS), remain scarce. The aim of the current study was to assess the effects of high Cu and Zn contents in a sandy acid soil from Southern Brazil on the physiological state and development of the root system in young grapevines

2. Materials and methods

2.1. Experiment description

The soil used in the experiment was classified as Typic Hapludalf (Soil Survey Staff, 2006); it was collected in natural non-anthropized field (30º47’23.5’’S and 55º22’7.0’’W), which presented naturally low Cu and Zn concentration. The area was located adjacent to a vineyard in Campanha Gaúcha region, Santana do Livramento County – RS (Southern Brazil). The soil was collected from the 0.00–0.15 m layer in August 2015. Subsequently, it was air dried, ground, sieved through 2mm mesh and reserved. Soil physicochemical characterization is shown in Table 1.

The soil was divided and placed in 10-kg containers. Soil acidity was corrected through the addition of 670 and 830 mg kg^{-1} of $CaCO_3$ and MgO, respectively. The total of 40 mg P kg^{-1} and 100 mg K kg^{-1} were added to the samples in their triple superphosphate and KCl forms, respectively. The correctives and fertilizers were added and mixed to the soil; next, distilled water (100 mL kg^{-1} of soil) was added to each container. The whole content in the containers was immediately homogenized and incubated for 60 days. The soil remained in

sealed plastic bags throughout the corrective and fertilizer incubation period to avoid water evaporation.

Subsequently, the total soil volume was dried, homogenized and separated in rizobox-type containers filled with 2.4 kg of dry soil; three repetitions were made per each treatment. The experiment design was completely randomized. The following doses were applied to the containers: 0 mg Cu kg^{-1} + 120 mg Zn kg^{-1}, 0 mg Cu kg^{-1} + 240 mg Zn kg^{-1}, 120 mg Cu kg^{-1} + 0 mg Zn kg^{-1}, 120 mg Cu kg^{-1} + 120 mg Zn kg^{-1} and 120 mg Cu kg^{-1} + 240 mg Zn kg^{-1}, besides the control treatment, which was not added with Cu and Zn. The Cu and Zn doses were based on previous studies conducted by Tiecher et al. (2016a; 2017). These doses resulted in the following Cu and Zn contents extracted through EDTA Contents of Cu and Zn available to plants were extracted with EDTA according to the methodology described by Chaignon et al., (2009): 3.8 mg Cu kg^{-1} and 95.3 mg Zn kg^{-1}, 1.5 mg Cu kg^{-1} and 197.0 mg Zn kg^{-1}, 102.4 mg Cu kg^{-1} and 12.9 mg Zn kg^{-1}, 114.3 mg Cu kg^{-1} and 100.2 mg Zn kg^{-1}, 119.2 mg Cu kg^{-1} and 203.5 mg Zn kg^{-1}, and 3.3 mg Cu kg^{-1} and 13.1 mg Zn kg^{-1}, respectively. The Zn doses were added to the soil in the form of a solution containing $ZnSO_4.7H_2O$ (60%) and $ZnCl_2$ (40%), whereas Cu doses were added to it in the form of $CuSO_4.5H_2O$. Salts were diluted in distilled water and individually applied to the soil in each container. The soil was, then, stirred to achieve homogenization. The soil was incubated in greenhouse for 60 days. The evaporated water was replaced every 2 days in order to keep humidity at 80% of the maximum water retention capacity (MWRC).

Young grapevine plants were obtained by micropropagation and acclimatized in sterile substrate. Explants were cultivated for 30 days in test tubes in a growth room with a temperature of 25±1 °C, photoperiod of 16 hours daylight and photosynthetically active radiation of 75 µmol photons m^{-2} s^{-1}. The plants were subsequently transferred into 200 mL plastic pots containing horticultural substrate and thin vermiculite (1:1 ratio) and cultivated for another 30 days in a growth room. Later, the plants were transferred for another 15 days into a greenhouse and kept with 50% interference of the radiation. Finally, in November 2015, one young grapevine plant (*Vitis vinifera*) was transplanted - after incubation - to each rizobox containing 2.4 kg of soil, and cultivated for 60 days. The rizoboxes were 20 cm wide, 32 cm tall and 4 cm deep, they had lateral wood frames and the inner sides were coated with acrylic. Both outer rizobox faces were covered with aluminum paper to avoid light incidence inside the soil mass, thus simulating real soil condition. Rizoboxes were allocated on wood supports, at inclination 45°, throughout the grapevine cultivation. During the experimental period, the temperature in greenhouse was kept at 25±5 ºC, the relative air humidity was

approximately 70%, and there was no interference in the photoperiod (natural conditions). Soil humidity was kept at 80% MWRC through daily irrigation to replace the evapotranspirated water. The total of 50 mL of a solution containing 50 mg N kg^{-1}, was added to the samples 15 days after transplant (DAT).

2.2. Dry matter production and total Cu and Zn content in the tissues

Roots were manually separated from the soil 60 DAT and washed in 0.02 mol L^{-1} EDTA. The shoot was cut right at soil surface and a sample of leaves was immediately conditioned in liquid N_2 and stored in ultrafreezer at -80°C up to the moment to perform the biochemical analyses. The rest of the shoot and root samples were dried in forced air circulation oven at ± 65°C, until they reached constant mass. Root and shoot dry matters were measured on precision scale.

The samples were ground in Wiley mill after drying; the total Cu and Zn content in the shoot and roots was set after the digestion of 0.1 g of tissue in 3.0 mL of HNO_3 65% P.A. (Vetec, ≤0.2 ppm of heavy metals) and 1 mL of $HClO_4$ 70% P.A. (Vetec, ≤0.1 ppm of Cu and Zn) (Embrapa, 2009). Sample digestion was conducted in open system by using the digester block Velp Scientifica (Milan, Italy), which was heated at 130°C for 4 hours. The total Cu and Zn content was analyzed in atomic absorption spectrophotometer with detection limit of 1.5 µg L^{-1} of Cu and Zn (AAS, Perkin Elmer AAnalyst 200, USA). To calibrate the AAS, a calibration curve was made from a standard Merck Certipur 1,000 mg L^{-1} of Cu and Zn.

2.3. Morphological analysis of the root system

Roots were manually separated from the soil during plant collection 60 DAT**.** Roots were added to transparent containers containing distilled water; the radicles were manually separated from one another. Next, roots were scanned in the WinRhizo Pro 2013 software, which was coupled to an EPSON Expression 11000 scanner equipped with additional light (TPU), at 600 dpi definition for roots. Root surface area length, diameter and volume were set.

2.4. Photosynthetic pigment extraction and quantification

Leaf discs were collected from the same leaf used to set the gas exchanges at 60 DAT in order to analyze the photosynthetic pigments in each treatment repetition. The discs were frozen in liquid N_2 and stored at -80°C. The chlorophylls *a* (Chl *a*) and *b* (Chl *b*), as well as carotenoid contents were quantified according to the methodology described by Hendry &

Price (1993). Subsequently, the leaf discs were macerated with liquid N_2 and the tissue was homogenized in 5 mL of 80% acetone; samples were then transferred to 15-mL falcon tubes and centrifuged at 4,000 g for 4 minutes at 25°C (3-18K Centrifuge, Sigma, Germany). Finally, supernatant absorbance was set at 480, 645 and 663 nm in order to determine the Chl *a*, Chl *b* and carotenoid contents, respectively, using spectrophotometer model SF325NM (Bel Engineering, Italy). The pigment contents were calculated according to the methodology suggested by Lichtenthaler (1987).

