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Edicarla Trentin

**SELEÇÃO DE PORTA-ENXERTOS DE VIDEIRAS E AMENIZANTES
COMO ESTRATÉGIAS PARA REDUZIR A FITOTOXIDEZ POR
COBRE**

Santa Maria, RS, Brasil
2020

Edicarla Trentin

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

Orientador: Prof. Dr. Gustavo Brunetto

Santa Maria – RS, Brasil
2020

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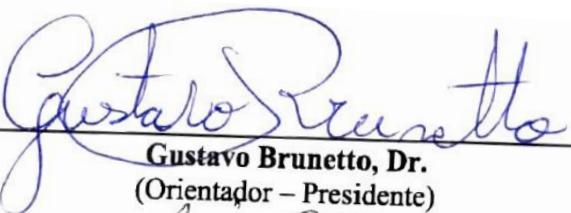
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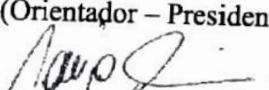
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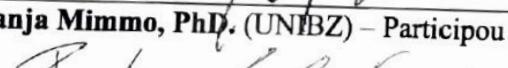
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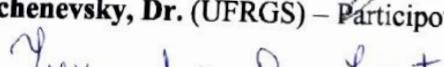
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Santa Maria, RS,
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“Lute com determinação, abrace a vida com paixão, perca com classe e vença com ousadia, porque o mundo pertence a quem se atreve e a vida é muito bela para ser insignificante!”

(Charles Chaplin)

RESUMO

SELEÇÃO DE PORTA-ENXERTOS DE VIDEIRAS E AMENIZANTES COMO ESTRATÉGIAS PARA REDUZIR A FITOTOXIDEZ POR COBRE

AUTOR: Edicarla Trentin
ORIENTADOR: Gustavo Brunetto

Frequentes aplicações de produtos químicos, como tratamento fitossanitário de prevenção e controle de doenças fúngicas foliares em vinhedos, podem ser responsáveis pela contaminação dos solos com elevados teores de cobre (Cu). O excesso de Cu no solo pode causar toxicidade aos cultivos e, consequentemente, perdas de produtividade. Desta forma, torna-se necessário desenvolver estratégias de remediação dos solos contaminados, no intuito de minimizar os efeitos deletérios causados pelo excesso de Cu e manter a viabilidade e produtividade das áreas de cultivo. Dentre essas estratégias de manejo, está o cultivo de genótipos de videira que apresentem mecanismos de tolerância à toxidez e a aplicação de amenizantes para reduzir a disponibilidade de Cu. O objetivo do trabalho foi verificar as respostas de plantas as altas concentrações de Cu, identificar genótipos de porta-enxerto de videira tolerantes a altos teores de Cu em solução, e avaliar o potencial de utilização de amenizantes como estratégia para reduzir a biodisponibilidade e toxidez de Cu em videiras jovens. Cinco estudos foram realizados. Os estudos foram conduzidos em condições controladas, em sala de crescimento (Estudos I e II) e casa de vegetação (Estudos III, IV e V). O solo utilizado no estudo V foi coletado na Campanha Gaúcha, em Santana do Livramento (RS), enquanto que os demais estudos foram conduzidos em solução nutritiva. No estudo I foram utilizadas três concentrações de Cu (0,2, 5 and 50 μM) e três condições de pH (4,5, 6,0 e 7,5), em duas plantas (*Cucumis sativus L.* e *Avena sativa* cv. Fronteira). No estudo II foram cultivados dois porta-enxertos de videira (*Vitis rotundifolia* cv. Magnolia e *Vitis vinifera* cv. Paulsen 1103) em solução nutritiva contendo concentrações crescentes de Cu (0,2; 20; 40 e 80 μM). No estudo III foram cultivados diferentes porta-enxertos de videira (Paulsen 1103, IAC 572, SO4, e Isabel) em solução nutritiva padrão e com excesso de Cu (80 μM). No estudo IV foram cultivados os mesmos porta-enxertos do estudo III, em solução nutritiva, com adição de amenizantes (fósforo e cálcio). No estudo V foi cultivado um porta-enxerto de videira (Paulsen 1103) em solo contaminado com Cu, com a aplicação de tratamentos amenizantes (calcário e vermicomposto). Em todos os estudos foram determinados parâmetros relacionados ao crescimento das plantas e parâmetros nutricionais. Também foram determinadas a atividade fotossintética (Estudos III, IV, V), fluorescência da clorofila *a* (Estudos II, III e V), concentração de pigmentos fotossintéticos (Estudos I, IV e V), análises bioquímicas (Estudos II e III), morfologia do sistema radicular (Estudos I e V), e exsudatos radiculares (Estudo I). Todas as plantas apresentaram redução nos parâmetros de crescimento e alterações nutricionais, com incremento na concentração de Cu no sistema radicular, quando cultivadas em condições de alta concentração de Cu. As plantas (Estudo I) cultivadas em solução com 50 μM de Cu e pH 4,5 sofreram maior efeito de toxicidade por excesso de Cu. Porém, houve maior exsudação de compostos orgânicos, flavonóides e compostos fenólicos, nestas plantas, o que pode atuar como uma estratégia para diminuir a biodisponibilidade e absorção de Cu pelas plantas. Porta-enxertos de videira apresentaram alterações na estrutura e funcionamento do aparato fotossintético e eficiência da atividade fotossintética, aumento de estresse oxidativo e síntese de enzimas antioxidantes, e alterações na morfologia do sistema radicular quando cultivadas em condições de alta concentração de Cu. As plantas de Paulsen 1103 e SO4 tenderam a apresentar melhor comportamento em relação ao excesso de Cu em solução, o que pode ser uma alternativa de cultivo em locais de vinhedos contaminados com excesso de Cu. A adição de Ca e P como tratamentos amenizante em solução nutritiva apresentaram efeito positivo sobre a atividade fotossintética de todos os PE em solução com alta concentração de Cu, porém, não foi suficiente para promover alterações nos parâmetros de crescimento das plantas. A aplicação de calcário foi eficiente como amenizante da fitotoxicidade de Cu no solo. Por outro lado, a aplicação de vermicomposto como tratamento amenizante não foi uma alternativa efetiva, ocasionando a morte das videiras, o que pode estar relacionado ao efeito de fitotoxicidade pelo excesso de Cu e Mn disponível em solução. Desta forma, a utilização de estratégias de manejo de forma integrada podem favorecer o cultivo e manutenção da produtividade em áreas de vinhedos contaminados por excesso de Cu.

Palavras-chave: Metal pesado. Porta-enxertos de videira. Amenizantes. Biodisponibilidade de cobre. Fitotoxicidade. Exudatos radiculares. Atividade fotossintética. Estresse oxidativo. Morfologia de raízes.

ABSTRACT

SELECTION OF GRAPEVINE ROOTSTOCKS AND AMENDMENTS AS STRATEGIES TO REDUCE COPPER PHYTOTOXICITY

AUTHOR: Edicarla Trentin
SUPERVISOR: Gustavo Brunetto

Frequent applications of chemical products, such as phytosanitary treatment to prevent and control leaf fungal diseases in vineyards, can be responsible for soil contamination with high copper (Cu) contents. Excess Cu in the soil can cause crop toxicity and, consequently, productivity losses. Thus, it is necessary to develop remediation strategies for contaminated soils in order to minimize the harmful effects caused by excess Cu and maintain the viability and productivity of the cultivation areas. Among these management strategies is the cultivation of grape genotypes that present mechanisms of tolerance to toxicity and the application of amendments to reduce Cu availability. The objective of this study was to verify plant responses to high Cu concentrations, to identify grape rootstock genotypes tolerant to high Cu contents in solution, and to evaluate the potential use of softeners as a strategy to reduce Cu bioavailability and toxicity in young vines. Five studies were conducted. They were conducted under controlled conditions, in a growing room (Studies I and II) and a greenhouse (Studies III, IV and V). The soil used in the study V was collected in the Campanha Gaúcha, in Santana do Livramento (RS), while the other studies were conducted in nutritive solution. In study I, three concentrations of Cu (0.2, 5 and 50 µM) and three pH conditions (4.5, 6.0 and 7.5) were used in two plants (*Cucumis sativus* L. and *Avena sativa* cv. Fronteira). In study II, two grapevine rootstocks (*Vitis rotundifolia* cv. Magnolia and *Vitis vinifera* cv. Paulsen 1103) were cultivated in nutritive solution containing increasing concentrations of Cu (0.2; 20; 40 and 80 µM). In study III, different grapevine rootstocks (Paulsen 1103, IAC 572, SO4 and Isabel) were grown in standard nutritive solution and with Cu excess (80 µM). In study IV, the same grapevine rootstocks used in study III were cultivated in nutritive solution with the addition of amendments (phosphorus and calcium). In study V, a grapevine rootstock (Paulsen 1103) was cultivated in soil contaminated with Cu, with the application of amendments treatments (limestone and vermicompost). In all studies, parameters related to plant growth and nutritional parameters were determined. It has also been determined photosynthesis activity (Studies III, IV, V), chlorophyll a fluorescence (Studies II, III and V), concentration of photosynthetic pigments (Studies I, IV and V), biochemical analyses (Studies II and III), morphology of the root system (Studies I and V), and root exudates (Study I). All plants presented reduced growth parameters and nutritional changes, with increased Cu concentration in the root system, when cultivated under high Cu concentration conditions. Plants (Study I) grown in solution with 50 µM Cu and pH 4.5 suffered greater toxicity effect from Cu excess. However, there has been greater exudation of organic compounds, flavonoids and phenolic compounds, in these plants, which can act as a strategy to decrease the bioavailability and absorption of Cu by plants. Grapevine rootstocks showed changes in the structure and functioning of the photosynthetic apparatus and efficiency of photosynthetic activity, increased oxidative stress and synthesis of antioxidant enzymes, and changes in the morphology of the root system when cultivated under conditions of high Cu concentration. Paulsen 1103 and SO4 plants have tended to perform better with excess Cu in solution, which can be an alternative to growing in vineyard sites contaminated with Cu excess. The addition of Ca and P as amendments treatments in nutritive solution had a positive effect on the photosynthetic activity of all rootstocks in solution with high Cu concentration, however, it was not enough to promote changes in plant growth parameters. The application of limestone was efficient as a soil amendment for Cu phytotoxicity. On the other hand, the application of vermicompost as an amendment treatment was not an effective alternative, causing the death of the grapevines, which may be related to the phytotoxic effect by the excess of Cu and Mn available in solution. In this way, the use of integrated management strategies can favour the cultivation and maintenance of productivity in areas of vineyards contaminated by Cu excess.

Keywords: Heavy metal. Grapevine rootstock. Amendments. Copper bioavailability. Phytotoxicity. Radicular exudates. Photosynthetic activity. Oxidative stress. Root morphology.

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1. INTRODUÇÃO GERAL

A viticultura é uma atividade agrícola de grande importância no Brasil. A área de produção vitivinícola brasileira ocupa cerca de 75.951 hectares, sendo produzidas 1.592.242 toneladas de uvas durante a safra de 2018 (MELLO, 2019). Da produção nacional de uvas em 2018, 51,39% foi destinada ao processamento (vinho, suco e derivados) e o restante foi consumido *in natura* (MELLO, 2019).

A maior parte da produção nacional está concentrada na região Sul do Brasil, principalmente no estado do Rio Grande do Sul, considerado como o maior produtor nacional de uva e vinhos (MELLO, 2019; INBRAVIN, 2020). As primeiras cultivares de videira, de origem americana, foram introduzidas no estado do Rio Grande do Sul em 1840. Entretanto, somente em 1875, com a chegada dos imigrantes italianos, a produção de uva e vinho começa a ganhar força e importância (INBRAVIN, 2007). Atualmente, a produção de uvas ocupa uma área de aproximadamente 47.383 hectares, sendo produzidos 822.689 toneladas de uva em 2018 (MELLO, 2019). O setor da vitivinicultura é responsável por 1% do Produto Interno Bruto (PIB) do Rio Grande do Sul (INBRAVIN, 2016). Dentro do estado do Rio Grande do Sul, as principais regiões produtoras de uva e vinho são a Serra e Campanha Gaúcha. A região da Serra Gaúcha é mais tradicional no cultivo e produção vitivinícola, sendo a principal área de produção do estado. Já a região do Campanha Gaúcha é resultante do processo de expansão da atividade no estado, em áreas anteriormente ocupadas com campo natural para pecuária de corte.

As condições climáticas das regiões produtoras de uva, principalmente na região da Campanha Gaúcha, com altos índices pluviométricos, propiciam a incidência de doenças fúngicas, como o míldio (*Plasmopara viticola*) (BRUNETTO et al., 2016). Assim, é necessária a aplicação periódica de produtos químicos, como tratamento fitossanitário preventivo e de controle de doenças fúngicas foliares. Dentre os produtos utilizados como tratamento fitossanitário em vinhedos, está a calda bordalesa ($\text{Ca}(\text{OH})_2 + \text{CuSO}_4$), a qual apresenta alta eficiência de controle e baixo custo econômico (MIOTTO et al., 2014). Entretanto, a calda bordalesa apresenta altas concentrações de cobre (Cu) em sua composição, que somadas às aplicações periódicas realizadas em vinhedos, eleva consideravelmente os teores de Cu no solo (FERNÁNDEZ-CALVIÑO et al., 2010; GIROTTI et al., 2014; BRUNETTO et al., 2014). O Cu aplicado durante os ciclos de produção das videiras ao longo dos anos de cultivo pode ser acumulado no solo no momento da aplicação, bem como após a

lavagem das folhas pela água das chuvas (KOMÁREK et al., 2010; BRUNETTO et al., 2014; COUTO et al., 2014; MIOTTO et al., 2014; BRUNETTO et al., 2016).

O excesso de Cu pode ficar acumulado no solo, e potencializa o risco de contaminação das águas superficiais e subsuperficiais (FERNÁNDEZ-CALVIÑO et al., 2008), e causar alterações na comunidade de microrganismos do solo (FERNÁNDEZ-CALVIÑO et al., 2010; FERNÁNDEZ-CALVIÑO et al., 2012; MACKIE et al., 2013). Além disso, os altos teores de Cu no solo podem causar problemas de toxidez para as videiras, bem como, para outras plantas que coabitam os vinhedos ou outras culturas que sejam empregadas nestes solos contaminados (YRUELA, 2005; TOSELLI et al., 2009; GIROTTA et al., 2014; AMBROSINI et al., 2015; CAMBROLLÉ et al., 2015; GUIMARÃES et al., 2016).

O Cu é um micronutriente, o qual atua como elemento estrutural na regulação da síntese de proteínas, participa no transporte de elétrons na fotossíntese, respiração mitocondrial, resposta ao estresse oxidativo, metabolismo de parede celular e sinalização hormonal (MARSCHNER, 2012; YRUELA, 2009). Entretanto, em quantidades excessivas pode produzir alterações anatômicas e morfológicas (LEQUEUX et al., 2010; ZAMBROSI et al., 2013; AMBROSINI et al., 2015; GUIMARÃES et al., 2016; TIECHER et al., 2018; DE CONTI et al., 2018a), fisiológicas (ROSA et al., 2014; ZHANG et al., 2014; CAMBROLLÉ et al., 2015; TIECHER et al., 2016; 2017; 2018; DE CONTI et al., 2018b), e nutricionais nas plantas (CAMBROLLÉ et al., 2015; TIECHER et al., 2016; DE CONTI et al., 2018a). Altos teores de Cu na solução do solo provocam danos ao sistema radicular, reduzindo a absorção de água e nutrientes e, consequentemente, diminui a taxa de fotossíntese e crescimento das plantas (MICHAUD et al., 2008; CAMBROLLÉ et al., 2015; TIECHER et al., 2018).