2.5. Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence analysis was conducted 60 DAT in pulse amplitude modulated (PAM) fluorometer JUNIOR-PAM (Walz, Germany). Fluorescence reading was performed in three repetitions for each treatment in the first completely expanded leaf of the plant between 08:00 and 10:00 am (Tiecher et al., 2016a). Leaves were pre-adapted to the dark for 30 minutes before the measurements were taken in order to set the initial fluorescence (Fo). Next, the sample was subjected to saturating light pulse (10,000 µmol m^{-2} s^{-1}) for 0.6s to find the maximum fluorescence (Fm). The maximum quantum yield of photosystem II (PSII) (Fv/Fm) was set through induction curve, as well as the basal quantum efficiency of PSII (Fv/Fo), the electron transport rate (ETR), non-photochemical energy dissipation (NPQ) and the effective quantum yield of PSII (Y(II)).

2.6. Superoxide dismutase (SOD) activity

Samples for the enzymatic analyses applied to leaf tissues were collected 60 DAT; completly expanded leaves from each treatment repetition were used in the experiment. These leaves were immediately conditioned in liquid N_2 and stored in ultrafreezer at -80°C up to the moment to perform the enzyme extraction. The total of 0.5 g of leaves, previously macerated in liquid N_2 and homogenized in 3 mL of sodium phosphate buffer solution 0.05 M (pH 7.8) containing 1 mM EDTA and 0.5 % Triton X-100, were used for the extract. The homogenized material was centrifuged at 13,000 g for 20 minutes at 4°C (3-18K Centrifuge, Sigma, Germany). The supernatant was used in the enzyme activity and protein content assays (Zhu et al., 2004; Bradford, 1976). The SOD activity (EC 1.15.1.1) was set according to the spectrophotometric method (Femto 800XI, Brazil) described by Giannopolitis and Ries (1977). One unit of SOD was defined as the amount of enzyme that inhibits nitroblue tetrazolium (NBT) photoreduction by 50% (Beauchamp and Fridovich, 1971).

2.7. Gas exchanges

Gas exchanges were measured 60 DAT with the aid of an open circuit system incorporating an infrared gas analyzer (LI-6400XT LI-COR, Inc., Lincoln, NE, EUA). The readings were applied to each treatment repetition in the first completely expanded leaf. Net photosynthetic rate (A) and intercellular CO_2 concentration (Ci) were measured at ambient CO_2 concentration 400 µmol mol^{-1}, temperature 20/25°C, relative humidity 50% ± 5% and photon flux density 1,000 µmol m^{-2} s^{-1}.

2.8. Statistical analysis

First, the experimental data were subjected to normality and variance homogeneity assessment through Shapiro-Wilk *W* test. All variables that were not normally distributed were tested again for normality after transformation using log, power, square root, cube root, inverse, and inverse square root functions. The best transformation for normality was selected, and the variables were transformed accordingly before entering to ANOVA. Next, they underwent variance analysis conducted by means of completely randomized two-factorial model:

$$Yijk = \mu + Ci + Zj + CZij + error\ (i, j)$$

Wherein: μ = general mean of the experiment; C = adopted Cu dose (i = 1, 2); Z = adopted Zn dose (j = 1, 2, 3) and error = experimental error. The means of Cu and Zn doses were compared through Tukey test at $P<0.05$ whenever factors were significant. All statistical analyses were performed using software R (R Core Team, 2013).

3. Results

3.1. Dry matter production and total Cu and Zn content in tissues

There was interaction between Cu and Zn in the root and shoot dry matter production of grapevine plants (Table 2). The higher the Cu and Zn doses added to the soil, the worse the decrease in dry matter production (Figures 1a and b). The lowest root and shoot dry matter production was recorded at dose 240 mg Zn kg^{-1}, which was not affected by the addition of the Cu doses.

There was interaction between the Cu and Zn contents in the roots (Table 2). Regarding treatments based on Cu addition, the higher the Zn dose, the higher the Cu contents

in the roots (Figures 1c, d). On the other hand, Cu contents in the shoot just responded to Cu addition to the soil (Figure 1e), whereas Zn contents in the shoot responded to Zn addition to the soil (Figure 1f); in both cases, the higher the dose applied to the soil, the higher the Cu and Zn contents in plant shoot.

3.2. Morphological analyses of the root system in grapevines

All the morphological analyses applied to the root system of young grapevines presented interaction between the Cu and Zn doses (Figure 2); there was reduction in variables “root length”, and “root area” and “root volume” due to Cu addition to the soil, given the interaction between doses 0 and 120 mg Zn kg^{-1}. On the other hand, treatments based on the addition of 240 mg Zn kg^{-1} did not present any difference resulting from Cu application (Figures 2a, b, d). Decreased values for these variables were also recorded due to the Zn addition to the soil, mainly in the treatment comprising the addition of 0 mg Cu kg^{-1} + 240 mg Zn kg^{-1}.

3.3. Photosynthetic pigments

The interaction between the Cu and Zn doses applied to the soil affected the Chl *a*, Chl *b* and carotenoid contents, as well as the Chl *a*/Chl *b* ratio (Figures 3 a, b, c, d), which presented the lowest values in the treatment recording interaction between doses 0 mg Cu kg^{-1} + 240 mg Zn kg^{-1} (Figure 3 d). It was possible observing reduction in pigment contents due to the addition of 120 mg Cu kg^{-1} when there was interaction with dose 120 mg Zn kg^{-1}. On the other hand, just the treatment using the addition of interaction between 0 mg Cu kg^{-1} and 240 mg Zn kg^{-1} showed diminished Chl *a* and Chl *b* contents due to Zn addition. The carotenoid contents were lower in treatments based on the addition of 120 mg Cu kg^{-1} + 120 mg Zn kg^{-1}, 0 mg Cu kg^{-1} + 240 mg Zn kg^{-1} and 120 mg Cu kg^{-1} + 240 mg Zn kg^{-1}, because of Zn addition to the soil.

3.4. Chlorophyll *a* fluorescence, (SOD) enzyme activity and gas exchanges

The maximum quantum yield of PSII (Fv/Fm) presented reduction due to Cu addition to the soil (Figure 4a), whereas the non-photochemical dissipation (NPQ) in grapevine leaves diminished due to increase in the Zn doses added to the soil (Figure 4b). The other variables - related to chlorophyll *a* - assessed for grapevine plants did not show differences between treatments. The SOD enzyme activity increased in treatments based on the addition of intermediate Zn doses (120 mg Zn kg^{-1}) (Figure 4c), whereas treatments using

the highest Zn doses showed reduction in such activity. On the other hand, the addition of Cu doses did not change the SOD enzyme activity.

Gas exchanges performed by grapevine plants did not evidence differences resulting from the addition of Cu doses to the soil (Figures 4 d). However, the net photosynthetic rate (A) were lower in the treatment using the addition of the highest Zn dose (240 mg Zn kg^{-1}) than in the other treatments, whereas the intercellular CO_2 concentration was not affected by the addition of both metals.

4. Discussion

The highest Cu and Zn availability in the soil recorded for treatments that have received the highest doses of these elements helped reducing root and shoot growth in grapevine plants (Figures 1a, b). Plants developed in environments presenting the highest metal concentration availability often showed toxicity symptoms, which led to reduction in dry matter production (Tiecher et al., 2016c; Tiecher et al., 2017). Such reduction may have resulted from the inhibition of plant cell elongation and division caused by the high Cu and Zn concentration in the soil solution (Hewit, 1983; Arduini et al., 1994; Jain et al., 2010). Moreover, the toxic effect may be attributed to Zn accumulation in the leaves (Cherif et al., 2011), fact that affects the normal ion homeostasis, because it influences the absorption, transport and regulation of essential ions (Wang et al., 2009). Such process results in the disruption of metabolic processes such as transpiration and photosynthesis, as well as in reduced growth (Sagardoy et al., 2009).

Leaf contents between 15-20 mg Cu kg^{-1} and 150-200 mg Zn kg^{-1} led to plant growth reduction in plant species sensitive to high heavy metal contents (Kloke et al., 1984; Kabata-Pendias, 2011). All plants cultivated in treatments added with Cu in the current study presented leaf content lower than 20 mg kg^{-1} (Figure 1e). Part of the absorbed Cu remained stuck in the plant roots (Figure 1c); the highest contents were recorded for treatments added with Zn. Such result may indicate that both elements presented the same absorption mechanisms and transport locations in plants (Kabata-Pendias, 2011). Besides, the high Cu and Zn concentrations in plant tissues induced changes in membrane properties, which affected the membrane transport function and the ionic channels (Janicka-Russak et al., 2008). These effects led to increased membrane non-specific permeability, fact that may be responsible for nutrient concentration imbalance in plants cultivated in environments presenting high heavy metal contents (Cambrollé et al., 2013), as well as favored higher Cu absorption.

On the other hand, the Zn content in the shoot of plants was higher than 200 mg kg^{-1} in all treatments added with Zn (Figure 1f). Such result may be attributed to the high Zn amounts found in the soil solution, along with similarity between the ionic radii of bivalent cations such as Cu, Manganese (Mn) and Iron (Fe). Thus, Zn ions can replace any of these divalent cations, be absorbed by the roots (Tewaru et al., 2008) and, subsequently, translocate to the shoot. Once these ions get inside the plant, the excess of Zn can change the physiological balance due to the competition with other cations found in different locations (Tewaru et al., 2008), such as the primary absorption sites or nutrient transport zones in the roots (Yang et al., 2011).

The Cu and Zn accumulation predominantly happened in grapevine roots presenting low translocation to the shoot (Figures 1c, d). This is observed by the translocation factor (TF = content in shoot/content in roots), which was 0.14, 0.13, 0.15, 0.12, 0.10, and 0.06 for Cu, and 0.10, 0.10, 0.12, 0.08, 0,15, and 0.10 for Zn, in the treatments control, 0 mg Cu kg^{-1} + 120 mg Zn kg^{-1}, 0 mg Cu kg^{-1} + 240 mg Zn kg^{-1}, 120 mg Cu kg^{-1} + 0 mg Zn kg^{-1}, 120 mg Cu kg^{-1} + 120 mg Zn kg^{-1} and 120 mg Cu kg^{-1} + 240 mg Zn kg^{-1}, respectively. It indicates that the root system in grapevine plants has mechanisms capable of preventing and/or reducing the translocation of the Cu and Zn excess to the shoot (Tiecher et al., 2017). The low Cu and Zn content in the shoot in comparison to the contents found in the roots may be associated with the interaction between these elements and the proteins and amino acids in root tissues (Kabata-Pendias, 2011). Moreover, Cu has strong interaction with the sulfhydryl groups of enzymes and proteins found in root cell apoplasts, which leads to enzyme activity inhibition or to nutrient replacement, thus resulting in deficit of other nutrients in plants (Yruela, 2005; Kabała et al., 2008).

The increased concentration of heavy metals in plant root cells derives from the intracellular production of chelating substances such as organic acids and phytochelatins. Metals are subject to chelation by organic molecules in the cytosol. Then, it can be sequestrated in the vacuole through a process known as compartmentalization (Souza et al., 2011), which helps diminishing the damages in the metabolic processes. Moreover, the higher Cu and Zn retention in grapevine roots may be a survival strategy, since plants keep lower metal concentrations in the most sensitive photosynthetic organs, such as the shoot, and store most of the metal excess in non-sensitive organs such as the roots (Yang et al., 2011; Ambrosini et al., 2015).

The toxicity symptoms resulting from Cu and Zn excess in the root growth environment may change between plant species. Overall, there is growth reduction in roots

presenting abnormal branches, thickening, dark color shades and elongation reduction (Ambrosini et al., 2015) similar to effects observed in the herein assessed young grapevine plants (Figures 2a, b, c, d). Among the morphological alterations, root mean diameter increase is often reported for plants cultivated in soil presenting heavy metal excess (Bochicchio et al., 2015). Such response may result from alterations in root development such as premature differentiation of the endoderm and cortical tissue lignification, or from root elongation reduction (Arduini et al., 1995). Besides, this response may be related to increased Zn availability, which stimulates auxin synthesis in plants (Henriques et al., 2012). Such process can cause auxin homeostasis disorder (Overvoorde et al., 2010) and have significant effect on root growth and development; moreover, it may result in considerable reduction in the number of lateral roots and root hair elongation reduction, as it was also recorded by Quint et al. (2009) in *Arabidopsis* IAR4 plants. It is worth highlighting that root thickening in plants cultivated in soil presenting high heavy metal contents is a defense strategy that often happens along with root shortening and lateral root number increase (Potters et al., 2007). Copper and zinc were less absorbed by the roots, besides being slowly translocated to the shoot (Sofo et al., 2013; Bochicchio et al., 2015).

The photosynthetic pigment decrease (Figures 3a, b, c), induced by the high Zn contents in the plant root-growth environment, can be attributed to the adverse effect from Zn excess on the electron transport during photosynthesis, which causes chlorophyll synthesis decrease or increase in its degradation (Cambrollé et al., 2012). Moreover, Cu excess can lead to degradation in the structure and in the inner content of the chloroplast (Ciscato et al, 1997; Ouzounidou, 1996). The magnesium central ion (Mg) can also be replaced by Cu in the chlorophyll molecule, and it can impair chlorophyll synthesis and energy capture (Küpper et al., 2002). The increased Chl *a*/Chl *b* ratio in some treatments (Figure 3d), in its turn, can be associated with PSII protection, since Chl *b* is found at high concentrations in the antenna complex (Pearcy and Yang, 1998). An efficient way to reduce the energy reaching PSII lies on the partial destruction of these pigments (Eckhardt et al., 2004). The Chl *b* absorbs the luminous energy at wavelength in the red range, consequently, it is more energetic than Chl *a*. Accordingly, the Chl *b* concentration reduction acts in such way that a smaller amount of energy is captured by the antenna complexes. Such process reduces the chlorophyll excitation state and prevents the formation of reactive oxygen species (ROS) capable of irreversibly damaging proteins, lipids and photosynthetic membrane pigments (Horton & Ruban, 2004). Carotenoids are also important protectors of the photosynthetic system. They can absorb the excess of energy from the excited chlorophylls and dissipate it in the form of heat, preventing

the production of ROS and, consequently, the degradation of the chloroplast membrane (Mittler, 2002; Havaux, 2014).

Young grapevine plant stress can be seen through the reduced maximum quantum efficiency of PSII (Fv/Fm) in treatments added with Cu doses (Figure 4a), and it characterizes a photoinhibition state (Alves et al., 2002). This Fv/Fm reduction can be related to chlorophyll content reduction in plant leaves (Cambrollé et al., 2015); moreover, due to Fv/Fm reduction, the smaller amount of energy captured by the plant through the antenna complex is used to transport electrons and to produce dry matter, fact that helps explaining the lower dry matter production in these treatments. Many authors relate Fv/Fm ratio reduction to the stress caused in many plant species cultivated in environments showing heavy metal excess (Cambrollé et al., 2015; Tiecher et al., 2017). The highest non-photochemical dissipation values (NPQ) in treatments based on non-additions and on the addition of intermediate Zn doses (Figure 4b) indicate that plants have dissipated light in the form of heat, thus protecting leaves from damages induced by luminosity (Maxwell & Johnson, 2000; Cambrollé et al., 2012), such as increased ROS formation. Besides, NPQ reduction in treatments added with the highest Zn doses can be linked to the lowest photosynthetic pigment contents recorded for leaves in this plant, fact that reduces the amount of energy absorbed by antenna complexes and, consequently, reduces the need of dissipating energy via heat.