O problema de contaminação do solo por altas concentrações de Cu em vinhedos é recorrente em vários países do mundo (MACKIE et al., 2012). Sendo mais intenso de acordo com as características edafoclimáticas do local. Na região da Campanha Gaúcha, devido as características dos solos mais arenosos e com menor capacidade troca de cátions (CTC), é maior a presença de formas de Cu trocáveis e solúveis, o que pode intensificar os riscos de fitotoxidez (MIOTTO, 2012; BRUNETTO et al., 2014; GIROTTA et al., 2014). Os valores de pH do solo e a quantidade e qualidade de carbono orgânico dissolvido (DOC) são os principais fatores determinantes da biodisponibilidade do Cu e de sua especiação (CHAIGNON et al., 2009; KIM et al., 2010; DE CONTI et al., 2016; 2018a). O Cu é absorbido pelas plantas principalmente na forma de Cu^{2+} e Cu^+ (MARSCHNER, 2012; BRUNETTO et al., 2016). Em condições de pH mais ácido, as espécies livres de Cu^{2+} são predominantes e estão disponíveis para absorção pelas plantas, aumentando o efeito de

toxicidade. Com o aumento do pH do solo, ocorre a formação de espécies ligadas com hidroxilas solúveis e insolúveis, bem como espécies poliméricas de Cu, o que reduz a biodisponibilidade e a fitotoxicidade do Cu (KIM et al., 2010; PÉREZ-ESTEBAN et al., 2014; DE CONTI et al., 2016; 2018a). As espécies de plantas em geral podem alterar características químicas, físicas e biológicas da rizosfera como resposta a estresses bióticos e/ou abióticos (HINSINGER et al 2005). Por exemplo, quando submetidas à toxicidade por excesso de Cu, as plantas podem aumentar o valor de pH da rizosfera através da liberação de OH⁻ e reduzir a biodisponibilidade do Cu e, consequentemente, inibir a toxicidade nas plantas (CHAIGNON et al., 2009; HINSINGER et al., 2009; BRAVIN et al., 2009a; 2009b; 2012). A capacidade de alterar o pH da rizosfera difere entre as espécies vegetais e de acordo com a concentração de Cu e o pH do solo (BRAVIN et al., 2009a).

Diante disso, estudos vêm sendo realizados com o intuito de entender os efeitos de elevadas concentrações de Cu sobre a morfologia, anatomia e fisiologia das plantas, especialmente de videiras (TOSELLI et al., 2009; YRUELA, 2009; CHEN et al., 2013; JUANG et al., 2012; CAMBROLLÉ et al., 2013; MIOTTO et al., 2014; ROSA et al., 2014; AMBROSINI et al., 2015; CAMBROLLÉ et al., 2015; TIECHER et al., 2017). Também é importante entender os mecanismos de defesa desenvolvidos pelas mesmas, como forma de adaptação em ambientes contaminados (YRUELA, 2009; MARTINS et al., 2012; MARTINS et al., 2014; LENG et al., 2015). Porém, temos pouca informação em relação ao potencial de tolerância ao excesso de Cu por porta-enxertos de videiras. Visto que, os porta-enxertos geralmente são selecionados considerando a compatibilidade entre materiais genéticos (porta-enxerto e variedade de produção); facilidade de propagação; vigor vegetativo; resistência à problemas fitossanitários (filoxera, fusariose, antracnose, míldio, nematóides e entre outros), e limitações do solo (pH, textura, profundidade, fertilidade, salinidade, temperatura, umidade do solo, acidez, teor de argila e salinidade) e efeitos sobre características de qualidade dos frutos (PROTAS, 2003; WARSCHEFSKY et al., 2016). Os porta-enxertos podem apresentar características intrínsecas e/ou de adaptação que conferem maior resistência quando cultivados em condições desfavoráveis no intuito de manter a homeostase, como em solos contaminados com Cu. Dentre estas características, as plantas podem exsudar ácidos orgânicos, os quais podem formar complexos com o Cu em solução diminuindo a disponibilidade deste para absorção pelo sistema radicular; compartimentalização do Cu no sistema radicular, no vacuolo ou apoplasto, reduzindo a translocação do metal para a parte aérea; síntese e/ou estímulo da bombas de efluxo do metal na membrana plasmática; formação de quelatos com o metal por fitoquelatinas, metalotioneínas, ácidos orgânicos e

proteínas nas raízes ou rizosfera (HALL, 2002; KRÄMER e CLEMENS, 2005; YRUELA, 2009; ADREES et al., 2015). As plantas podem apresentar diferentes mecanismos de defesa de acordo com o genótipo, a concentração de Cu e tempo de exposição das plantas ao metal (YRUELA, 2003; 2009; FIDALGO et al., 2013; MARASTONI et al., 2019a).

Somado à isso, estratégias de manejo, como a utilização de produtos amenizantes vêm sendo estudados no intuito de reduzir a disponibilidade de Cu no solo e seu efeito fitotóxico (PIETRZAK e UREN, 2011; CHEN et al., 2013; JUANG et al., 2014; MACKIE et al., 2014; AMBROSINI et al., 2015; BALDI et al., 2018a). A aplicação de amenizantes visa a indução de processos químicos com ligantes inorgânicos e orgânicos com o objetivo de reduzir a biodisponibilidade do Cu na solução do solo e seu efeito fitotóxico (BRUNETTO et al., 2016). Dentre as alternativas de amenizantes estudados estão a aplicação de P, Ca, calcário, e compostos orgânicos (LAGOMARSINO et al., 2011; JORIS et al., 2012; MACKIE, 2012; CHEN et al., 2013; BRUNETTO et al., 2016; GUIMARÃES et al., 2016; SANTANA et al., 2015; AMBROSINI et al., 2015; 2017; 2018; BALDI et al., 2018a; 2018b; 2018c).

A aplicação de P pode atuar como estratégia para diminuir a disponibilidade de Cu em áreas contaminadas, através da formação de moléculas estáveis (CAO et al., 2002; MIRETZKY and FERNANDEZ-CIRELLI, 2008; BALDI et al., 2018a). Maiores teores de P também podem minimizar os efeitos tóxicos do Cu no sistema radicular das plantas, uma vez que o P forma complexos metal-fosfato com o Cu, os quais apresentam baixa mobilidade, reduzindo o translocação de Cu para a parte aérea (SOARES e SIQUEIRA, 2008; ZAMBROSI et al., 2013). Além disso, a adição de P no sistema contribui para a nutrição mineral, favorecendo processo de crescimento e desenvolvimento das plantas (MARSCHNER et al., 2012). A adição de Ca também pode atuar como amenizante a fitotoxicidade por Cu, favorecendo o aumento da espessura da parede celular, reduzindo os sintomas de engrossamento e encurtamento do sistema radicular, além disso, promovendo um maior acúmulo de Cu no sistema radicular e menor translocação para a parte aérea, reduzindo sintomas de toxicidade (CHEN et al., 2013). Além de ser um macronutriente, favorece a nutrição mineral das plantas e, consequentemente, o crescimento e desenvolvimento vegetal (MARSCHNER et al., 2012; CHEN et al., 2013).

O calcário promove o aumento dos valores de pH do solo, com isso ocorre a desprotonação dos grupos funcionais ácidos das partículas do solo e aumenta a CTC, o que consequentemente diminui a disponibilidade de Cu no solo e absorção pelas plantas (JORIS et al., 2012; AMBROSINI et al., 2015; BRUNETTO et al., 2016). Lagomarsino et al. (2011) observou que a aplicação de calcário e composto orgânico contribui para a estabilização do

Cu, minimizando sua disponibilidade em solução. A adição de resíduo orgânico no solo favorece o aumento da disponibilidade de nutrientes para absorção pelas plantas, e pode limitar a mobilidade e biodisponibilidade do Cu, visto que o mesmo tem alta afinidade pelos grupos carboxílicos de superfície da MO (PARK et al., 2011; BRUNETTO et al., 2016).

Desta forma, o objetivo do trabalho foi verificar as respostas de plantas as altas concentrações de Cu, identificar genótipos de porta-enxerto de videira tolerantes a altos teores de Cu em solução, e avaliar o potencial de utilização de amenizantes como estratégia para reduzir a biodisponibilidade e toxidez de Cu em videiras jovens.

2. HIPÓTESES

I - Baixos valores de pH em solução e altas concentrações de Cu disponíveis induzem a ativação de mecanismos de defesa pelas plantas no intuito de evitar fitotoxidez por Cu.

II - Porta-enxertos de videira podem apresentar diferentes respostas ao excesso de metal, sendo que plantas com maior crescimento podem apresentar maior tolerância.

III - Existe variação genética de porta-enxertos de videira na tolerância às concentrações elevadas de Cu em solução, os quais podem desenvolver diferentes estratégias para manter a homeostase do Cu no tecido.

IV - A utilização de adubação fosfatada como estratégia amenizante em solução nutritiva é eficiente para diminuir o efeito fitotóxico causado pelo excesso de Cu em plantas de videira.

V - A aplicação de calcário é eficaz como estratégia amenizante da disponibilidade do Cu na solução do solo e do problema de toxidez às videiras jovens em solos contaminados.

3. OBJETIVOS

3.1 OBJETIVO GERAL

O objetivo do presente trabalho foi verificar as respostas de plantas as altas concentrações de Cu, identificar genótipos de porta-enxerto de videira tolerantes a altos teores de Cu em solução, e avaliar o potencial de utilização de amenizantes como estratégia para reduzir a biodisponibilidade e toxidez de Cu em videiras jovens.

3.2 OBJETIVOS ESPECÍFICOS

I - Verificar a influência do pH e alta concentração de Cu nas respostas fisiológicas e morfológicas de duas espécies vegetais, pepino (*Cucumis sativus L.*) e aveia (*Avena sativa L.*).

II - Avaliar o crescimento, respostas bioquímicas e fisiológicas de porta-enxertos de videira ao excesso de Cu em solução nutritiva.

III - Identificar porta-enxertos de videira que apresentem potencial de tolerância à elevadas concentrações de Cu em solução nutritiva.

IV - Verificar a tolerância de porta-enxertos de videiras ao excesso de Cu e a eficiência do P e Ca na redução da fitotoxidez de Cu.

V - Avaliar o crescimento, estado fisiológico e as alterações na morfologia radicular de videiras jovens cultivadas por 12 meses em solo contaminado com Cu, com e sem aplicação de amenizantes.

4. REVISÃO BIBLIOGRÁFICA

4.1 VITICULTURA: UM PANORAMA GERAL

A viticultura é uma atividade de importância econômica mundial, onde os dez principais países produtores de vinho são Itália, França, Espanha, Estados Unidos, Argentina, Chile, Austrália, Alemanha, África do Sul e China, segundo a Organização Internacional da Videira e do Vinho (OIV, 2019). A Espanha foi o principal exportador de vinho, seguida pela Itália, França e Chile. Em termos de importações, a Alemanha é o principal país importador, seguido pelos Inglaterra, EUA, China, França, Canadá e Holanda (OIV, 2019). Neste cenário mundial, o Brasil ocupa a décima quinta posição em produção de vinhos (OIV, 2019).

No Brasil, a área de produção vitivinícola corresponde a cerca de 75.951 hectares, sendo produzidas 1.592.242 toneladas de uvas durante a safra de 2018 (MELLO, 2019). Da produção nacional de uvas em 2018, 51,39% foram destinadas ao processamento (vinho, suco e derivados) e, o restante foi consumido *in natura* (MELLO, 2019). Os principais estados produtores de uva são Rio Grande do Sul, Pernambuco, São Paulo, Santa Catarina, Paraná e Bahia. Porém, do total da área nacional plantada com vinhedos, a maior parcela, 48.830 hectares, está localizada no estado do Rio Grande do Sul (MELLO, 2019).

O Rio Grande do Sul é o maior estado produtor de uvas, com uma produção de vinhos, suco e derivados de 605,96 milhões de litros, em 2017 (MELLO, 2019). O setor vitivinícola gaúcho é responsável pela movimentação de cerca de R\$ 3,5 bilhões anualmente, empregando cerca de 15 mil famílias em atividades relacionadas com a produção de uvas (IBRAVIN, 2020). Dentro do estado do Rio Grande do Sul, as principais regiões produtoras de uva e vinhos são a Serra Gaúcha, Campanha Gaúcha (IBRAVIN, 2020).

A região da Serra Gaúcha foi a pioneira no cultivo de videiras no estado. Ela compreende os municípios de Bento Gonçalves, Caxias do Sul e arredores, sendo a principal área de produção vitivinícola do Rio Grande do Sul (IBRAVIN, 2020). Já a região da Campanha Gaúcha é resultante do processo de expansão da atividade no estado, em áreas anteriormente ocupadas com campo natural, utilizadas para pecuária de corte, e convertidas à vinhedos a partir da década de 70. Destaca-se o município de Santana de Livramento, sede de vinícolas importantes para o desenvolvimento da atividade nesta região (IBRAVIN, 2020).

As condições edafoclimáticas das regiões produtoras de uva podem contribuir para a incidência de doenças fúngicas, o que compromete a quantidade e qualidade da produção. Na região sul, predomina uma maior quantidade de precipitações, distribuídas ao longo do

crescimento vegetativo da videira, o que favorece o aparecimento de doenças fúngicas foliares, como míldio (*Plasmopara viticola*) (SÔNEGO et al., 2005). Desta forma, é necessário a aplicação de tratamentos fitossanitários, para a prevenção e controle de doenças fúngicas foliares. Estes produtos normalmente possuem Cu e Zn em sua composição, contribuindo para o aumento da concentração destes elementos no solo (MACKIE et al., 2012; BRUNETTO et al., 2014; GIROTTA et al., 2014; MIOTTO et al., 2014).

4.2 APLICAÇÃO DE FÚNGICAS CÚPRICOS E NÍVEIS DE COBRE NO SOLO

No estado do Rio Grande do Sul as principais doenças fúngicas que atacam a cultura da videira são: na parte aérea das videiras, o míldio (*Plasmopara viticola*), a antracnose (*Elsinoe ampelina*), a podridão cinzenta (*Botryotinia fuckeliana*), o oídio (*Uncinula necator*), as podridões do cacho causadas por *Melanconium fuligineum* e *Glomerella cingulata*, a escoríose (*Phomopsis viticola*), a mancha das folhas (*Isariopsis clavigpora*) e a ferrugem (*Phakopsora euvitis*); e no sistema radicular, a fusariose (*Fusarium oxysporum* f. sp. *herbemontis*) e o “pé-preto” (*Cylindrocarpon destructans*) (SÔNEGO et al., 2003; SÔNEGO et al., 2005). Dentre estas doenças, o míldio tem maior importância para a viticultura brasileira, pois provoca a perda total ou parcial das inflorescências e frutos e, queda prematura das folhas, causando perdas na qualidade e quantidade de produção no ano e dos anos seguintes (SÔNEGO et al., 2005).