Although Zn does not present redox reaction, its excess can induce ROS formation, which damages organic molecules. The ROS naturally form inside the cells, mainly in the chloroplasts and mitochondria, where the electron transport takes place (Ferreira et al., 2015). However, ROS can have their production significantly increased under high heavy metal contents in the soil. When ROS formation increases, the strategy used by plants lies on activating the enzymatic antioxidant system (Gill & Tuteja, 2010); SOD, and its isoforms, belong to the primary defense system in plants and work to eliminate O_2^- radicals. With regard to the present study, the increased SOD activity in plants subjected to treatments added with intermediate Zn dose (Figure 4c) indicates the activation of an enzymatic response to prevent oxidative damages caused by Zn toxicity (Jain et al., 2010). It suggests that the produced O_2^- is predominantly degraded in the cell as an attempt to avoid oxidative stress in plant cells. However, reduction in the activity of this enzyme, in treatments added with higher Zn doses, indicates that higher Zn concentrations do not protect SOD in grapevine plants. Besides, it can lead to ROS increase in the tissues, as well as potentiate oxidative damages, fact that leads to

plant growth reduction and to lack of protection to structures such as chloroplast, as well as to photosynthetic rate reduction.

The photosynthetic activity is highly sensitive to several types of stress in plants, and most heavy metals are known to inhibit this process at varying levels (Sheetal et al., 2016). Therefore, the reduced net photosynthesis rate can be attributed to different effects caused by heavy metals on the integrity or in the photochemical function, as well as on its impact on chlorophyll concentration in leaves (Cambrollé et al., 2015). The high Zn concentrations in tissues can negatively influence gas exchanges performed by plants (Figures 4d), since they often induce significant effects on net photosynthesis rates (Cambrollé et al., 2012). Another factor able to explain photosynthesis rate reduction is the excess of heavy metals, which can diminish the activity of enzymes involved in carbon fixation, such as ribulose–1,5–bisphosphate carboxylase oxygenase, where excess of Zn can reduce enzyme activity by competition with Mg at the enzyme site of action, and then inhibit PSII activity by replacing Mn in the membranes of thylakoids (Mysliwa-Kurdziel et al., 2004; Broadley et al., 2012).

5. Conclusions

Young grapevines presented mechanisms to accumulate Cu and Zn in the root system and lower translocation of these elements to the shoot, where the cytotoxic effects from the excess of heavy metals could be maximized. Even though, the combination between high Cu and Zn contents in the soil resulted in reduced dry matter production in young grapevine plants due to morphological changes in their root system, to the length of photosynthetic apparatus functioning, to alterations in the enzyme activity, and to net photosynthesis rate by plants. Thus, the increase in Zn contents caused by the use of fungicides (made of this element) in sandy soil of low MOS content from vineyards in the *Gaúcha* Campaign region, in which high Cu contents were already recorded, can maximize the toxic effects of these metals to young grapevines cultivated in this soil.

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Table 1. Physical and chemical characteristics of the 0.0-0.15 m layer in a Typic Hapludalf soil under natural field (data from Tiecher et al. (2016b)). Values represents a mean of three replications.

Physical and chemical characteristics	Natural field
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 ($cmol_c$ 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})	132
Available P by Mehlich-1 (mg kg^{-1})	7.0
Available Fe by EDTA (mg kg^{-1})	21.9
Available Mn by EDTA (mg kg^{-1})	40.7
Exchangeable Ca ($cmol_c$ kg^{-1})	0.5
Exchangeable Mg ($cmol_c$ kg^{-1})	0.2
CEC_{ef} *, $cmol_c$ kg^{-1}	1.4
$CEC_{pH\ 7.0}$ **, $cmol_c$ kg^{-1}	3.2

*CEC_{ef} = Effective cation exchange capacity; **$CEC_{ph\ 7.0}$ = Cation exchange capacity at pH 7.0.

Table 2. Significance of the effects of the experimental factors and their interactions resulting from analysis of variance (ANOVA), and the effects of the Cu doses.

Variable	Effects of ANOVA				Shapiro-Wilk test	
	Cu doses	Zn doses	Cu × Zn	CV (%)	*W*-value	*p*-value
Production parameters						
Shoot dry matter	****	****	***	17.6	0.83	**0.004**
Root dry matter	**	****	*	27.9	0.92	0.135
Soil parameters						
Available Cu content	****	ns	ns	11.0	0.72	**<0.001**
Available Zn content	ns	****	ns	6.3	0.84	**0.007**
Element concentration						
Cu in shoot dry matter	****	ns	ns	22.5	0.85	**0.007**
Cu in root dry matter	****	****	****	11.8	0.84	**0.005**
Zn in shoot dry matter	ns	****	ns	23.9	0.88	**0.029**
Zn in root dry matter	ns	****	***	12.5	0.91	0.071
Roots parameters						
Length (cm)	****	****	****	16.6	0.79	0.001
Surface area (cm^2)	****	****	****	18.5	0.85	0.007
Average Diameter (mm)	**	ns	*	8.3	0.92	0.122
Lenght/Volume (cm/m^3)	ns	*	**	21.8	0.98	0.919
Volume (cm^3)	****	****	***	20.2	0.89	0.036
Photosynthetic pigment parameters						
Chl *a*	**	***	*	22.8	0.94	0.271
Chl *b*	**	***	**	22.6	0.94	0.306
Carotenoid	*	***	*	20.5	0.95	0.471
Total Chl	**	***	*	22.7	0.94	0.262
Chl *a*/Chl *b*	**	****	***	3.6	0.66	**<0.001**
Carotenoid/Total Chl	*	ns	ns	9.3	0.95	0.445
Chlorophyll a *fluorescence parameters*						
F_o	ns	ns	ns	21.0	0.95	0.415
Fm	ns	ns	ns	19.1	0.85	**0.007**
Fv/Fm	**	ns	ns	3.3	0.88	**0.031**
Fv/F_o	ns	ns	ns	23.3	0.99	0.993
Y (II)	ns	ns	ns	23.3	0.98	0.926
ETR	ns	ns	ns	21.0	0.98	0.926
NPQ	ns	**	ns	25.3	0.96	0.536
Activity of antioxidant enzyme						
SOD	ns	*	ns	38.7	0.55	**<0.001**
Gas exchange parameters						
A	ns	*	ns	31.7	0.94	0.274
Ci	ns	ns	ns	13.5	0.96	0.606

[a] Cu doses followed by the same letter are not significantly different according to Tukey test at P <0.05.
ns, not significant.
* Significant at P<0.05; ** Significant at P<0.01; *** Significant at P<0.001; **** Significant at P<0.0001.
Bold values indicate variables with non-normal distribution by the Shapiro-Wilk *W* test. All variables that were not normally distributed were tested again for normality after transformation using log, power, square root, cube root, inverse, and inverse square root functions. The best transformation for normality was selected, and the variables were transformed accordingly before entering to ANOVA.