O surgimento de doenças fúngicas nos vinhedos no Rio Grande do Sul é favorecido pelas condições climáticas (BRUNETTO et al., 2014; BRUNETTO et al., 2016). O clima da região da Campanha Gaúcha é classificado como Cfa, segundo a Köppen e Geiger, apresenta temperatura média de 18.4 °C e precipitação média anual de 1467 mm (CPTEC, 2017). É um clima subtropical, com pluviosidade bem distribuída durante o ano e temperaturas amenas favorecem o desenvolvimento de doenças fúngicas nas videiras (SÔNEGO et al., 2005; BRUNETTO et al., 2014; BRUNETTO et al., 2016).

Assim, é necessária a aplicação periódica de produtos químicos, como tratamento fitossanitário preventivo e de controle de doenças fúngicas foliares (MACKIE et al., 2012; BRUNETTO et al., 2014; GIROTTA et al., 2014; MIOTTO et al., 2014). Dentre estes produtos utilizados como tratamento fitossanitário em vinhedos, estão a calda bordalesa ($\text{Ca(OH)}_2 + \text{CuSO}_4$), oxicloreto de cobre [$\text{CuCl}_2 \cdot 3\text{Cu}(\text{OH})_2$] e hidróxido de cobre [$\text{Cu}(\text{OH})_2$] (MAIA et al., 2003). Estes produtos são aplicados periodicamente nos vinhedos, e por conter altas concentrações de metais pesados em sua composição, como o Cu, podem apresentar

efeito acumulativo no solo, principalmente nas camadas mais superficiais do solo, e distribuindo-se ao longo do perfil (FERNÁNDEZ-CALVIÑO et al., 2010; KOMÁREK et al., 2010; BRUNETTO et al., 2014; COUTO et al., 2014; MIOTTO et al., 2014). O excesso de elementos químicos pode causar problemas de poluição ambiental, pela contaminação de recursos hídricos e no solo produzir efeito de fitotoxicidade para os cultivos (KOMÁREK et al., 2010).

A calda bordalesa, suspensão coloidal de sulfato de cobre, hidróxido de cálcio e água, é o produto mais utilizado para a prevenção e controle de doenças fúngicas em vinhedos, por apresentar uma alta eficiência de controle e baixo custo econômico (GIROTTI, 2010; MIOTTO et al., 2014). Entretanto, a calda bordalesa apresenta concentração de 0,5 a 1% de CuSO₄.5H₂O em sua composição, que somadas às aplicações periódicas realizadas em vinhedos, eleva consideravelmente os teores de Cu no solo (FERNÁNDEZ-CALVIÑO et al., 2009; BRUNETTO et al., 2014). Embora os vinhedos de produção orgânica utilizem maior quantidade de fungicida à base de Cu, os vinhedos de produção convencional também utilizam estes produtos. De acordo com a região produtora, são aplicadas taxas de 1 - 2 kg ha⁻¹ano⁻¹ na Europa, 0-20 kg ha⁻¹ano⁻¹ na Austrália, suplementando outras substâncias químicas aplicadas (NACHTIGALL et al., 2007). Na região Sul do Brasil o uso continuado de produtos químicos como tratamento fitossanitário pode somar até 30 kg de Cu ha⁻¹ano⁻¹ que chegam na superfície do solo (BRUNETTO et al., 2014; COUTO et al., 2014).

A contaminação das áreas por metais pesados ocorre quando os níveis considerados como referência de qualidade são excedidos. O Conselho Nacional do Meio Ambiente (CONAMA), o qual dispõe sobre critérios e valores limítrofes para a presença de substâncias químicas, bem como normas de gerenciamento ambiental de áreas contaminadas com as mesmas em decorrência da ação antrópica, expõe que os seguintes valores de referência para o teor de Cu no solo: 200 mg kg⁻¹ para solos agrícolas, 400 mg kg⁻¹ para áreas residenciais e 600 mg kg⁻¹ para áreas industriais. Sendo que, concentrações de Cu no solo superiores de 60 mg kg⁻¹ sinalizam a necessidade de realizar práticas preventivas para garantir a manutenção da funcionalidade do solo e a proteção da qualidade das águas superficiais e subterrâneas (CONAMA 420, 2009).

O Cu aplicado durante os ciclos de produção das videiras ao longo dos anos de cultivo, pode ser acumulado no solo no momento da aplicação, devido a unidirecionalidade das aplicações, ao escoamento foliar mediado pela chuva ou pela queda de folhas senescentes ricas em Cu (BRUNETTO et al., 2014; COUTO et al., 2014; GIROTTI et al., 2016). A aplicação periódica de fungicidas contendo metais pesados como Cu durante muitos anos

resultou em altos níveis de acumulação destes na camada superior de solo em vinhedos de todo o mundo. A concentração de Cu em vinhedos apresenta grande variação, devido às condições climáticas, características físico-químicas do solo, frequência da incidência de doenças fúngicas e aplicação de produtos fitossanitários contendo Cu em sua composição (KOMÁREK et al., 2010). Estudos realizados demonstram que o teor de Cu em vinhedos podem ser de 100 – 1500 mg kg⁻¹ de Cu, o que excede cerca de 300 vezes a concentração mínima encontrada no solo de 5–30 mg kg⁻¹ (CHAIGNON et al., 2003). Na Austrália, a concentração de Cu no solo varia de 40 a 250 mg kg⁻¹ (WIGHTWICK, et al., 2008), na região central de Taiwan, o teor médio de Cu em vinhedos oscila de 9,1 - 100 mg kg⁻¹ (LAI e JUANG, 2010), de 100 a 210 mg kg⁻¹ na Grécia (VAVOULIDOU et al., 2005), 50 a 300 mg kg⁻¹ na Itália (TOSELLI et al., 2009); 35 a 600 mg kg⁻¹ na Espanha (NÓVOA-MUÑOZ et al., 2007); 100 a 1500 mg kg⁻¹ em França (FLORES-VÉLEZ et al., 1996; BRUN et al., 2001). Na região sul do Brasil, Mirlean et al., (2007) encontrou a concentração máxima de 3200 mg kg⁻¹ de Cu no solo de vinhedos. Casali et al., (2008), observou que na região da Serra Gaúcha, os solos de vinhedos podem apresentar concentrações de Cu total de até 665 mg kg⁻¹.

Assim, a aplicação de produtos fitossanitários contendo Cu em sua composição podem representar uma fonte de contaminação indireta do solo e da água, causando alterações na comunidade de organismos, bem como, problemas de fitotoxicidade aos cultivos e riscos à saúde humana.

4.3 EFEITO DO EXCESSO DE COBRE SOBRE AS PLANTAS E MECANISMOS DE DEFESA

O Cu é um micronutriente com importante função nos processos de fotossíntese, respiração, metabolismo do C e N, e na proteção contra danos por estresse oxidativo (MARSCHNER, 2012; YRUELA, 2005; YRUELA, 2009). Tanto condições de déficit como de excesso de Cu disponível para absorção pelas plantas podem ser prejudiciais para o crescimento e desenvolvimento vegetal (MARSCHNER, 2012).

Em tecido de folhas maduras, concentrações de Cu entre 20 e 100 mg Cu kg⁻¹ de matéria seca são consideradas como tóxicas (KABATA-PENDIAS e PENDIAS, 2001). O excesso de Cu pode provocar alterações anatômicas, morfológicas e fisiológicas nas plantas, como prejudicar o crescimento do sistema radicular e brotos, redução da taxa fotossintética, causar sintomas de clorose, e em casos mais graves pode provocar a necrose de tecidos e

morte das plantas (MARSCHNER, 2012; MICHAUD et al., 2008; LEQUEUX et al., 2010; CAMBROLLÉ et al., 2015).

No sistema radicular das plantas, altas concentrações de Cu podem provocar a redução no taxa de divisão mitótica das células, prejudicando o crescimento das raízes (JIANG et al., 2000; LEQUEUX et al., 2010; AMBROSINI et al., 2015). As alterações produzidas no sistema radicular das plantas resultam em menor superfície de exploração do solo pelas raízes, consequentemente, menor absorção de água e nutrientes (DE VOS et al., 1989; KOPSELL e KOPSELL, 2007; AMBROSINI et al., 2015; GUIMARÃES et al., 2016). Altos teores de Cu provocam alterações no ápice radicular, com modificações nas paredes celulares e no arranjo do tecido do ápice radicular fazendo com que ocorra o encurtamento e aumento da espessura da raiz. Somado a isso, aumenta o número de raízes laterais, e no tecido da epiderme pode ocorrer a plasmólise de algumas células, consequentemente, resultando em coloração escura das raízes e redução da densidade radicular (POTTERS et al., 2007; LEQUEUX et al., 2010; JUANG et al., 2012; CHEN et al., 2013; ZHANG et al., 2014; AMBROSINI et al., 2015). Alterações na biossíntese e translocação de hormônios, como a citocinina, giberilina, auxina e etileno, envolvidos no processo de divisão e elongação celular, podem ser produzidas pelo excesso de Cu nos tecidos (POTTERS et al., 2007; LEQUEUX et al., 2010; CHEN et al., 2013). Videiras cultivadas em solo com altas concentrações de Cu podem apresentar acúmulo de compostos fenólicos nas células da endoderme e do córtex radicular (AMBROSINI et al., 2015). A presença de compostos fenólicos pode atuar como uma estratégia de defesa das plantas contra os danos causados pelo estresse oxidativo, pela presença das ROS (MICHALAK, 2006). Além disso, compostos fenólicos podem atuar como intermediários para a biossíntese de lignina nas raízes, a qual é catalisada por lacases e peroxidases, que são glicoproteínas que contém Cu em sua composição. A lignificação pode limitar o crescimento radicular pelo aumento da rígidez das paredes celulares e efluxo de metais do cilindro vascular via floema (MICHALAK, 2006; LEQUEUX et al., 2010).

O elemento Cu pode ser encontrado nas plantas em dois estados de oxidação, Cu^+ e Cu^{2+} (YRUELA, 2005; YRUELA, 2009). O ciclo redox do Cu^{2+} e Cu^+ , catalisa a produção de radicais hidroxila (OH^-), a partir de uma reação química não-enzimática entre o superóxido (O_2^-) e o peróxido de hidrogênio (H_2O_2) (Reação de Fenton e Haber–Weiss). A absorção em excesso de Cu pode causar estresse oxidativo devido ao aumento na concentração de espécies reativas de oxigênio (ROS), como os radicais superóxido (O_2^-), oxigênio singuleto (${}^1\text{O}_2$), peróxido de hidrogênio (H_2O_2) e radicais hidroxil (OH^-) (BRIAT e LEBRUNB, 1999; APEL e HIRT, 2004; GIROTTTO et al., 2013; TIECHER et al., 2016). As ROS podem causar danos

à biomoléculas nas células, sendo a peroxidação lipídica das membranas celulares um dos principais efeitos observados durante o estresse oxidativo, resultando em menor seletividade da membrana, o que pode causar sua ruptura e extravasamento do conteúdo celular (DE VOS et al., 1989; YRUELA, 2005). Em resposta ao estresse oxidativo pelo excesso de Cu na célula são ativadas enzimas antioxidantes, como a ascorbato peroxidase (APX), catalase (CAT), dehidroascorbato redutase (DHAR), guaicol peroxidase, glutathione redutase (GR), monodehydroascorbato redutase (MDHAR) e superóxido dismutases (SODs) (LOMBARDI e SEBASTIANI, 2005).

O excesso de Cu pode interferir na biosíntese do complexo fotossintético, através da redução no teor de pigmentos fotossintetizantes, como a clorofila e carotenóides, e alterações na estrutura do cloroplasto e composição da membrana do tilacóide (CAMBROLLÉ et al., 2013; CAMBROLLÉ et al., 2015). O Cu pode formar complexos com a clorofila, através da substituição do Mg central da clorofila pelo Cu, nos cloroplastos das células, prejudicando o processo de conversão de luz em energia química na forma de NADPH e ATP, e liberação de O₂ (YRUELA, 2005, 2009; KABATA-PENDIAS, 2011). Além disso, através da peroxidação lipídica ocorre o aumento da fluidez da membrana dos tilacóides, influenciando a atividade dos fotossistemas, prejudicando a fase fotoquímica da fotossíntese. O fotossistema II (FSII) apresenta maior sensibilidade à toxidez por Cu em relação ao fotossistema I (FSI), inibindo o complexo de evolução de oxigênio e causando perdas por fluorescência (YRUELA, 2005, 2009; KABATA-PENDIAS, 2011).

As plantas podem apresentar diferentes mecanismos de defesa, relacionados ao estresse por excesso de metal disponível em áreas de cultivo (YRUELA, 2005; 2009; ADREES et al., 2015), o que varia de acordo com o genótipo, a concentração de Cu, e tempo de exposição das plantas ao metal (YRUELA, 2003; 2009; FIDALGO et al., 2013; MARASTONI et al., 2019a). Algumas espécies podem acumular ou hiperacumular o metal no tecido sem expressar sintomas de fitotoxicidade (YRUELA, 2009; PIETRZAK e UREN, 2011). Para manter a homeostase de metais, as plantas podem apresentar maior vigor e aporte nutricional, o que favorece o crescimento e desenvolvimento das plantas; diminuir a absorção de Cu e armazenar o excesso de Cu no sistema radicular no vacúolo ou ligado à parede celular, evitando a translocação para a parte aérea; manutenção da estrutura e funcionamento do aparato fotossintético; síntese de enzimas antioxidantes para combater as espécies reativas de oxigênio (YRUELA 2005; 2009; ADREES et al., 2015).

Somado a isso, as plantas podem promover alterações em características químicas, físicas e biológicas da rizosfera como resposta a estresses bióticos e/ou abióticos

(HINSINGER et al 2005). Por exemplo, quando submetidas à toxicidade de metais, como o Cu, as plantas podem modificar os valores de pH da rizosfera, através da liberação de OH⁻, reduzindo a biodisponibilidade do Cu e, consequentemente, a toxicidade às plantas (CHAIGNON et al., 2009; HINSINGER et al., 2009; BRAVIN et al., 2009a; 2012). Além disso, a exsudação de compostos orgânicos por raízes e microorganismos pode alterar a biodisponibilidade de metais na rizosfera (HINSINGER et al., 2009; MARSCHNER, 2012; BRAVIN et al., 2012). A exsudação de compostos orgânicos aumenta a complexação de espécies químicas livres de metais pesados na solução do solo, especialmente do Cu⁺², consequentemente diminuindo a absorção e translocação destes elementos pelas plantas (CHAIGNON et al., 2009; KIM et al., 2010; KABATA-PENDIAS, 2011; DE CONTI et al., 2018a). Dentre os exsudatos radiculares estão os compostos orgânicos de baixo peso molecular, como compostos fenólicos, ácidos orgânicos, aminoácidos, fitossideróforos, açúcares, etc. (MONTIEL-ROZAS et al., 2016; ZAFARI et al., 2016). Ácidos orgânicos são altamente reativos ao Cu e, portanto, influenciam a distribuição de espécies químicas na rizosfera e disponibilidade destas para absorção pelas plantas, bem como, compostos específicos podem complexar o Cu²⁺ no apoplasto, impedindo a translocação para a parte aérea (MARSCHNER, 2012; SESHADRI et al., 2015; MONTIEL-ROZAS et al., 2016; DE CONTI et al., 2018a). Alterações no pH da rizosfera e exsudação radicular são respostas altamente específicas de espécies vegetais a alta concentração de metais nos solos (MEIER et al., 2012; MONTIEL-ROZAS et al., 2016).

Desta forma, diferentes genótipos podem apresentar um comportamento particular em resposta às elevadas concentrações de Cu no solo, sendo mais tolerante ou sensível ao excesso de Cu no solo. Porém, ainda temos poucas informações referentes a tolerância de diferentes porta-enxertos de videira aos altos teores de Cu no solo. Sendo assim, fica evidente a importância de obter uma maior gama de informações em relação ao material vegetal utilizado, bem como, a utilização de substâncias amenizantes que podem atuar na redução da disponibilidade de Cu para absorção pelas plantas, diminuindo assim o potencial fitotóxico aos cultivos.

4.4 ESTRATÉGIAS DE CULTIVO PARA REDUÇÃO DA BIODISPONIBILIDADE, ABSORÇÃO E FITOTOXIDEZ POR EXCESSO DE COBRE

Estratégias para amenizar o efeito fitotóxico ocasionado pelo excesso de Cu no solo vêm sendo estudadas nos últimos anos, visto que além de prejudicar a produtividade agrícola,

altos teores de Cu no solo podem representar risco de contaminação ao ambiente. Assim, é necessária a aplicação de práticas de manejo agrícola no intuito de restringir as frações de Cu disponíveis no solo para absorção pelas plantas e reduzir a problemas de toxidez.

O uso de porta-enxertos de videira com potencial tolerância a altas concentrações de Cu pode atuar como uma estratégia para a manutenção da capacidade produtiva em áreas de vinhedos antigos. A seleção de porta-enxertos de videira está alicerçada em características de compatibilidade entre porta-enxerto e copa, vigor vegetativo, resistência a insetos, doenças e nematoïdes, facilidade de propagação, influência sobre qualidade da produção, adequação a características de solo (pH, textura, profundidade, fertilidade, salinidade, umidade) e clima (temperatura e pluviosidade) (WARSCHEF SKY et al., 2016). No entanto, existe pouca informação em relação a tolerância de PE de videiras ao excesso de metal pesado no solo, especialmente de Cu.

Além disso, o uso de substâncias amenizantes que tem como princípio a indução de processos químicos de sorção do Cu, através da adsorção a superfícies minerais, formação de complexos estáveis com ligantes orgânicos, precipitação superficial e troca iônica, podem reduzir a biodisponibilidade de Cu no solo e consequentemente fitotoxidez (BRUNETTO et al., 2016). Em contrapartida, pode ocorrer a lixiviação do Cu no perfil do solo, desta forma a aplicação de compostos amenizantes deve ser monitorada (BRUNETTO et al., 2016). De forma geral, é usual a aplicação de substâncias como amenizantes ao excesso de Cu no solo e para melhorar o crescimento e o rendimento das culturas (BOLAN et al., 2003; BRUNETTO et al., 2016). Dentre estas alternativas, é possível utilizar compostos orgânicos e inorgânicos, como o calcário, P e Ca (TERZANO et al., 2005; KUMPIENE et al., 2008; LAGOMARSINO et al., 2011; MACKIE et al., 2012; AMBROSINI et al., 2015; BALDI et al., 2018 a; 2018 b; 2018c; FERREIRA et al., 2018).

A adição de calcário no solo pode reduzir a disponibilidade de Cu no solo e absorção pelas plantas. O manejo de adição de calcário no solo, geralmente, é realizado antes da implantação das videiras. O calcário promove o aumento dos valores de pH do solo e, com isso, ocorre a desprotonação de grupos funcionais ácidos das partículas do solo, aumentando a capacidade de troca de cátions (CTC), consequentemente, diminuindo a disponibilidade de Cu no solo e absorção pelas plantas (JORIS et al., 2012; BRUNETTO et al., 2016). A maior disponibilidade de Cu na solução do solo ocorre quando os valores de pH estiverem mais ácidos, abaixo de 5,5 (NOVAIS et al., 2007). Com o aumento do pH, acontece a elevação da concentração de ânions na solução, carbonato de cálcio (CaCO_3^{-2}), hidroxilas (OH^-) e fosfato (HPO_4^{-2}), que podem se ligar ao Cu formando complexos, os quais precipitam, reduzindo sua

disponibilidade na solução do solo (JORIS et al., 2012; WANG et al., 2012; BRUNETTO et al., 2016). Aliado a isso, a calagem pode aumentar a disponibilidade de macronutrientes, como o Ca e Mg, facilitando sua absorção pelas plantas (SOUSA et al., 2007; BRUNETTO et al., 2016). O aumento da absorção de Ca e Mg pelas videiras contribui para reduzir os efeitos fitotóxicos do Cu no sistema radicular, uma vez que o Ca e Mg competem pelos sítios de absorção na superfície das raízes, diminuindo o transporte de Cu para a parte aérea, e fornece Mg para as moléculas de clorofila favorecendo a atividade fotossintética, e incremento na biomassa vegetal (YRUELA, 2009; KOPITTKE et al., 2011; CHEN et al., 2013; JUANG et al., 2014; OLIVEIRA et al., 2015; AMBROSINI et al., 2015; 2017). Portanto, a aplicação de calcário no solo pode ser uma importante estratégia para reduzir os efeitos tóxicos do Cu para as videiras jovens.

Estudos vêm sendo realizados utilizando a aplicação de calcário como alternativa para reduzir o efeito fitotóxico do Cu no solo. Ambrosini et al. (2015) observou que a aplicação de calcário em solo contaminado com Cu diminui o efeito da toxicidade do Cu, através de parâmetros relacionados a anatomia radicular de videiras jovens. O calcário, principalmente na dose de 3 Mg ha⁻¹, elevou o valor de pH do solo e diminuiu a concentração de Cu no solo, bem como houve incremento de Ca e Mg nas plantas, o que limitou a ocorrência de alterações anatômicas nas raízes em resposta às altas concentrações de Cu no solo. O mesmo efeito foi observado por Ambrosini et al. (2017) em estudo realizado com plantas de aveia-preta (*Avena strigosa*) e em videiras jovens (*Vitis* sp.). Os autores observaram que a aplicação de calagem no solo com excesso de Cu foi adequada para amenizar os efeitos de toxidez de Cu e permitir o crescimento e desenvolvimento das duas espécies. Resultados similares foram encontrados por Oliveira et al. (2015) avaliando o efeito da calagem como estratégia de amenizar a toxidez de Cu em solos contaminados. Os autores demonstraram o efeito negativo do aumento da concentração de Cu sobre o crescimento e desenvolvimento de videiras, e que aplicação de calcário foi eficiente em minimizar os efeitos causados pelo excesso de Cu adicionado.

O íon fosfato pode interagir com o Cu na solução do solo, reduzindo sua mobilidade e disponibilidade para absorção pelos cultivos (BRUNETTO et al., 2016; BALDI et al., 2018a). Maiores teores de P no solo podem minimizar os efeitos tóxicos do Cu no sistema radicular das plantas, uma vez que o P forma complexos metal-fosfato com o Cu, os quais apresentam baixa mobilidade, reduzindo o transporte de Cu para a parte aérea (SOARES e SIQUEIRA, 2008; ZAMBROSI et al., 2013). Além disso, o P é um importante nutriente, o qual promove o crescimento radicular, otimiza a taxa fotossintética e estimula o crescimento das plantas (MARSCHNER et al., 2012; ZAMBROSI et al., 2011; BALDI et al., 2018a; 2018b; 2018c).

Este efeito também foi observado por Guimarães et al. (2016) em plantas de aveia preta (*Avena strigosa* Schreb.), onde a aplicação de P no solo contribuiu para a redução dos sintomas de toxidez por Cu, observado através de parâmetros de anatomia do sistema radicular e produção de matéria seca das plantas. A aplicação de P (0,5 mmol L⁻¹) melhorou a estrutura da parede celular e da lamela média em estudo realizado por Zambrosi et al. (2013), cultivando Citrus, *Sunki mandarin* e *Swingle citrumelo*, em solução nutritiva com 50 µmol L⁻¹ de Cu. Estudos realizados por Baldi et al. (2018a; 2018b; 2018c) em videiras cultivadas em solo com alta concentração de Cu e aplicação de P como amenizante também observaram um efeito benéfico da adição de P sobre a eficiência do aparato fotossintético, aquisição de nutrientes e, consequentemente, sobre o crescimento das plantas.

A adição de Ca também pode atuar como amenizante a fitotoxidez por Cu, além de favorecer o crescimento das plantas (CHEN et al., 2013). Em condições de alta disponibilidade de Cu, um maior nível de Ca pode contribuir como amenizante através do aumento da espessura da parede celular, reduzindo os sintomas de engrossamento e encurtamento do sistema radicular pela toxidez por Cu, além disso, promovendo um maior acúmulo de Cu no sistema radicular e menor translocação para a parte aérea, reduzindo sintomas de toxicidade (CHEN et al., 2013). Além disso, o Ca é um macronutriente, favorecendo o crescimento e desenvolvimento vegetal (MARSCHNER et al., 2012). Chen et al., (2013) verificaram que a aplicação de 5 mM de Ca reduziu os sintomas de toxidez por excesso de Cu, favorecendo o acúmulo do excesso de Cu no sistema radicular das plantas, reduzindo a translocação de Cu para a parte aérea. Estudos reportados por Hippler et al. (2018) verificaram o aumento dos efeitos de toxidez por excesso de Cu sobre atividade fotossintética de plantas de citrumelo, cultivadas em baixa disponibilidade de Ca em solução nutritiva.

A mobilidade e biodisponibilidade de Cu pode ser limitada pela adição de resíduos orgânicos no solo, devido à alta afinidade do Cu pelos grupos carboxílicos de superfície da matéria orgânica (MO) (BRUNETTO et al., 2016). Compostos de alto peso molecular e insolúveis da MO podem reter o Cu por complexação, diminuindo sua disponibilidade na solução do solo. Em contrapartida, a presença de compostos de baixo peso molecular e solúveis, os quais mantém o Cu em solução favorecendo sua lixiviação no perfil (PARK et al., 2011; BRUNETTO et al., 2016). Lagomarsino et al. (2011) observou que a aplicação de calcário e composto orgânico contribui para a estabilização do Cu, minimizando sua disponibilidade em solução. Porém, de acordo com a especiação química e distribuição nas

frações argila, o Cu ligado a MO pode ser liberado com a decomposição da MO e/ou lixiviado no perfil.

Em estudo realizado por Casali et al. (2015) com aplicação de doses de composto orgânico na linha e entrelinha em videiras (Niágara Rosada) cultivadas em um Neossolo Litólico, os autores observaram que houve pouco efeito da aplicação do composto orgânico sobre o estado nutricional das plantas, componentes de produção e composição das uvas. Cárdenas-Aguiar et al. (2017), observou a redução na mobilidade do Cu e incremento da biomassa microbiana do solo com a aplicação de biochar e biochar acompanhado por composto orgânico, em um solo com até 1000 mg Cu kg⁻¹, cultivado com mostarda, agrião e azevém. As plantas de mostarda e agrião só apresentaram crescimento no solo contaminado com Cu após a adição do tratamento com biochar e composto orgânico. Uchimiya et al. (2011) destacou que a ligação eletrostática que ocorre entre o cátion Cu e a superfície do biochar carregada negativamente resulta na imobilização do Cu no solo. Entretanto, a mobilidade e/ou disponibilidade do Cu no solo é determinada pelo teor de carbono orgânico dissolvido (COD) no biochar, de forma geral, o processo de queima realizado com temperaturas menores que 500°C resulta em um material com alto teor de COD, o que facilita a formação de complexos de maior solubilidade do Cu (BEESLEY et al., 2011; PARK et al., 2011); e quando o processo de queima do resíduo orgânico é realizado com temperaturas acima de 600°C, o produto apresenta menores concentrações de COD, consequentemente podendo causar a imobilização do Cu no solo (UCHIMIYA et al., 2011).

Diversos rejeitos orgânicos podem ser utilizados na confecção de composto e vermicomposto, como o bagaço de uva remanescente do processo de produção de vinho (FERNÁNDEZ-BAYO et al., 2007). A utilização deste resíduo contribui para a reposição de uma parcela dos nutrientes absorvidos pela videira, além de melhorar características físicas, químicas e biológicas do solo (FERNÁNDEZ-BAYO et al., 2007). Santana et al. (2015) estudou o efeito da interação entre a inoculação com o fungo micorrízico arbuscular (FMA), *Rhizophagus clarus*, e a adição de vermicomposto, produzido a partir de bagaço de uva, como estratégia de amenização por *Canavalia ensiformis* de um solo arenoso com alto teor de Cu. Os autores observaram que a fitoestabilização do Cu no solo por *C. ensiformis* foi incrementada pela adição de teores de vermicomposto, equivalentes a 20 mg P kg⁻¹, e inoculação com *R. clarus*.

Sendo assim, a aplicação de amenizantes pode contribuir na redução da biodisponibilidade de Cu na solução do solo, e somado a seleção de porta-enxertos mais

tolerantes às altas concentrações de Cu no solo representam um importante avanço e estratégia de cultivo em áreas contaminadas por metais pesados.

5. RESULTADOS

No intuito de compreender as respostas de plantas as altas concentrações de Cu disponíveis, identificar genótipos de porta-enxertos de videiras tolerantes a altos teores de Cu em solução, e avaliar o potencial de utilização de amenizantes como estratégia para diminuir a biodisponibilidade e toxidez de Cu em videiras jovens, foram realizados cinco estudos:

I - Espécies de plantas e respostas dependentes do pH à toxicidade do cobre;

II - Crescimento, respostas bioquímicas e fisiológicas de porta-enxertos de videiras ao excesso de cobre em solução nutritiva;

III - Identificação de porta-enxertos de videira tolerantes à altas concentrações de cobre;

IV - Tolerância de porta-enxertos de videiras ao excesso de cobre e uso de cálcio e fósforo como amenizantes da sua fitotoxicidade;

V - Potencial do vermicomposto e calcário na redução da toxicidade do cobre em videiras jovens cultivadas em solo de vinhedos contaminado com cobre.

5.1 ESTUDO I

Plant species and pH dependent responses to copper toxicity¹

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Abstract

High copper (Cu) levels in soil caused by anthropic activity, as in vineyards, can cause phytotoxicity and reduce plant productivity. However, the distribution of Cu chemical-species in the soil and then, the level of its availability for crops can be considerably altered by pH and root exudates in the rhizosphere. This study aimed at assessing the effect of the plant species cultivated and the pH values of the growing medium on the physiological and morphological adaptation strategies to Cu stress. The experiment was conducted in controlled conditions with two plant species, cucumber (*Cucumis sativus* L.) and oat (*Avena sativa* L.), three pH levels (4.5, 6.0 and 7.5) and three Cu concentrations (0.2, 5 and 50 µM). During plant cultivation, pH changes and chlorophyll contents have been monitored. At harvest, shoot and root fresh biomass, the ionomic profile of shoots and roots, root morphology and root exudates have been assessed. Both plant species showed the capacity to change the pH of the solution, but oat plants showed a higher capacity to alkalinize the nutrient solution than cucumber plants to reduce Cu availability. Furthermore, oat plants exude more organic compounds and mainly phenolic compounds, while cucumber plants release more flavonoid compounds. Cucumber and oat plants supplied with 50 µM Cu and pH 4.5 exhibited the lowest growth, shoot and root biomass, root length and development because of higher Cu availability and toxicity effects. Copper interfered with the accumulation of both macro-and micronutrients, yet the synergisms and/or antagonisms resulted to be species and pH dependent. Overall, oat plants showed more resistance to high Cu concentrations in solution over a wide range of pH exhibiting a better plant development and more Cu-induced synergistic responses to nutrients than cucumber plants. Oat plants can be used in Cu-contaminated soils as phytostabilizer plant.