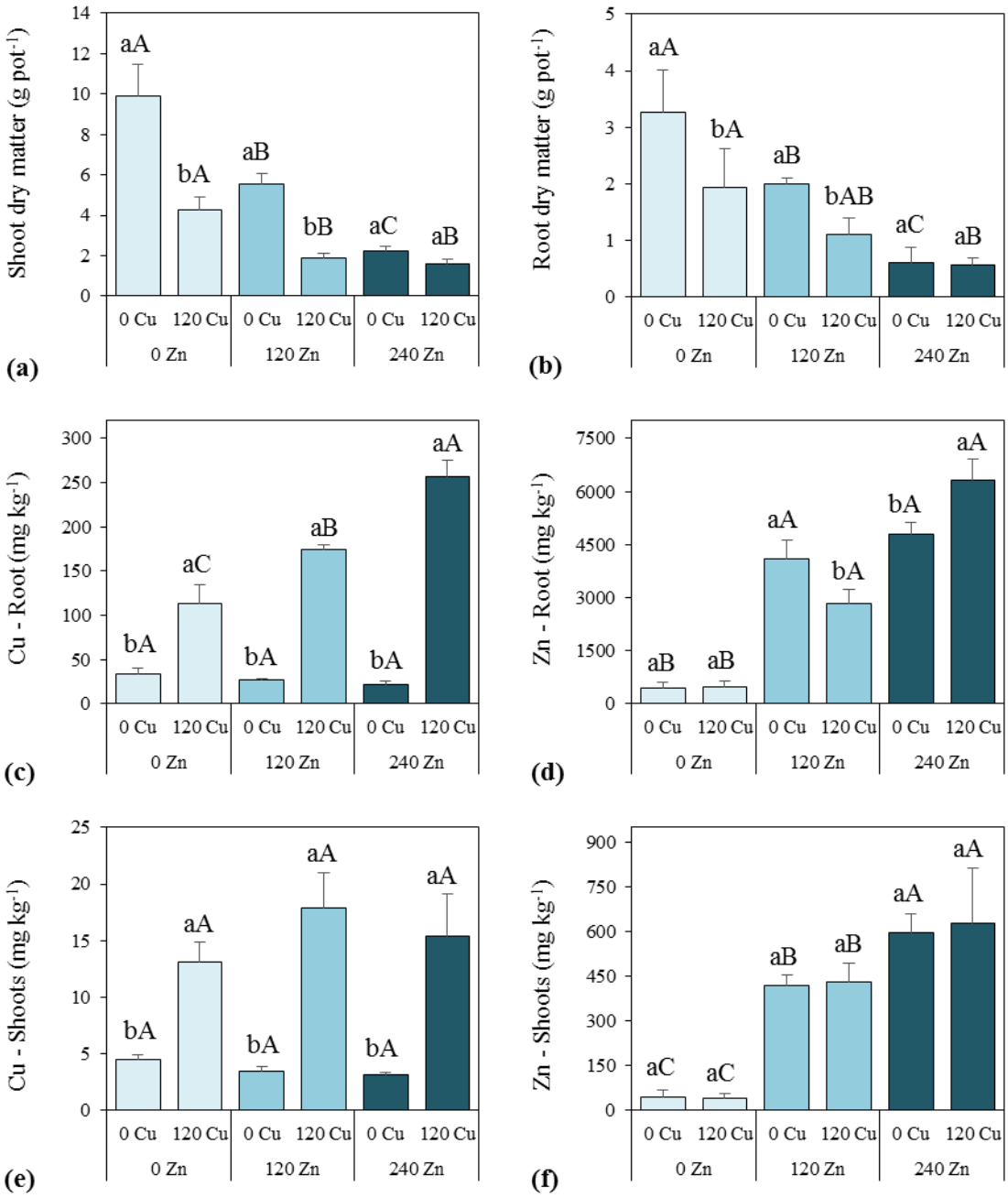

Fig. 1. Dry matter production of the shoots (a) and roots (b), total Cu content (c) and Zn content (d) in the roots of young grapevine in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn, and upper-case letters compare doses of Zn within each dose of Cu. Bars followed by the same letter are not significantly different at P <0.05 by Tukey test. Mean and standard deviation presented in each bar refer to the three replicates per treatment (n = 3).

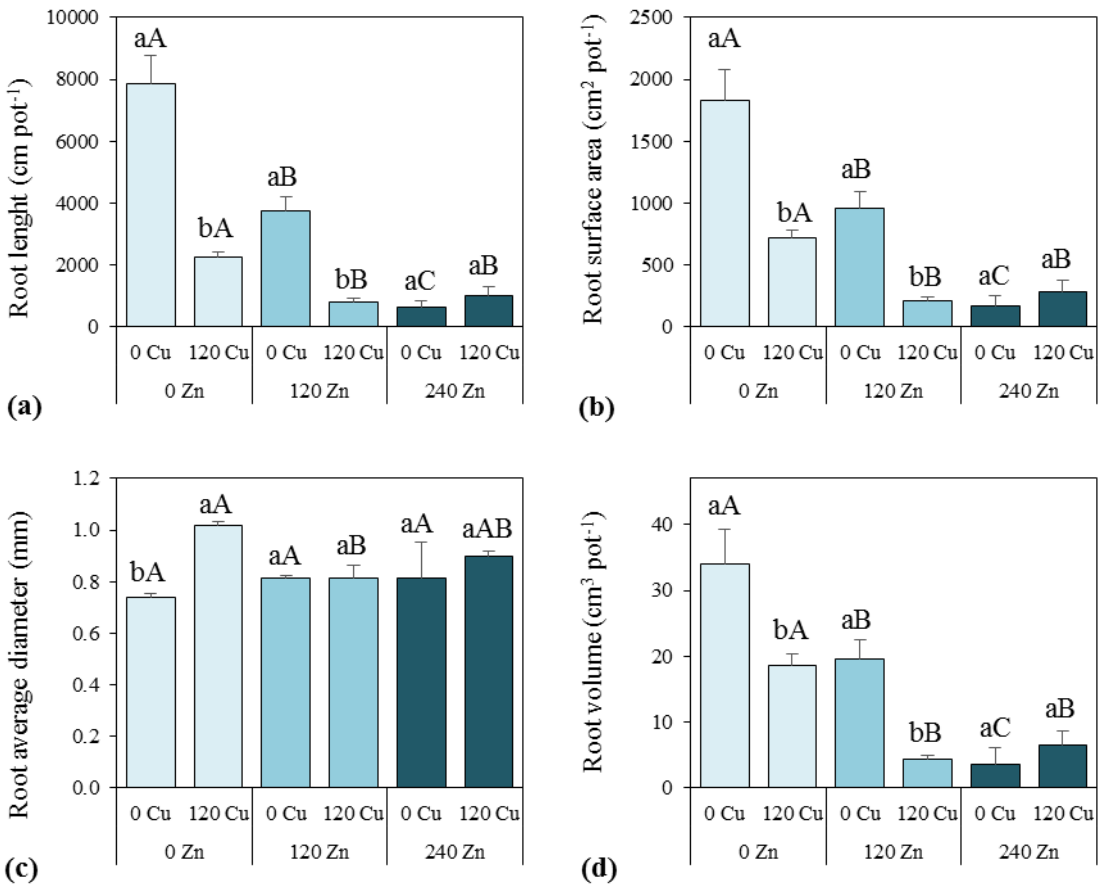

Fig. 2. Length (a), surface area (b), average diameter (c), and volume (d) in roots young grapevine in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn, and upper-case letters compare doses of Zn within each dose of Cu. Bars followed by the same letter are not significantly different at P <0.05 by Tukey test. Mean and standard deviation presented in each bar refer to the three replicates per treatment (n = 3).