¹ Artigo elaborado de acordo com as normas de formatação da revista Environmental Pollution

Keywords: *Cucumis sativus L.*, *Avena sativa* cv. Fronteira, Cu availability, root exudates.

1. Introduction

Copper (Cu) is an essential element for plant growth and development. It is a structural component for the regulation of protein synthesis, participates in electron transport in the photosynthesis process, mitochondrial respiration, oxidative stress response, cell wall metabolism and hormonal signaling (Yruela, 2009; Marschner, 2012). However, excessive Cu availability in the soil can cause anatomical and morphological (Lequeux et al., 2010; Zambrosi et al., 2013; Ambrosini et al., 2015), physiological (Rosa et al., 2014; Zhang et al., 2014; Cambrollé et al., 2015), and nutritional alterations in plants (Cambrollé et al., 2015). Excessive concentrations of Cu in plant tissues influence the photosynthetic complex biosynthesis by reducing photosynthetic pigment content such as chlorophyll and carotenoids and change the chloroplast structure and thylakoid membrane composition (Cambrollé et al., 2013; Cambrollé et al., 2015). Copper can form complexes with chlorophyll by replacing the chlorophyll central Mg with Cu, impairing the process of converting light in chemical energy in the form of NADPH and ATP, and releasing O₂ (Yruela, 2005, 2009; Kabata-Pendias, 2011).

Too high availability of Cu in the rhizosphere also causes alterations in the root apex, with modifications in the cell walls and in the arrangement of the root apex tissue causing shortening and thickening of the roots (Potters et al., 2007; Lequeux et al., 2010). In addition, root darken, the number of lateral roots increases, and in the epidermis tissue can be plasmolyzed, thereby reducing the root density (Juang et al., 2012; Chen et al., 2013; Zhang et al., 2014; Ambrosini et al., 2015). The Cu-induced changes produced in the root cause a decrease in the mitotic division rate of cells, impairing root growth. As a result, a smaller soil surface is explored by the roots, and absorption of water and nutrients are hampered (De Vos et al., 1989; Juang et al., 2000; Kopsell e Kopsell, 2007; Lequeux et al., 2010; Ambrosini et al., 2015).

An exceptional accumulation of Cu is often observed in agricultural soils like in vineyards, because of the periodic application of Cu-containing agrochemicals (Brunetto et al 2016). Soil pH and both the quantity and quality of dissolved organic carbon (DOC) are the main drivers determining the bioavailability of Cu and its speciation (Chaignon et al., 2009; Kim et al., 2010; De Conti et al., 2016). Copper is mainly acquired by plants in the form of Cu²⁺ and Cu⁺ (Marschner, 2012; Brunetto et al., 2016). The free Cu²⁺ species predominates at acidic pH. For this reason, acid soils are more prone to induce Cu toxicity phenomena in

crops. With increasing soil pH, the formation of soluble and insoluble hydroxyl-species, as well as polymeric Cu species, shrink the extent of the available Cu²⁺ fraction and, in turn, the level of phytotoxicity (Kim et al., 2010; Pérez-Esteban et al., 2014; De Conti et al., 2016).

Plants actively change the chemical, physical and biological characteristics of the rhizosphere as a response to a myriad of biotic and/or abiotic stressors (Hinsinger et al 2005). For instance, when subjected to metal toxicity like Cu, plants increase the rhizosphere pH (Chaignon et al., 2009; Hinsinger et al., 2009; Bravin et al., 2009a; 2012). This possibility is plant-species dependent and strongly affected by the Cu contents, soil pH values and the soil buffer capacity (Bravin et al., 2009b). Moreover, roots' and microorganisms' exudation of organic compounds can affect the metals bioavailability (Hinsinger et al., 2009; Marschner, 2012; Bravin et al., 2012) via the formation of metal chelates/complexes (Chaignon et al., 2009; Kim et al., 2010; Kabata-Pendias, 2011; De Conti et al., 2018). Root exudates comprise a plethora of low molecular weight organic compound classes as phenolics, organic acids, amino acids, phytosiderophores, sugars, etc. (Montiel-Rozas et al., 2016; Zafari et al., 2016). Organic acids, for instance, are highly reactive with Cu and thus influence the distribution of chemical species in the rhizosphere, root apoplasm and root symplast (Michalak, 2006; Seshadri et al., 2015; Montiel-Rozas et al., 2016). Root exudation might represent a strategy to restrain plant Cu uptake as a metal tolerance response. Furthermore, specific compounds showed Cu²⁺ complexation capacity within the apoplasm preventing the Cu entrance into the symplast (Marschner, 2012; Seshadri et al., 2015; Montiel-Rozas et al., 2016; De Conti et al., 2018). As response to higher levels of available Cu, plants can also store excessive Cu at the root level, adsorbed to the wall cells or compartmentalized in the vacuole, thereby decreasing the concentration of Cu translocated to the shoot and the toxicity effects (Adrees et al., 2015; Printz et al., 2016). Yet, alterations in rhizosphere pH and root exudation pattern can be likely considered plant species-specific responses to the high concentration of metals in soils, as postulated by (Meier et al., 2012; Montiel-Rozas et al., 2016). Thus, the present study aimed at investigating the influence of both pH and excessive Cu concentrations on physiological and morphological responses of two different plant species, cucumber (*Cucumis sativus* L.) and oat (*Avena sativa* L.).

2. Material and methods

2.1 Plant material and growing conditions

The experiment was conducted with two plants, *Cucumis sativus* L. cv Chinese Long and *Avena sativa* cv. Fronteira. The plants were cultivated in a climatic growth chamber with

a light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 70% of relative humidity, a day/night cycle of 14/10 h and 24°C/19°C.

Seeds of cucumber and oat were germinated in darkness on moistened filter paper with 0.5 mM CaSO₄ for 5 and 7 days, respectively (Nikolic et al., 2012; Marasatoni et al., 2019b). The plants were then transferred to a full nutrient solution, for acclimatization, for 7 days. The full nutrient solution for cucumber had the following composition (mM): 2 Ca(NO₃)₂, 0.7 K₂SO₄, 0.1 KH₂PO₄, 0.1 KCl, 0.5 MgSO₄, and (μM): 10 H₃BO₃, 0.5 MnSO₄, 0.2 CuSO₄, 0.1 ZnSO₄, 0.01 (NH₄)₆Mo₇O₂₄, 100 Fe(III)-EDTA. The full nutrient solution for oat had the following composition: 0.5 KH₂PO₄, 1 M KNO₃, 2 M Ca(NO₃)₄H₂O, 1 M MgSO₄·7H₂O, a micronutrient solution with 0.2 mM CuCl₂·2H₂O, 0.01 M H₃BO₃, 2 mM MnCl₂·4H₂O, 1 mM ZnSO₄·7H₂O and 0.05 mM Na₂MoO₄·2H₂O, and 0.1 M NaFe(III)-EDTA. The nutrient solutions were changed every three days. The plants were cultivated in pots with a capacity of 1.5 L; we grew 10 and 28 plants per pot of cucumber and oat, respectively.

After the acclimatization period (*i.e.* 7 days in full nutrient solution), the plants were cultivated for another 7 days at three different pH values: 4.5, 6.0 (control) and 7.5. The pH of the solutions was measured and adjusted every day with either 1 M KOH or 1M HCl. Each treatment had 5 replicates. Afterward, each pH nutrient solution was supplemented with different Cu concentrations (0.2 (control), 5 and 50 μM) and plants were grown for an additional 7 days. Again, the pH of the solutions was measured and adjusted every day with either 1 M KOH or 1M HCl. The treatment 0.2 μM Cu was considered as control. The Cu source was CuSO₄ 5H₂O (ACS reagent, $\geq 98.0\%$, Sigma-Aldrich, Italy). Copper speciation depending on the pH of the nutrient solution has been computed with the Hydrochemical Equilibrium-Constant Database (HYDRA) and Medusa Software (Royal Institute of Technology in Stockholm, Sweden).

2.2 SPAD index

After 7 days of cultivation in nutrient solutions at different pHs supplied with different levels of Cu, the light transmittance of fully expanded leaves was determined using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan); the values were presented as SPAD (single-photon avalanche diode) index values. The average of five measurements was taken in each cucumber plant, measuring three plants per pot. The average of three measurements was taken in each oat plant, measuring five plants per pot.

2.3 Plant biomasses and tissue mineral composition

Plants were collected at the end of the experiment to determine the biomasses and mineral composition of the tissues. Roots and shoots were separated, roots were washed three times in distilled water and dried at 60°C until a constant weight was reached. Afterward, the shoot and root dry weight were measured. The samples were ground to a fine powder by a ball mill (MM400, Retsch, Germany), acid digested with concentrated nitric acid (HNO_3 , 65 % (v/v), Carlo Erba) using a single reaction chamber (SRC, UltraWave, Milestone Inc, Shelton, CT, USA). The element concentrations were determined by Inductively Coupled Plasma – Optic Emission Spectroscopy (ICP-OES, Arcos Ametek Spectro, Germany).

2.4 Root morphology

At harvest of the experiment (7 days after Cu addition, i.e. 21 days old plants), the root systems were scanned with an Epson Perfection V8000 Photo scanner. The images were analyzed by WinRHIZO software (EPSON 1680, WinRHIZO Pro2003b, Regent Instruments Inc., Quebec, Canada) for the determination of root length (cm plant^{-1}), surface area ($\text{cm}^2 \text{ plant}^{-1}$), volume ($\text{cm}^3 \text{ plant}^{-1}$), diameter (mm) and the number of tips.

2.5 Root exudate collection and analysis

Root exudates were collected after 14 days of cultivation in the nutrient solutions at different pHs and supplied with different levels of Cu. The root exudates were collected as described by Valentinuzzi et al. (2015a): three plants were removed from the nutrient solution and the roots were washed several times with distilled water; the roots were transferred to small pots covered by aluminium foil to keep roots in the dark and avoid oxidation, and submerged in 20 mL of milliQ water with aeration for 24 hours. Afterward, the plants were removed, the trap solution was filtered at 0.45 μm , freeze-dried, and resuspended in 2 mL of methanol: Milli-Q water (60:40 v/v) and stored at -20 °C until the analysis. The roots were weighed after collecting the exudates (fresh matter) and dried at 60°C until a constant weight was reached (dry matter).

The concentration of total phenolic compounds in root exudates was determined using a modified colorimetric method (Folin and Ciocalteu, 1927). The absorbance of the total phenolic compounds was measured at 765 nm and the concentration was expressed as nmol equivalent of Gallic acid per g of root fresh weight. The flavonoids concentrations were determined as described by Miliauskas et al. (2004). The absorbance of the flavonoids

compounds was measured at 415 nm and the concentration was expressed as nmole equivalent of Rutin per g of root fresh weight.

2.6 Statistical analysis

The experimental data were submitted to analysis of variance (ANOVA) in Sisvar software, version 4.0 (Ferreira, 2008). When the effect of treatments was significant, the means were separated by the Tukey test at P<0.05.

In addition to the analysis of variance, a multivariate principal component analysis (PCA) was performed, using Canoco software version 4.5 (Ter Braak and Smilauer, 2002). The PCA was performed for both cultures, cucumber and oats, using data of elements concentration in the roots and shoots (P, Ca, Mg, S, Cu, Mn, Fe and Zn), root exudates (phenolic and flavonoids compounds), root parameters (total roots length and root tips) and plant growth parameters (roots and shoots fresh matter, and SPAD index). Thus, PCA was performed using a set of principal components (PC), of which, PC1 and PC2 were used. These were the reflection of standardized linear combinations, orthogonal, which explain the original total variability of the results.

3. Results

3.1 Time course of rhizosphere pH changes

The capability of cucumber and oat plants to modify the rhizosphere pH was assessed by measuring the pH of the nutrient solution before and after starting the Cu supply (Supplementary Figure 1). At pH 4.5 plants of cucumber and oat increased the pH to approx. 5.2 and 6.0, respectively, when they were cultivated with 0.2 µM Cu (Supplementary Figure 1a, d). After the addition of higher Cu concentrations (5 and 50 µM), the plant's capacity for changing the pH decreased, especially for cucumber plants (Supplementary Figure 1a, d). This effect is more evident in the treatment with 50 µM Cu, where cucumber and oat plant's capability of alkalinizing the rhizosphere pH is almost impaired (Figure 1a, d at supplementary material).

At pH 6.0 the plants increased slightly the pH of the rhizosphere of cucumber plants treated with low Cu concentrations (0.2 and 5 µM), while in plants treated with 50 µM Cu, the pH decreased down to 5.2 – 5.4 after the Cu supply to the nutrient solution (Supplementary Figure 1b). Similarly, oat plants, increased the pH of their rhizosphere to 6.5 – 6.7 when treated with low Cu concentration (0.2 and 5 µM). At 50 µM Cu, oat plants first acidified the rhizosphere to pH 5.4 and then alkalinized it to 6.5 (Supplementary Figure 1e).

Cucumber and oat plants grown in nutrient solutions at pH 7.5 tend to decrease the pH (Supplementary Figure 1c, f). Cucumber plants treated with the highest Cu concentration, 50 μM Cu, acidified only slightly the rhizosphere to pH 6.9 (Supplementary Figure 1c) while oat plants treated with 5 and 50 μM Cu presented pH rhizosphere values between 6.9 and 7.1, respectively (Supplementary Figure 1f).

3.2 Copper speciation

The ionic speciation of the nutrient solution showed that 100% of Cu is available as Cu^{2+} at pH 4.5 at all the Cu concentrations used (Supplementary Table 3). At pH 6.0, 98% of Cu is available as Cu^{2+} and CuOH^+ represents the remaining 2% (Supplementary Table 3). Cu^{2+} availability is greatly reduced at pH 7.5, where Cu forms monomeric and polymeric hydroxy-species as CuOH^+ , $\text{Cu}_2(\text{OH}_2)^{2+}$, $\text{Cu}(\text{OH})_2$ and $\text{Cu}_3(\text{OH})_4^{2+}$. At pH 7.5, the reduction in Cu^{2+} distribution is also Cu concentration dependent: it decreases from 70 to 59 and 30 % at Cu 0.2, 5 and 50 μM , respectively (Supplementary Table 3).

3.3 Plant biomass allocation

At harvest, plants fresh weight was assessed and a species-dependent effect of both the pH and the different Cu levels was observed (Figure 1). Cucumber plants grown at pH 4.5, presented a decreasing shoot and root growth with increasing Cu concentration (Figure 1a, b). When cucumber plants were grown at higher pHs, *i.e.* 6.0 and 7.5, the intermediate Cu concentration (5 μM) seemed to have a plant growth promoting effect leading to the highest shoot and root biomass while, as expected, plants treated with the highest Cu concentration (50 μM) exhibited the lowest root and shoot biomass (Figure 1a, b). Comparing the same Cu doses, control plants revealed decreasing shoot biomass with increasing pH of the nutrient solution, no effect was observed at the root level (Figure 1a, b). At intermediate Cu concentrations (5 μM Cu), root fresh weight increased with increasing pH, while shoot biomass resulted highest in cucumber plants grown at pH 6, followed by pH 7.5 and 4.5 (Figure 1a, b). When cucumber plants were exposed to the highest Cu dose (50 μM), shoot and root biomass increased with increasing pH, thus Cu exhibited reduced toxicity at the highest pH studied (Figure 1a, b).