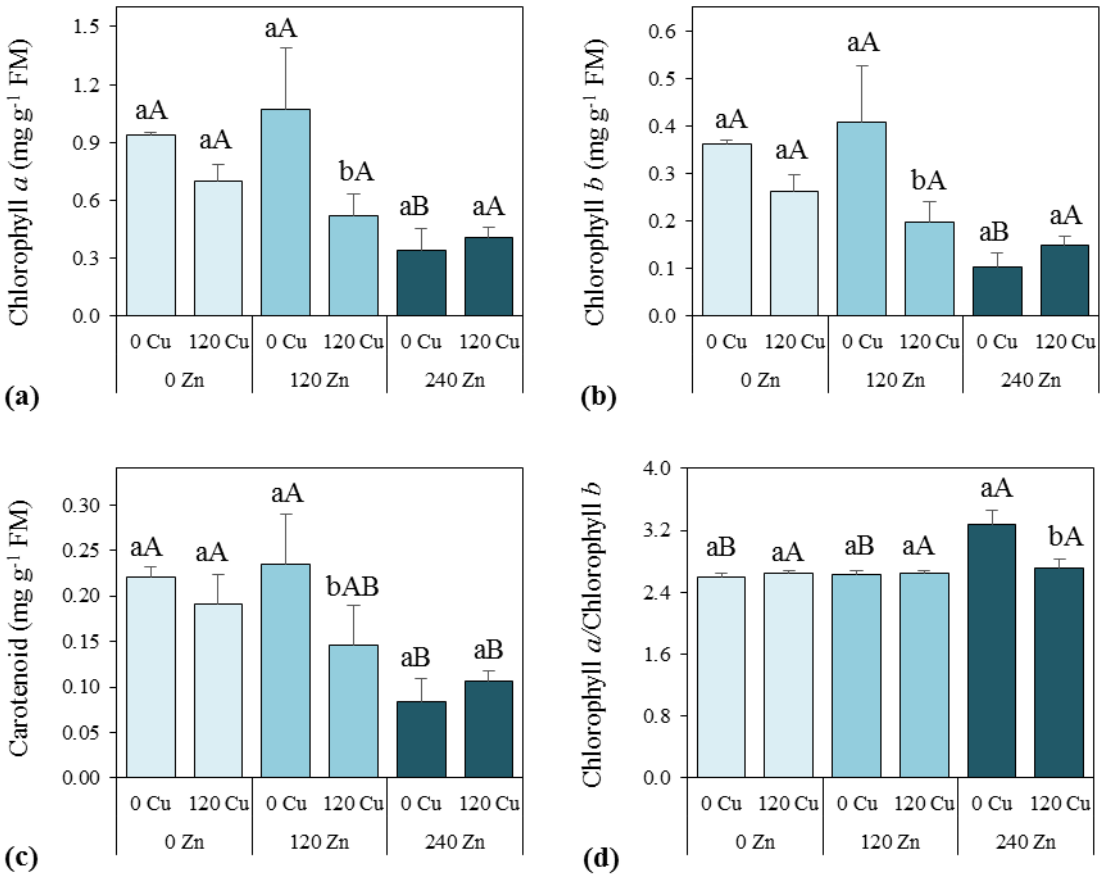

Fig. 3. Chlorophyll *a* (a), chlorophyll *b* (b), and carotenoid (c) content, and chlorophyll *a*/chlorophyll *b* ratio (d) in young grapevine leaves in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn, and upper-case letters compare doses of Zn within each dose of Cu. Bars followed by the same letter are not significantly different at $P < 0.05$ by Tukey test. Mean and standard deviation presented in each bar refer to the three replicates per treatment ($n = 3$).

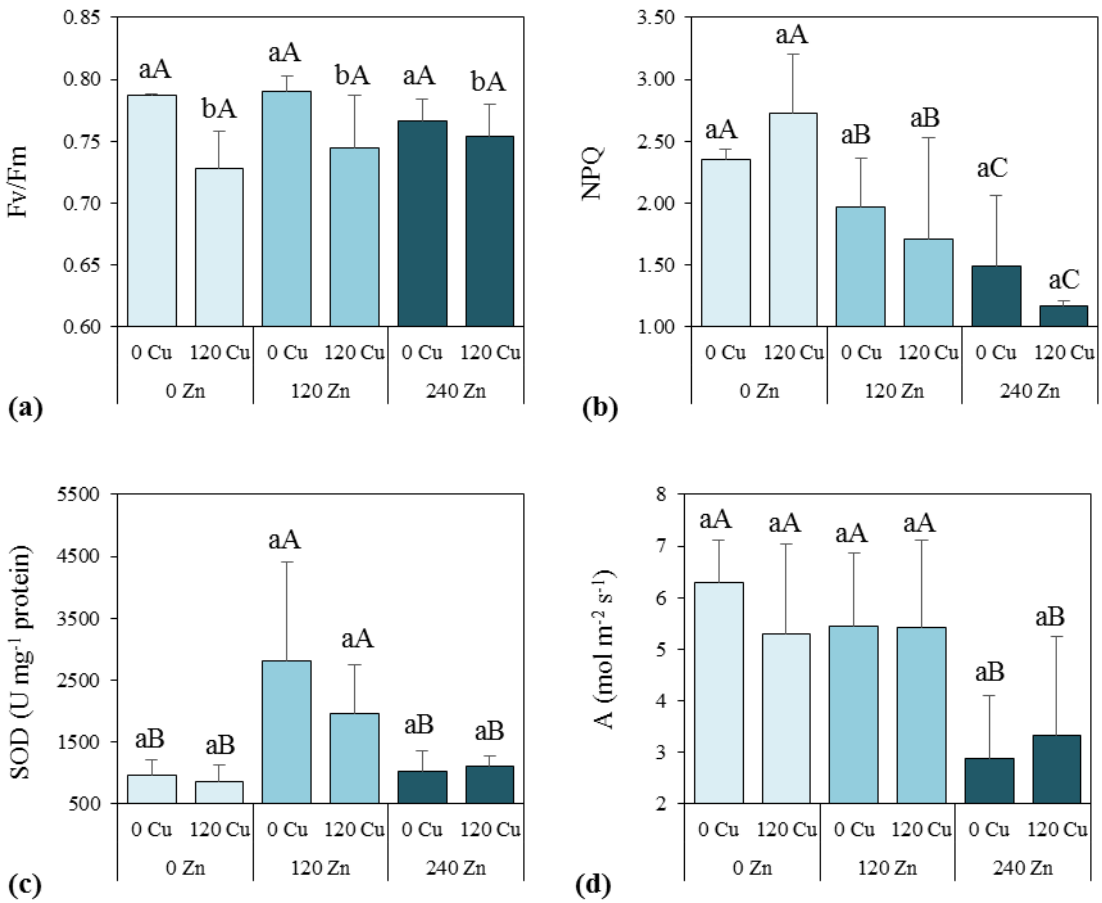

Fig. 4. Maximum quantum yield of PSII (Fv/Fm) (a), non-photochemical quenching (NPQ) (b), superoxide dismutase (SOD) (c) activity, and net photosynthetic rate (A) (d) in young grapevine leaves in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn, and upper-case letters compare doses of Zn within each dose of Cu. Bars followed by the same letter are not significantly different at P <0.05 by Tukey test. Mean and standard deviation presented in each bar refer to the three replicates per treatment (n = 3).

6. DISCUSSÃO GERAL

A utilização intensiva de fungicidas de contato que possuem Cu e Zn em sua composição, para o controle de doenças fungicas em vinhedos, invariavelmente ocasiona aumento nos teores destes metais pesados no solo. Este acúmulo pode desencadear uma série de respostas nos microrganismos e organismos vivos que crescem e se desenvolvem nestes ambientes, principalmente as plantas, devido a sua estaticidade. Desta forma, torna-se imprescindível conhecer os efeitos combinados entre os elevados teores de Cu e Zn no solo, bem como os eventuais efeitos que uma interação entre eles pode ocasionar às plantas cultivadas em solos de vinhedos contaminados, em especial as videiras, mas também as plantas de cobertura que são utilizadas nas entre-linhas dos vinhedos.

No estudo I, realizado com plantas de aveia preta, observou-se que os teores de Cu disponível no solo foram maiores quanto maior a dose de Cu adicionada ao solo, enquanto os teores de Zn disponível no solo aumentaram conforme a dose de Zn adicionada ao solo. Não foi observada diferença na disponibilidade de Cu em função da adição de Zn no solo nem na disponibilidade de Zn em função da adição de Cu no solo, embora tenham sido observadas diferenças na absorção dos elementos pelas plantas. Por outro lado, no estudo II, além do aumento nos teores de Cu e Zn em função da adição destes elementos no solo, o teor de Cu disponível no solo também aumentou em função do aumento da dose de Zn adicionada ao solo, enquanto o teor de Zn disponível no solo também foi incrementado com aumento da dose de Cu aplicada, especialmente para as doses de Zn maiores que 120 mg kg^{-1}.