The growth stimulatory effect of the intermediate Cu concentration was observed also in oat roots and shoots (Figure 1c, d), particularly at pH 4.5 and 6.0. On the other hand, contrary to what observed for cucumber plants, control oat plants exhibited an increasing shoot and root biomass with increasing pH. At higher Cu doses, oat plants behaved again

similar to cucumber plants: the higher the pH, the higher the root and shoot biomass (Figure 1c, d).

Shoot and root biomasses of both cucumber and oat plants were confirmed by the visual observation of the plant health status (Figure 2 and 3). Indeed, cucumber plants grown at pH 7.5 showed the least Cu toxicity symptoms compared to plants grown at pH 4.5 and 6.0 (Figure 2 a, b, c). The highest Cu concentration (50 µM) Cu caused a strong effect on plants cultivated at pH 4.5 and 6.0; particularly at pH 4.5, plants showed yellowing, withering and drying of leaves, and to some extent even leave death (Figure 2 a, b, c).

In oat plants, the effects of Cu were not so evident, especially in the solution at pH 6.0 and 7.5 (Figure 3 a, b, c). Oat plants cultivated in solutions at pH 4.5 and 50 µM Cu showed slight withering and yellowing of leaves (Figure 3 a, b, c).

3.4 SPAD index

The chlorophyll content determined as light transmittance and represented as SPAD index values revealed plant-species dependent responses to both pH and Cu levels (Table 1). At all pHs studied, cucumber plants exhibited an increasing SPAD index with increasing Cu concentration supplied in the nutrient solution. Comparing the behavior within the same Cu doses, we observed only an effect in cucumber plants exposed to the highest Cu concentration (50 µM): plants grown at pH 7.5 showed the lowest SPAD values (Table 1).

Oat plants, on the other hand, displayed decreasing SPAD index values with increasing Cu concentrations supplied in the nutrient solutions at all the three pHs studied (Table 1). No pH effects were observed at the intermediate Cu concentrations. In control conditions and at the highest Cu concentration supplied, the highest chlorophyll content was determined in plants grown at alkaline pH (7.5, Table 1).

3.5 Root architecture

Both pH and Cu toxicity affected the root morphology of both plant species studied (Figure 4a, b, c and 6a, b, c). Cucumber plants grown at pH 4.5 presented decreasing root length and root tips with increasing Cu concentration in the nutrient solution (Figure 5a, b), no significant effects were observed at pH 6.0. At pH 7.5 cucumber plants increased root length and root tips with increasing Cu concentration in solution (Figure 5a, b). In control conditions, cucumber plants displayed the highest root length and the greatest number of tips when grown in acidic conditions (pH 4.5). When exposed to toxic concentrations, cucumber

plants enhanced their root length and their number of tips with increasing pH of the nutrient solutions (Figure 4a, b, c and 5a, b).

Oat plants behaved again differently: in very acidic conditions (pH 4.5) plants exposed to the intermediate and highest Cu concentration reported the highest and lowest root length and the number of tips, respectively (Figure 7a, b). In slightly acidic (pH 6.0) and alkaline (7.5) conditions, oat plants showed the lowest root length and number of tips, when they were exposed to the highest Cu concentration.

Comparing the root morphology of oat plants within the same Cu doses, the highest root traits were determined in plants grown in alkaline conditions (pH 7.5, Figure 6a, b, c).

3.6 Root exudate analysis

Cucumber and oat root exudates collected during the cultivation period was further analyzed to determine the qualitative and quantitative composition in terms of phenolic compounds and flavonoids (Figure 8). Interestingly, root exudation was higher in oat plants ($84.3 \text{ nmol g}^{-1} \text{ FW}$ in $50\mu\text{M Cu}$ and pH 4.5). Again, both pH and Cu supply affected the quali-quantitative composition of the root exudates of both plant species. In cucumber plants grown with intermediate and high Cu concentrations, flavonoid release resulted highest at pH 7.5 (Figure 8a). The same occurred for the release of phenolics, *i.e.* the highest release was observed in control conditions and in cucumber plants exposed to high Cu concentrations (Figure 8b). Comparing the different pHs of the nutrient solutions, the highest release of flavonoids was determined in the exudates of cucumber plants exposed to the highest and intermediate Cu concentration at pH 6.0 and 7.5, respectively (Figure 8a). The release of phenolics resulted significant only at acidic pHs: again, the highest Cu concentration induced the highest release of phenolics (Figure 8b).

Oat plants did not reveal a clear trend in terms of flavonoid and phenolics release both in function of pH and Cu concentration (Figure 8c, d). In control conditions, flavonoid release resulted highest when plants were grown in nutrient solutions at pH 6.0 and 7.5, while no effect was observed for the phenolics in the same conditions. When oat plants were exposed to the highest concentration of Cu, both flavonoid and phenolics release resulted highest at pH 4.5 and 7.5 compared to the exudates collected from oat plants grown at pH 6.0 (Figure 8c, d). Comparing the exudation pattern within the same pH, at pH 4.5, the highest Cu concentration led to the highest flavonoid and phenolics release (Figure 8c, d), while no significant differences could be observed at pH 6 for the two compound classes. At pH 7.5, we observed

a significant effect only for the phenolics, being highest in plants grown at the highest Cu concentration (Figure 8d).

3.7 Effect of pH and Cu toxicity on the mineral composition of cucumber and oat plants

Cucumber plants grown at all the three different pH conditions showed major Cu concentrations in shoots and roots when plants were supplied with the highest Cu concentration (50 µM Cu) (Figure 9a, b). The different pHs of the nutrient solutions led to a significant effect only at the highest Cu concentration supplied: shoot and root Cu concentration resulted highest when plants were cultivated at pH 4.5 (Figure 9a, b).

The main effect on Zn concentration was observed at the root level: in all pH conditions, Zn root content decreased with increasing Cu supply in the nutrient solution (Figure 9c, d). Also, the pH influenced Zn allocation in the roots, which resulted highest at pH 7.5. The Zn shoot content revealed an opposite trend: indeed, the highest Zn accumulation in cucumber shoots has been observed in plants grown at acidic pH (4.5, Figure 9c, d). Within each pH condition, Zn shoot content decreased with increasing Cu supply, yet resulted significant only at pH 6.0 and 7.5.

Iron shoot and root concentration of cucumber plants resulted highest in plants supplied with the highest Cu dose, independently from the pH (Figure 9e, f). When plants were grown with the intermediate and highest Cu concentration, the revealed the highest Fe root and shoot content at highly acidic pH (4.5). The only exception was observed for control plants, which exhibited the highest Fe root content at pH 6.0 (Figure 9e, f).

Manganese root and shoot content of cucumber plants displayed an interesting trend both in function of pH and Cu dose, completely different than the other micronutrients (Figure 9g, h). Independently from the pH, Mn plant content (roots and shoots) decreased with increasing Cu dose applied in the nutrient solution. Furthermore, at all Cu doses, the highest Mn plant content was observed at pH 7.5.

In addition, the macronutrients showed specific responses in function of pH and Cu dose applied (Supplementary Table 4). In particular, P shoot and root content decreased with increasing Cu concentration, yet significantly only at pH 6.0 and 7.5, respectively. Also, Ca concentration was hampered when increasing Cu doses were applied, yet only at the root level and at pH 4.5 and 7.5. On the contrary, at the shoot level, at pH 4.5, Ca concentration increased with increasing Cu concentrations (Supplementary Table 4).

Copper root content in oat plants resulted highest at pH 4.5 when plants were grown in control conditions and with intermediate Cu doses. At the highest Cu dose, Cu root and shoot content were highest at pH 7.5 and 4.5, respectively (Figure 10a, b).

Similar to what observed in cucumber plants, Zn shoot content displayed the highest concentrations in acidic conditions (pH 4.5.) and at all Cu doses (Figure 10c, d). At the root level, Zn concentration resulted highest at pH 6.0, when plants were subjected to low and intermediate Cu concentrations. Such a clear trend was not visible at high Cu concentrations (Figure 10c, d). In addition, in all pH conditions, Zn root content decreased with increasing Cu concentration in the nutrient solution.

Interestingly, Fe plant accumulation in oat plants displayed a completely different behavior than in cucumber plants (Figure 10e, f). When Cu was applied at low and intermediate concentrations, Fe root and shoot content resulted highest at pH 7.5. At higher Cu doses, i.e. 50 µM, the highest Fe root and shoot content was observed at pH 6.0 and 4.5, respectively. At all three pH conditions, the highest Fe root content was reached when oat plants were cultivated with the highest Cu dose. At shoot level, the same behavior was detected only at pH 4.5 (Figure 10e, f).

Also Mn plant content exhibited similar behavior as the one observed for cucumber plants (Figure 10g, h). When oat plants were cultivated with intermediate and high Cu concentrations, the highest Mn root and shoot content was detected at pH 7.5 (Figure 10g, h). Furthermore, as for the other micronutrients, Mn root content decreased significantly with increasing Cu concentrations. Manganese shoot accumulation revealed a similar trend, yet only at acidic pH conditions (4.5 and 6.0). In alkaline conditions Mn shoot accumulation displayed the exact opposite behavior: the highest Mn shoot content was obtained with the intermediate Cu concentration, the lowest in control conditions (Figure 10g).

Considering the macronutrients of oat plants, pH and Cu dose exerted the main effect on P and Ca depending on the plant organs (shoots vs. roots, Supplementary Table 5). As expected, P shoot content decreased with increasing Cu concentrations in all pH conditions. Calcium, on the other hand, increased its shoot content with increasing Cu concentration, again in all pH conditions (Supplementary Table 5). This very clear behavior were not reflected at the root level. At the root level, Mg accumulation decreased with increasing Cu concentrations, yet only at pH 6.0 and 7.5 (Supplementary Table 5).

3.8 Principal component analysis (PCA)

The principal component analysis (PCA) carried out from the data obtained with cucumber used only components 1 and 2, which explained around 60% of the original variability of the results (Figure 11a). Of this amount, 43% was explained by PC1, which was mainly influenced by the concentration of Cu, Fe, Mg, P, S and Zn in the roots, Cu, Fe and Mn in the shoots, as well as fresh matter of roots and SPAD index. The principal component 1 was efficient in separating the treatments into two large groups, the positive group of PC1 (orange dashed ellipse), in which the cucumber samples submitted to 50 µM Cu at pH 4.5 and 6.0 were grouped, in addition to those subjected to 5 µM Cu at pH 4.5. The other group, negative on the PC1 axis (brown dashed ellipse), was composed of the remaining treatment combinations. Thus, we can see that only at pH 7.5 the Cu doses were grouped equally. In addition, principal component 2 explained around 16%, being influenced only by the Ca and Mg concentrations in the shoots. And as in PC1, it separated the treatments at pH 7.5 below the central line, as well as, all observations made when applying 50 µM Cu at any pH. The pink, blue and green ellipses indicate the allocation of different concentrations of Cu at each pH. The concentrations of Cu and Fe in roots and shoots correlated positively with each other. In addition, these parameters were negatively correlated with the concentration of Mg in the roots, and root parameters. In particular, root parameters, such as total root length and root tips, had a positive correlation with the concentrations of Ca, Mg and S in the roots. Fresh root matter correlated positively with the concentration of P in roots. The concentration of Zn in the shoots was inversely correlated with the production of flavonoids, which is correlated with the concentrations of Ca and Mg in shoots.

Principal component analysis (PCA) for data obtained from oats also used components 1 and 2, and added together also explained around 60% of the original data variability (Figure 11b). PC1 explained 45% of the variability, being influenced by the concentrations of Cu, Fe, Mn and S in the roots, Cu and P in the shoots, in addition to the number of root tips. The principal component 1 was efficient in separating the treatments into two large groups, the positive group of PC1 (pink ellipse), in which only the oats samples submitted to 50 µM Cu were grouped at all pH levels. The other group, located on the left of the PC1 axis (blue ellipse), was composed of treatments 0.2 and 5 µM Cu at all pH tested. Finally, the main component 2 explained about 14%, being influenced only by the concentrations of Mg in roots and Mn in shoots, besides the fresh matter of roots. PC2 was not efficient in clearly separating groups. Thus, following correlations that also occurred in the study with cucumbers, the concentrations of Cu and Fe in the roots correlated positively. In addition, the

concentration of Zn in roots showed a negative linear correlation with the production of flavonoids and concentration of Cu in roots. In addition, there was a positive correlation between total root length, root tips and concentration of S in roots. Especially, the total root length was inversely related to the Cu concentration in the shoots. The fresh matter parameters of roots and shoots, in addition to the concentration of Mn in shoots, correlated positively with each other.

4. Discussion

Copper availability levels and chemical species distribution in soil are dependent on the pH and the quasi-quantitative dynamic of rhizosphere organic compounds (Chaignon et al., 2009; Kim et al., 2010; De Conti et al., 2016). Copper is mainly acquired by plants in the forms of Cu^+ and Cu^{2+} , which are both available in the soil solution (Marschner, 2012; Brunetto et al., 2016). However, the levels of Cu availability exceed very often the optimum required for plants resulting in different physiological responses (Brunetto et al., 2016), which are strictly concentration-, time of exposition and plant-species dependent (Marastoni et al., 2019a; 2019b). Copper toxicity can promote alterations in ion homeostasis in plants through the antagonism, synergism and/or competition between elements for uptake and translocation (De Conti et al., 2018; Marastoni et al., 2019a). In fact, the specific response of plants to this Cu disorder may present different changes in nutritional homeostasis and root activities in terms of pH changes and root exudation (Marastoni et al., 2019a; 2019b). Indeed, the present study aimed at evaluating the physiological responses of cucumber and oat plants in function of pH and Cu concentrations.

It is well known that Cu solubility and thus its bioavailability and toxicity increase with decreasing pH being the acidic soils more prone to induce the disorder (McBride, 1994; Chaignon et al., 2009). Yet, plants have developed an adaptation strategy counteracting metal toxicity by alkalinizing their rhizosphere (Bravin et al. 2008; Hinsinger et al., 2003; 2009; Bravin et al., 2012). Indeed, in the present study, plants grown at acidic pH, *i.e.* 4.5, increased their rhizosphere pH during the cultivation period (Supplementary Figure 1), yet with a species and Cu dose dependent response. Oat plants seemed to be more efficient in this sense (Marastoni et al., 2019c) exhibiting the capability to alkalinize their rhizosphere up to an intermediate concentration of Cu (*i.e.* 5 μM). At very high Cu concentrations, both plant species revealed an impaired alkalinizing effect most likely due to high Cu shoot and root content causing severe damages to important cellular processes. Again, oat plants performed better presenting a 3-fold lower root Cu content than cucumber plants (Figure 9b and 10b).

Interestingly, both plant species alkalinized their rhizosphere when grown at slightly acidic pH (*i.e.* 6.0) and supplied with low and intermediate Cu concentrations. At high Cu supply, the two plants behaved differently: cucumber plants even acidified the growing medium down to almost one pH unit while oat plants first acidified and then alkalinized the rhizosphere after the second Cu supply reaching pH values close to the other treatments (Supplementary Figure 1). This recovery capacity exhibited by oat plants is correlated with a much lower Cu accumulation in both roots and shoots than the one detected in cucumber plants (Figure 9a, b and 10a, b). Indeed, particularly at shoot level, oat plants exhibited a 2-fold lower Cu concentration than cucumber plants when supplied with 50 µM Cu. Also, oat plants cultivated with high Cu concentrations showed similar behavior at all the pH values (Figure 11b). These results highlight the strong adaptation capability of oat plants to excessive Cu concentrations and confirm that rhizosphere effects (*i.e.* acidification and alkalinization processes) are not always constant over time (Faget et al., 2013) and are strongly plant species dependent (Bravin et al., 2009a; Faget et al., 2013). Furthermore, pH modifications through the release of H⁺ or OH⁻ in the growing medium depend also on the plant's nutritional needs and on Cu-induced imbalances in cations/anions uptake rate (Hinsinger et al., 2003; 2009; Bravin et al., 2012; Marschner et al., 2012). Differences in this latter might also reflect the simple genotypical difference between oat and cucumber plants, which is most likely connected to a differential calcium demand for the cell wall biosynthesis observed in monocots and dicots (Marschner et al., 2012).

It is interesting to note, that both cucumber and oat plants adopted an opposite behavior when grown in alkaline conditions (Figure 1, 2 and 3). Indeed, in both plant species an acidification of the growing medium has been detected, particularly pronounced at high Cu levels. In these conditions, *i.e.* at alkaline pH and excessive Cu, oat plants exhibited almost a two-fold higher Cu root and shoot concentration than cucumber plants (Figure 9a, b and 10a, b). Interestingly, even though oat accumulated more Cu than cucumber, oat resulted to be the more Cu tolerant species in terms of shoot and root development (Figure 1a, b, c, d) as already observed in previous studies (Marastoni et al 2019b). Indeed, oat plants have been described to tolerate excessive Cu concentrations via an external exclusion mechanism favoring Cu accumulation in the roots and particularly in the apoplasm to avoid damages at cellular level (Marastoni et al., 2019b). Indeed, the accumulation of Cu in the root system might occur thanks to the affinity for Cu of the ligands present at the root cell wall and apoplasm. Consequently, this phenomenon reduces the extent of the free Cu fraction within the plant limiting the Cu translocation to the shoots and the onset of toxicity symptoms

(Ambrosini et al., 2017; Comin et al., 2018; Juang et al., 2012; Ambrosini et al., 2015). As a whole this phenomenon can be considered a component of a more complex plant-defense strategy aimed at containing the toxic effects linked to an excessive availability of the metal.

In cucumber plants, Cu supply to the growing medium affected the chlorophyll content of leaves (in terms of SPAD units, Table 1) most likely due to the Cu-induced P deficiency (Toselli et al., 2009; Marschner et al., 2012; Baldi et al., 2018). Interestingly, the changes in SPAD index values did not vary with changing pH of the growing solution. Phosphorus deficiency inhibits leaf expansion, but proteins and chlorophyll are still being synthesized. Therefore, plants tend to increase the concentration of chlorophyll per unit leaf area and the leaves present a darker green color (Marschner et al., 2012; Valentinuzzi et al., 2015b) with consequent increased SPAD index values (Table 1). In oat plants, the SPAD index has slightly decreased with increasing Cu supply (Table 1), yet again without significant differences among the pH values used. Indeed, excess of Cu can reduce the biosynthesis of chlorophyll and produce structural alterations in the photosynthetic apparatus, limiting plants photosynthetic activity (Adrees et al., 2015). Marastoni et al. (2019a) described a similar phenomenon caused by Cu in two oat cultivars.

With respect to the root tissue, it is well known that plants can modify the architecture and the development of the roots as a consequence to the exposure to high Cu availability and pH of the growing medium. Cucumber and oat growing in the solutions at pH 4.5 with 50 µM Cu supply showed the lowest root growth and development (Figure 4, 5, 6 and 7). This behavior can be explained by the high availability of free Cu ions (Supplementary Table 3) and the consequent phytotoxic effect in the root system. High Cu concentration can in fact reduce the rate of cell division in the root apex, consequently diminishing the further root apex development and increasing the number of lateral roots. These phenomena together impair considerably the root development (Ouzounidou et al., 1992, Potters et al., 2007; Guimarrães et al., 2016). The Cu-toxicity induced shortening of the main roots as well as the increase of root thickness and of the secondary root number cause cells plasmolysis, which consequently result in a smaller soil holding surface by the roots and an impaired water and nutrients acquisition by plants (Juang et al., 2012; Chen et al., 2013; Zhang et al., 2014; Ambrosini et al., 2015).

Alkaline growing conditions improved root growth containing thereby the severity of Cu toxicity (Figure 4c and 6c) in both plant species. In particular, the increased growth and ramification of the roots might be correlated with the restraint of the free ion species Cu²⁺

(Supplementary Table 3). Particularly at high Cu concentrations, the extent of free Cu ions species is shorten of 30 %.

Abiotic stress conditions, such as the lower availability of nutrients or the presence of higher levels of toxic ions in the solution, like Cu, induce the release of organic exudates (Vives-Peris et al., 2019). Organic acids and flavonoids are released by dicotyledonous and non-gramineous monocotyledonous plants to increase nutrients availability and promote the root acquisition of some barely available nutrients like Fe and Zn (Broadley et al., 2011). Differently, for the same purpose gramineous plants release non proteinogenic amino acids named phytosiderophores (Broadley et al., 2011). Results here presented show that cucumber and oat plants triggered the exudation of flavonoids and phenolic compounds as a consequence to the exposure to high levels of Cu in the growing solution (Figure 8). Cucumber and oat plants grown in alkaline pH conditions released more flavonoids and phenolic compounds. Furthermore, the quantity and quality of organic exudates differed between the two plant species. Oat roots exuded higher levels of phenolic compounds, whereas cucumber plants more flavonoids. Indeed, previous studies (Marastoni et al. 2019a) revealed that the release of phenolic compounds was fundamental in the Cu tolerance through the over expression of phenolic transporters (PEZ-like genes). Phenolic and flavonoids reduce the Cu availability through the binding with Cu²⁺ in the rhizosphere soil solution and in the root apoplasm, limiting thus the metal acquisition by the plants (Jung et al., 2003). This phenomenon postulated by (Michalak, 2006) has been also described in grapevine (Ambrosini et al., 2015) and peach (Somavilla et al., 2018).

With respect to the ionomic profile of the plant tissue, Cu excess is known to affect plant nutrient uptake and ion homeostasis through the synergic, antagonist and competitive relation in solution (De Conti et al., 2018; Marastoni et al., 2019). In the present study, Cu and Fe concentration were positively correlated with each other in both species (Figure 11a, b). This synergic relation between Cu and Fe has been observed in roots and shoots of cucumber and oat plants and at almost all pH values used (Marastoni et al. 2019). Indeed, shoots of both plant species revealed a Cu-Fe antagonism at pH 7.5 (Figure 9e and 10e). Cucumber and oat plants showed a decrease in Mn and Zn shoot and root concentration with increasing Cu concentration (Figure 9c, d, g, h and 10c, d, g, h). Such antagonisms were already observed in oat plants (Marastoni et al., 2019b) and in ryegrass plants (De Conti et al., 2020). However, the present study highlighted that nutrient interactions are highly species and pH value dependent. Indeed, the Cu/Mn antagonisms were observed at all pH in cucumber plants, while in oat plants, only at pH 6.0 and 7.5, a Cu-concentration dependent interaction

has been observed: a synergism at low Cu supply (5 µM) and an antagonism at high Cu supply (50 µM). Furthermore, the Cu/Zn antagonisms was also confirmed in both species and at all pH values used in the present work, except at pH 6.0 in oat shoots, which exhibited a synergistic Cu/Zn interaction.

Considering the macronutrient accumulation in both roots and shoots of cucumber and oat plants, Cu induced again species and pH value dependent nutrient interactions (Supplementary Table 4 and 5). Phosphorus accumulation was reduced with increasing Cu supply in both species at all pH, as widely described in literature (De Conti et al., 2018; Marastoni et al., 2019b), yet with the exception of oat plants grown at pH 6.0 and 7.5. These plants exhibited a significantly increased P root accumulation when supplied with the highest Cu concentration (50 µM). Such an increase at the root level might be due to the co-precipitation of Cu and P favored at alkaline and sub-acidic pH and inhibiting a further translocation of P to the shoots (De Conti et al., 2018). Considering S, a Cu-induced decrease has been observed at the shoot and root level in cucumber plants while only at the root level in oat plants, independently from the pH value of the growing medium (Supplementary Table 4 and 5). An impaired root accumulation of anionic nutrients as S and P might suggest that cationic adsorption and binding sites are predominant hindering anionic nutrient accumulation. On the other hand, oat shoots exhibited even an enhanced S accumulation with increasing Cu supply, which resulted significant at pH 4.5 and 7.5. Also Ca did not reveal a common trend in function of plant species and pH values. For instance, a synergistic Cu/Ca effect was observed for both plant species at the shoot level at all the pH studied. At the root level, on the other hand, cucumber plants exhibited an antagonistic Cu/Ca interaction at all pHs studied as described for several other plant species (Kopittke and Menzies, 2006; Lequeux et al., 2010). The Cu/Ca interaction in oat plants at the root level revealed no clear trend: at pH 4.5 it resulted antagonistic at low and synergistic at high Cu concentrations; at pH 6.0 it resulted synergistic as in its shoots and at pH 7.5 it resulted synergistic at low and antagonistic at high Cu concentrations. These results highlight that the impact on ion homeostasis induced by Cu toxicity is strictly dependent on the plant species and the pH of the growing medium (Marastoni et al., 2019a; 2019b). In all the pH conditions studied and in both plant species, Mg shoot concentration was not affected by Cu supply (Supplementary Table 4 and 5). On the contrary, Mg root accumulation has been significantly reduced in both plant species and all pHs with increasing Cu supply suggesting a competitive absorption in favor of Cu (Marastoni et al., 2019b).

5. Conclusion

Results here presented show that plants are able to modify the extent of Cu availability as well as the magnitude of the metal acquisition and translocation to the shoot by modifying the rhizosphere. These changes concern its pH and foresee the root release of specific low molecular weight organic compounds. Yet the intensity of these changes varies according to the plant species, the initial pH of the growing medium and the Cu availability. In this study, oat plants exhibited a greater capacity to alkalize the growing medium than cucumber plants. Furthermore, oat plants release more organic compounds (mainly phenolics), while cucumber plants exude more flavonoids. Data here presented showed that the Cu-induced alteration of ion homeostasis in roots and shoots is extremely species- and pH -dependent. Therefore, the effects of Cu toxicity in crops is rather complex to predict. With respect to the two plant species here considered, oat plants seem to be more prone to tolerate Cu toxicity conditions than cucumber ones. Therefore, oat plants could represent a valid alternative in Cu-contaminated soils over a wide range of pH to phytostabilize Cu in the growth medium in favor of other more sensitive crops in a context of intercropping system.

6. Contributions

Edicarla Trentin: study design, accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Stefano Cesco, Youry Pii, Tanja Mimmo and Gustavo Brunetto: study design, interpretation of results and writing of the article. Fabio Valentiniuzzi, Silvia Celletti, Sebastian Benedikt Feil and Mónica Yorlady Alzate Zuluaga: accomplishment of the experiment, laboratory analysis and interpretation of results. Paulo Ademar Avelar Ferreira, Felipe Klein Ricachenevsky, Lincon Oliveira Stefanello da Silva and Lessandro De Conti: analysis the data, interpretation of results and writing of the article.

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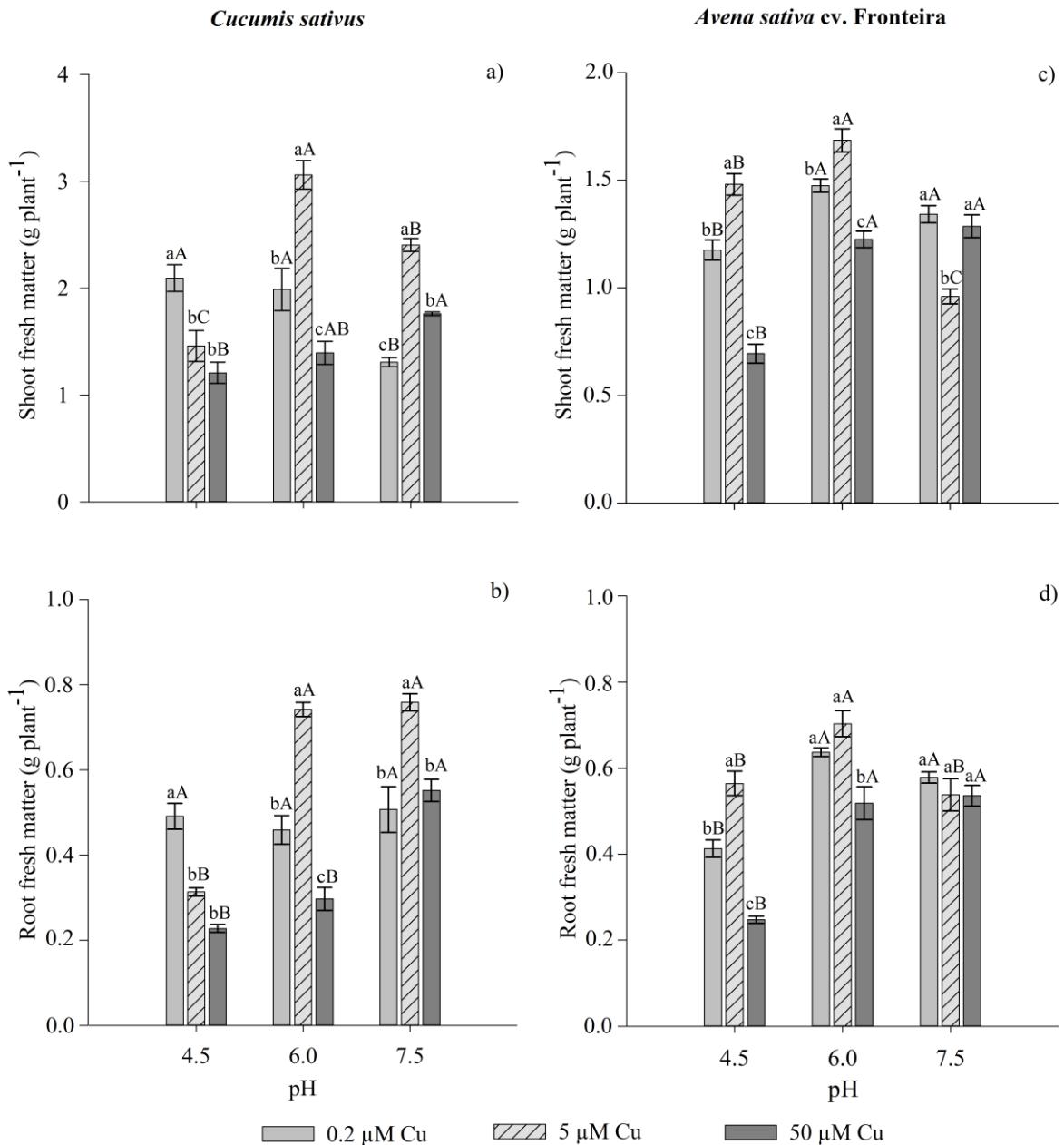


Figure 1. Shoot and root fresh matter of *Cucumis sativus* L. (a, b) and *Avena sativa* L. cv. Fronteira (c, d) grown in nutrient solutions at different pH and Cu concentrations. Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$); Data are expressed as mean \pm SD, $n = 5$.