Isto demonstra que a disponibilidade destes dois metais pesados no solo depende da interação competitiva deles pelos sítios de adsorção (PÉREZ-NOVO et al., 2009, 2011). Embora se saiba que o Cu é adsorvido principalmente na matéria orgânica do solo (TIECHER et al., 2013), enquanto o Zn é adsorvido aos grupos funcionais da fração mineral (PÉREZ-NOVO et al., 2011), em concentrações elevadas de ambos pode ocorrer a competição pelos mesmos sítios sortivos, com o consequente aumento da disponibilidade de Cu e Zn no solo. Isso ficou evidente no estudo realizado por Tiecher et al. (2016b), que adicionaram juntamente Cu e Zn a um Argissolo Vermelho e encontraram redução de 14,7% na capacidade máxima de adsorção (CMA) de Cu e 71,0% na CMA de Zn.

Além disso, foi observado aumento nos teores de Cu e Zn disponíveis no solo dos tratamentos do estudo com as plantas de videira (estudo II) em relação ao estudo com as plantas de aveia preta (estudo I). Isto pode ter ocorrido principalmente devido à atividade

microbiana do solo que pode ter resultado em mineralização da MOS e, consequentemente, em aumento na disponibilidade de Cu e Zn, visto que foi utilizado o mesmo solo para ambos os estudos e, após o cultivo da aveia preta, o solo permaneceu incubado até o transplante das mudas de videira e a condução do segundo experimento.

Os estudos I, II e III, realizados com as plantas de videira e aveia preta, apresentaram redução na produção de matéria seca e acúmulo de Cu e Zn nos tecidos das plantas. A redução na produção de matéria seca pode ser resultado da inibição do alongamento e divisão celular causada pela elevada concentração de Cu e Zn na solução do solo (ARDUINI et al., 1994; HEWIT, 1983; JAIN et al., 2010). Além disso, o efeito tóxico pode ser atribuído à acumulação de Zn nas folhas (CHERIF et al., 2011), que afeta a homeostase iônica normal, por interferir na absorção, transporte e regulação de íons essenciais (WANG et al., 2009), resultando na ruptura dos processos metabólicos, como a transpiração e fotossíntese, o que reduz o crescimento das plantas (SAGARDOY et al., 2009).

Embora as plantas de videira e de aveia preta tenham apresentado incremento nos teores de Cu e Zn nos tecidos, as videiras apresentaram mecanismos mais eficientes para diminuir a translocação, especialmente de Zn, das raízes para a parte aérea. Enquanto as plantas de aveia preta cultivadas nos solos dos tratamentos com adição de Zn continham nas raízes pouco mais de 60% do total de Zn absorvido pelas plantas, as videiras cultivadas nestes mesmos tratamentos apresentaram mais de 80% do metal absorvido pelas plantas retido nas raízes. Esta menor translocação pode ocorrer devido à produção intracelular de substâncias quelantes, como ácidos orgânicos e fitoquelatinas. Desta forma, os metais são ligados a quelantes no citosol e, posteriormente, o quelato é sequestrado no vacúolo, através do processo conhecido como compartimentalização (SOUZA et al., 2011). Além disso, pode ter ocorrido uma maior lignificação dos tecidos das raízes das plantas de videira, bem como maior diferenciação das células da endoderme, ocasionando o espessamento das paredes celulares destas células, resultando em maior retenção do metal nas raízes.

O acúmulo de Cu ocorreu predominantemente nas raízes das plantas de videira e aveia preta, sem que tivesse ocorrido uma translocação proporcional para a parte aérea. Isto indica que o sistema radicular dessas plantas possuem mecanismos que podem prevenir e/ou reduzir a translocação do excesso de Cu para a parte aérea (GIROTTO et al., 2014). O menor teor de Cu em relação ao teor de Zn na parte aérea pode estar associado à baixa mobilidade do Cu nas plantas, uma vez que o mesmo possui forte interação com os grupos sulfidrila de enzimas e proteínas nos apoplastos das células da raiz, além de apresentar forte interação com o

nitrogênio (N) das proteínas (KABAŁA et al., 2008; KABATA-PENDIAS, 2011; YRUELA, 2005).

O estresse nas plantas de todos os experimentos pode ser observado também pela redução na eficiência quântica máxima do PSII (Fv/Fm), bem como nos demais parâmetros da fluorescência da clorofila *a* avaliados, o que caracteriza um estado de fotoinibição crônica (ALVES et al., 2002). Além disso, indica uma redução na proporção de centros de reação abertos (MAXWELL; JOHNSON, 2000), possivelmente por causa da diminuição dos teores de clorofila das folhas (CAMBROLLÉ et al., 2012). Devido a isso, uma menor quantidade da energia captada pela planta, através dos complexos antena, é utilizada para reduzir o carbono e produzir matéria seca, pois os processos de dissipação da energia absorvida são complementares. Assim, quando há aumento de dissipação da energia na forma de fluorescência, por consequência ocorrerá redução da dissipação da energia via processo fotoquímico, com redução na formação de ATP e NADPH e da assimilação de carbono (KRAUSE; WEIS, 1991).

As plantas de aveia preta e videira apresentaram redução nos teores de pigmentos fotossintéticos nas folhas. Segundo Cambrollé et al. (2012), a diminuição dos pigmentos fotossintéticos, induzida pelas elevadas concentrações de Zn no meio de crescimento das raízes das plantas, pode ser atribuída ao efeito adverso do excesso do Zn no transporte de elétrons na fotossíntese, causando diminuição da síntese de clorofila ou incremento da sua degradação. Aliado a isto, o Cu em excesso pode provocar a degradação da estrutura e do conteúdo interno do cloroplasto (CISCATO et al, 1997; OUZOUNIDOU, 1996), além de substituir o íon central de Mg por Cu na molécula de clorofila, prejudicando a síntese de clorofila (KÜPPER et al., 2002).

O aumento na atividade das enzimas avaliadas nos tratamentos com a adição de doses intermediárias de Cu e Zn, indica a ativação de uma resposta enzimática para prevenir danos oxidativos, ocasionados pelos metais pesados (JAIN et al., 2010) e sugere que as EROs estão sendo predominantemente eliminadas da célula. Contudo, nos tratamentos com a adição das doses mais elevadas de Cu e Zn foi observada diminuição da atividade enzimática, indicando que as mesmas podem não proteger suficientemente as plantas dos danos oxidativos. Devido a isso, podem ser observados sintomas de toxicidade induzida pelas elevadas concentrações de Cu e Zn no solo, os quais podem estar relacionados ao aumento acima dos níveis considerados normais tanto de Cu, como de Zn, nas folhas das plantas, ocasionando estresse oxidativo e propiciando menor crescimento e acúmulo de biomassa pelas plantas.

As análises morfológicas do sistema radicular das videiras jovens apresentaram redução do comprimento, área e volume de raízes devido à adição de Cu e Zn no solo. Plantas cultivadas em ambientes com elevadas concentrações de metais pesados, geralmente apresentam redução no crescimento das raízes com ramificações anormais, espessamento, coloração escura e redução do alongamento (AMBROSINI et al., 2015). Estas alterações morfológicas podem ser observadas na Figura 5, que compara o efeito das doses de Cu e Zn sobre o crescimento radicular de videiras jovens. O aumento do diâmetro médio das raízes é um dos principais sintomas observados em plantas sob condições de estresse ocasionado por metais pesados, sendo frequentemente relatado em estudos científicos (BOCHICCHIO et al., 2015). Isto pode ser consequência de alterações no desenvolvimento da raiz, como a diferenciação prematura da endoderme e a lignificação dos tecidos corticais, ou consequência da redução do alongamento radicular (ARDUINI et al., 1995).

Figura 5: Ápice radicular de videiras jovens cultivadas durante 60 dias no solo do tratamento controle (sem adição de Cu e Zn) (a), com a adição de 120 mg Cu kg^{-1} e 120 mg Zn kg^{-1} (b) e 120 mg Cu kg^{-1} e 240 mg Zn kg^{-1} (c).

As plantas de videiras jovens também apresentaram redução na taxa fotossintética, taxa de transpiração e condutância estomática de CO_2 em função do excesso de Zn no solo. As elevadas concentrações Zn nos tecidos das plantas podem reduzir a taxa de fotossíntese líquida devido a alterações na função fotoquímica, bem como à redução da concentração de clorofila nas folhas (CAMBROLLÉ et al., 2015). Outro fator que pode explicar a redução da taxa de fotossíntese é que o excesso de metais pesados pode diminuir a atividade de enzimas envolvidas na fixação de carborno (MYSLIWA-KURDZIEL et al., 2004), além de promover o fechamento estomático e consequentemente a redução da condutância estomática (PAULA et al., 2015). Desta forma, uma menor quantidade de carbono é assimilada, diminuindo o crescimento das plantas cultivadas sob condição de estresse ocasionado pelo excesso de Cu e Zn no solo. Estas respostas observadas nas plantas de aveia preta e videiras jovens indicam

que algumas ações práticas devem ser adotadas pelos viticultores nos vinhedos da região da Campanha Gaúcha.

Dentre as medidas a serem implementadas está a necessidade da adoção de práticas de manejo que beneficiem manutenção ou mesmo o incremento dos teores de MOS nos solos dos vinhedos, algo fundamental para reduzir a disponibilidade do Cu, o que pode minimizar o potencial de toxidez às videiras ou plantas de cobertura (BRUNETTO et al., 2014; MIOTTO et al., 2014). Para isto, podem ser adotadas medidas simples, como a utilização de adubação orgânica a partir de recomendações técnicas, a introdução de plantas de cobertura nas linhas e entrelinhas dos vinhedos, juntamente com a deposição de folhas e resíduos da poda de inverno das videiras, o que contribui para o aporte de material orgânico no solo (TIECHER et al., 2016a). Embora seja difícil de ser alcançado nos solos da região da Campanha Gaúcha devido às características físicas dos solos, bem como às características climáticas da região, o incremento da MOS pode contribuir ainda na conservação dos solos da região da Campanha Gaúcha, os quais são extremamente frágeis e arenosos, alguns com pouco mais de 5% de argila. Este baixo teor de argila propicia também baixos teores de óxidos de ferro, que são um dos principais adsorventes de metais pesados no solo, o que contribui para a maior disponibilidade de Cu e Zn no solo. Sendo assim, a manutenção do pH do solo próximo ao ideal para a cultura da videira, que é de 6,0 (CQFS-RS/SC, 2016) é fundamental. Isto porque valores de pH mais elevados contribuem para a redução da disponibilidade de Cu e Zn no solo, contribuindo para a redução a toxicidade destes elementos, além de melhorar a disponibilidade de outros nutrientes às plantas.

Também se faz necessário reduzir o aporte de Cu e Zn no solo via fungicidas, o que contribui para não aumentar os teores disponíveis destes elementos no solo, mantendo-os abaixo de concentrações que podem ocasionar toxidez às plantas. Para isso, deve-se lançar mão, primeiramente, do controle preventivo de doenças, adotando-se medidas que melhorem a aeração e insolação da copa das plantas, com o intuito de reduzir o tempo de molhamento foliar, que afeta diretamente na incidência de doenças. Para tanto é importante a utilização de espaçamento entre plantas e entre linhas adequados, evitar o cultivo em áreas de baixada, proporcionar adubação equilibrada às plantas, realizar podas verdes para a retirada do excesso de ramos e folhas, dentre outros tratos culturais que contribuem para a redução na incidência de doenças. Através disso, pode-se reduzir substancialmente o número de aplicações de fungicidas a cada ciclo da cultura.

Em um segundo momento, em caso de necessidade de utilização de fungicidas para o controle de doenças, deve-se adotar a rotatividade de princípios ativos e, especialmente, de

modos de ação dos fungicidas. Para isto, o produtor poderá utilizar os tradicionais fungicidas de contato, que possuem Cu e Zn na sua composição, como a calda bordalesa e o Mancozeb, respectivamente. Contudo, é importante que sejam incluídos no manejo do controle de doenças também fungicidas sistêmicos, os quais muitas vezes apresentam maior efeito residual, o que reduz o número de aplicações, e controle satisfatório das doenças. Através da adoção destas práticas de manejo, as quantidades de Cu e Zn adicionadas aos solos de vinhedos poderiam ser significativamente reduzidas, evitando a ocorrência de altos teores destes metais pesados no solo.

7. CONCLUSÕES GERAIS

As plantas de aveia preta e videiras jovens apresentaram tolerância às elevadas concentrações de Cu e Zn no solo, o que pode ter ocorrido devido à retenção destes metais nas suas raízes, diminuindo a translocação para a parte aérea, onde os efeitos tóxicos poderiam ser mais severos. Ainda assim, as doses mais elevadas de Cu e, especialmente, de Zn no solo proporcionam teores foliares acima dos níveis considerados normais nas plantas, resultando em toxicidade às plantas de aveia preta e videiras jovens. Já a combinação da dose mais elevada de Cu (60 mg Cu kg^{-1}) com as doses mais baixas de Zn (15 e 30 mg Zn kg^{-1}), pode ter contribuído para amenizar os efeitos tóxicos do Cu, o que foi observado especialmente nas plantas de aveia preta, onde a adição de Zn no solo pode ter contribuído para suprir as exigências nutricionais da cultura.

A combinação de altos teores de Cu e Zn no solo resulta em redução da produção de matéria seca das plantas de aveia preta devido ao comprometimento do funcionamento do aparato fotossintético e à alteração da atividade enzimática das plantas. Além disso, as plantas de videiras jovens apresentaram alterações morfológicas no sistema radicular e nas trocas gasosas realizadas pelas plantas. Desta forma, solos ácidos e arenosos de vinhedos, com baixo teor de MOS e contaminados com Cu, poderão ter seu efeito tóxico às plantas de videiras jovens e aveia preta incrementados à medida que o teor de Zn for aumentando no solo, devido à utilização de fungicidas à base de Zn, em substituição aos tradicionais fungicidas cúpricos.

8. PERSPECTIVAS DE ESTUDOS FUTUROS

i) Estudar os efeitos do Cu e Zn sobre a anatomia e morfologia do sistema radicular de videiras jovens e plantas que coabitam os vinhedos, bem como avaliar as alterações na atividade de enzimas antioxidantes nas raízes e suas relações com as alterações morfoanatômicas;

ii) Validar os estudos realizados nesta obra em experimentos realizados a campo, com plantas de videiras jovens e que coabitam os vinhedos, a fim de confirmar se as respostas obtidas em condições controladas refletem as condições de campo;

iii) Realização de estudos a campo com a utilização de amenizantes da toxidez de metais pesados no solo, como o calcário, composto orgânico, silicato de cálcio e a aplicação de doses de fósforo em solos já contaminados e as respostas no crescimento e desenvolvimento de videiras jovens;

iv) Entender os efeitos que o excesso de Cu e Zn no solo, bem como a aplicação foliar de ambos os elementos via fungicidas, podem ocasionar no metabolismo e morfologia do sistema radicular de plantas adultas de videiras, uma vez que mesmo em solos contaminados as plantas adultas mantém-se produtivas;

v) Esclarecer se a ocorrência de elevados teores de Cu e Zn no solo, bem como a aplicação destes elementos via foliar, através da aplicação de fungicidas de contato, ao longo do ciclo da videira, podem conferir aumento nas concentrações de Cu e Zn nos frutos das videiras ou nos produtos oriundos do seu beneficiamento;

vi) Compreender a dinâmica da interação entre os elevados teores de Cu e Zn em diferentes tipos de solo, bem como as inter relações destes metais pesados com as frações orgânicas e mineral do solo;

vii) Avaliar a mobilidade do Cu e Zn em solos contaminados e os riscos de contaminação de águas superficiais e subsuperficiais, a partir do monitoraramento das

transferências para ambientes aquáticos destes metais pesados e dos demais nutrientes aplicados em solos de vinhedos.

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