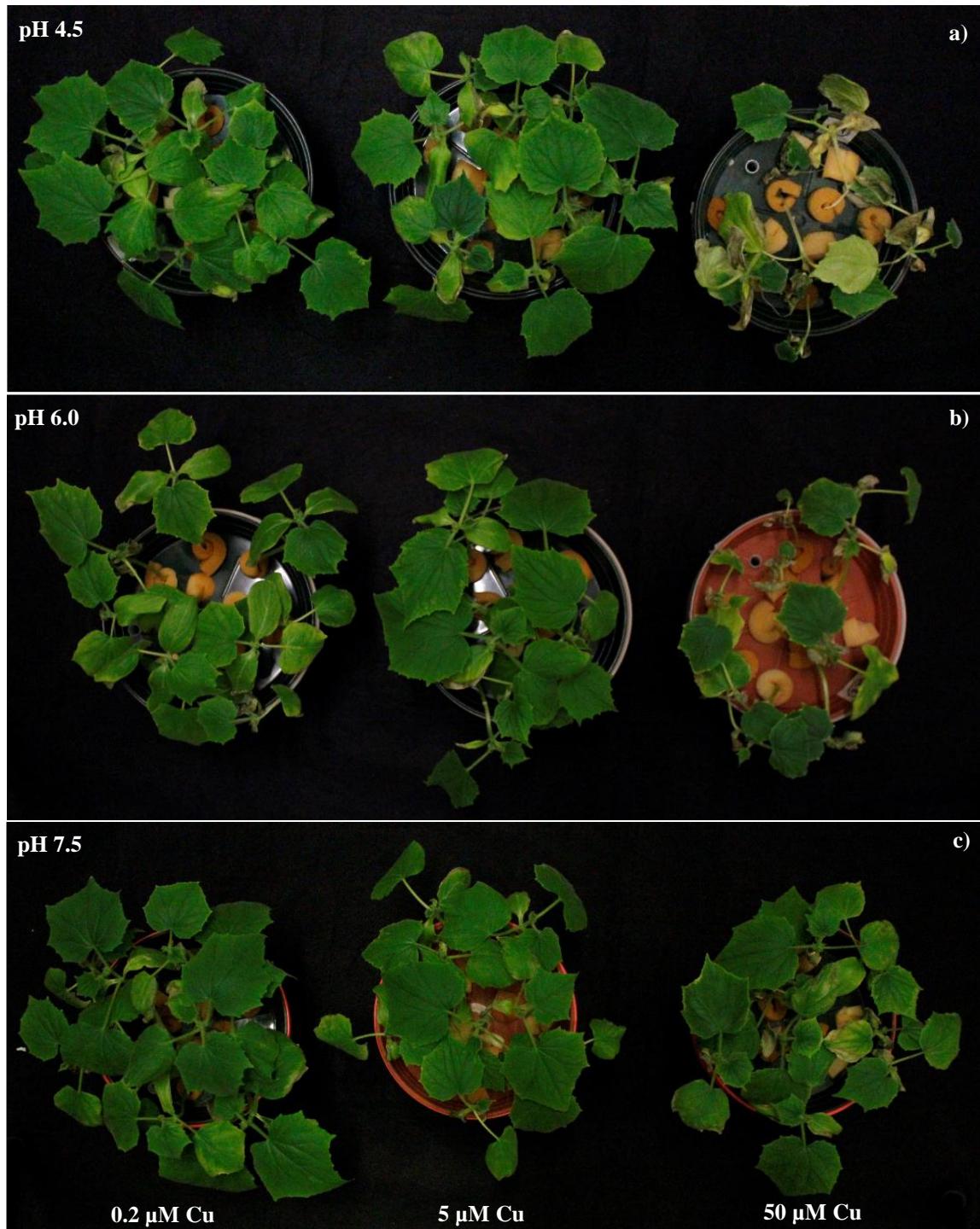


Figure 2. *Cucumis sativus* L. plants (21 days old plants) grown in nutrient solutions at different pH and Cu concentrations.

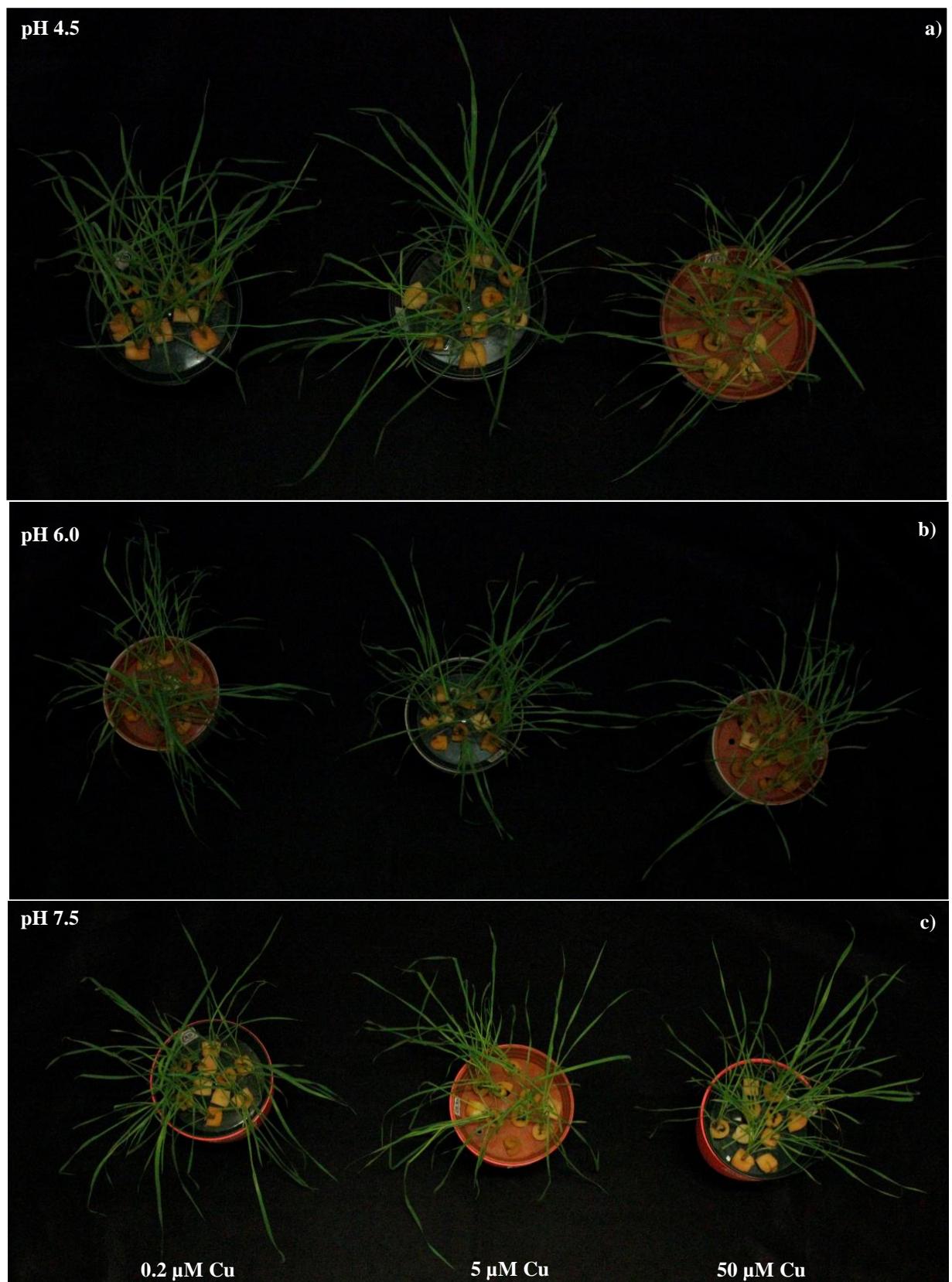


Figure 3. *Avena sativa* L. cv. Fronteira plants (21 days old plants) grown in nutrient solutions at different pH and Cu concentrations.

Table 1. SPAD index in *Cucumis sativus* L. and *Avena sativa* L. cv. Fronteira grown in nutrient solution with different pH values and with different pH values and Cu concentrations.

pH	Cu (µM)	SPAD (<i>Cucumis sativus</i>)	SPAD (<i>Avena sativa</i> cv. Fronteira)
4.5	0.2	31.92 ± 1.23 bA ⁽¹⁾	31.80 ± 0.28 aB
	5	32.38 ± 0.85 bA	32.30 ± 0.50 aA
	50	40.55 ± 1.17 aA	29.56 ± 0.28 bB
6.0	0.2	31.62 ± 0.99 bA	32.53 ± 0.43 aAB
	5	33.49 ± 0.22 bA	31.66 ± 0.22 abA
	50	41.52 ± 0.32 aA	30.77 ± 0.17 bA
7.5	0.2	30.92 ± 0.24 bA	33.08 ± 0.37 aA
	5	33.53 ± 0.91 abA	32.61 ± 0.43 abA
	50	34.14 ± 0.78 aB	31.79 ± 0.29 bA

⁽¹⁾Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test (p <0.05); Data are expressed as mean ± SD, n = 5.

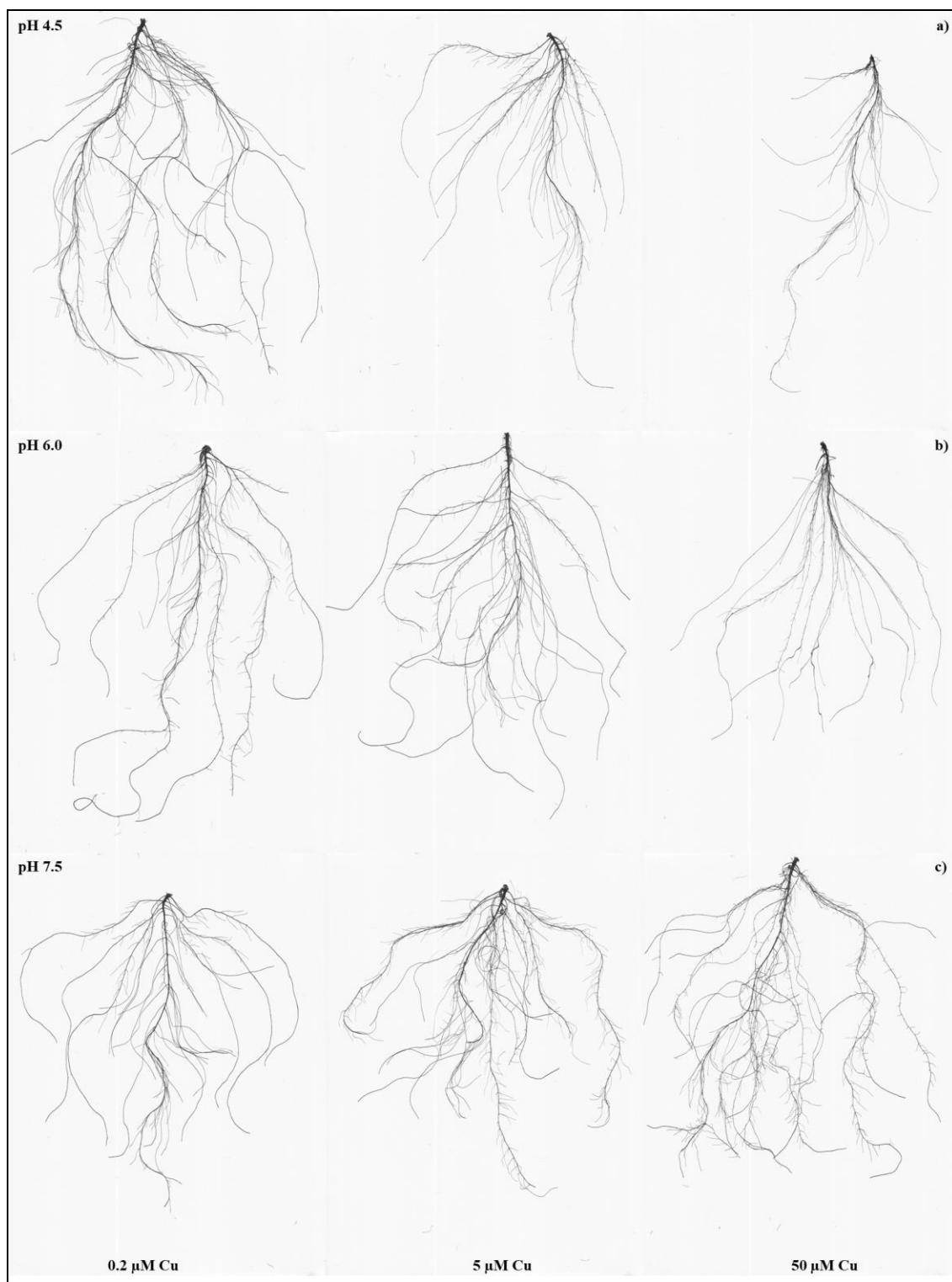


Figure 4. Root system of *Cucumis sativus* L. plants (21 days old plants) grown in nutrient solutions at different pH and Cu concentrations.

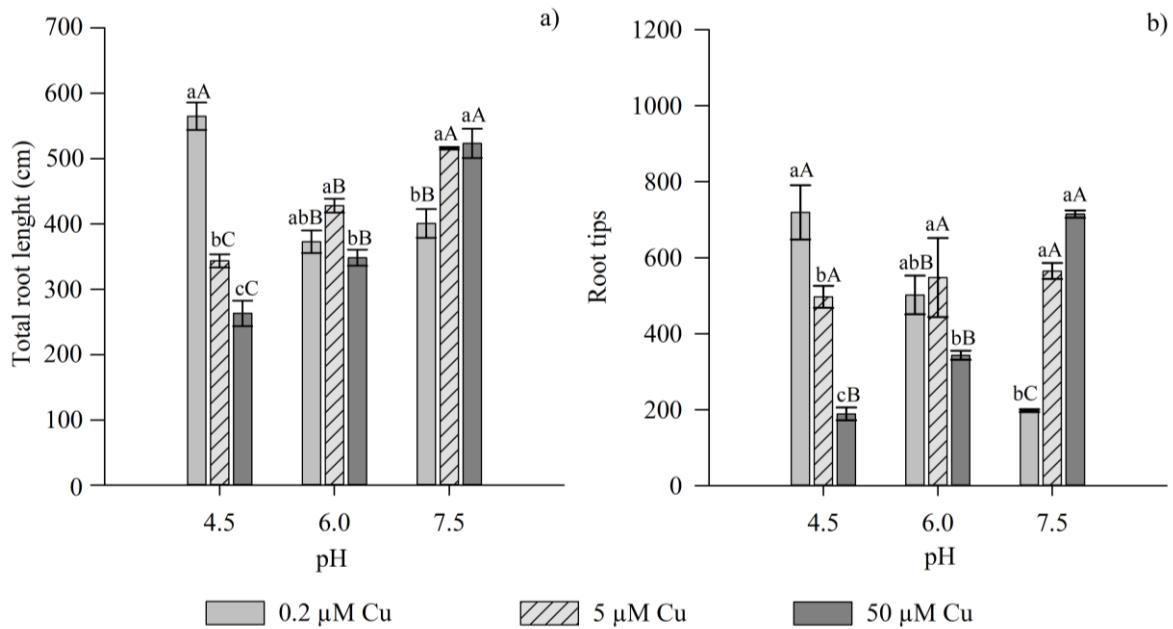


Figure 5. Total root length (a) and root tips (e) of *Cucumis sativus* L. grown in nutrient solution with different pH values and Cu concentrations. Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$); Data are expressed as mean \pm SD, $n = 5$.

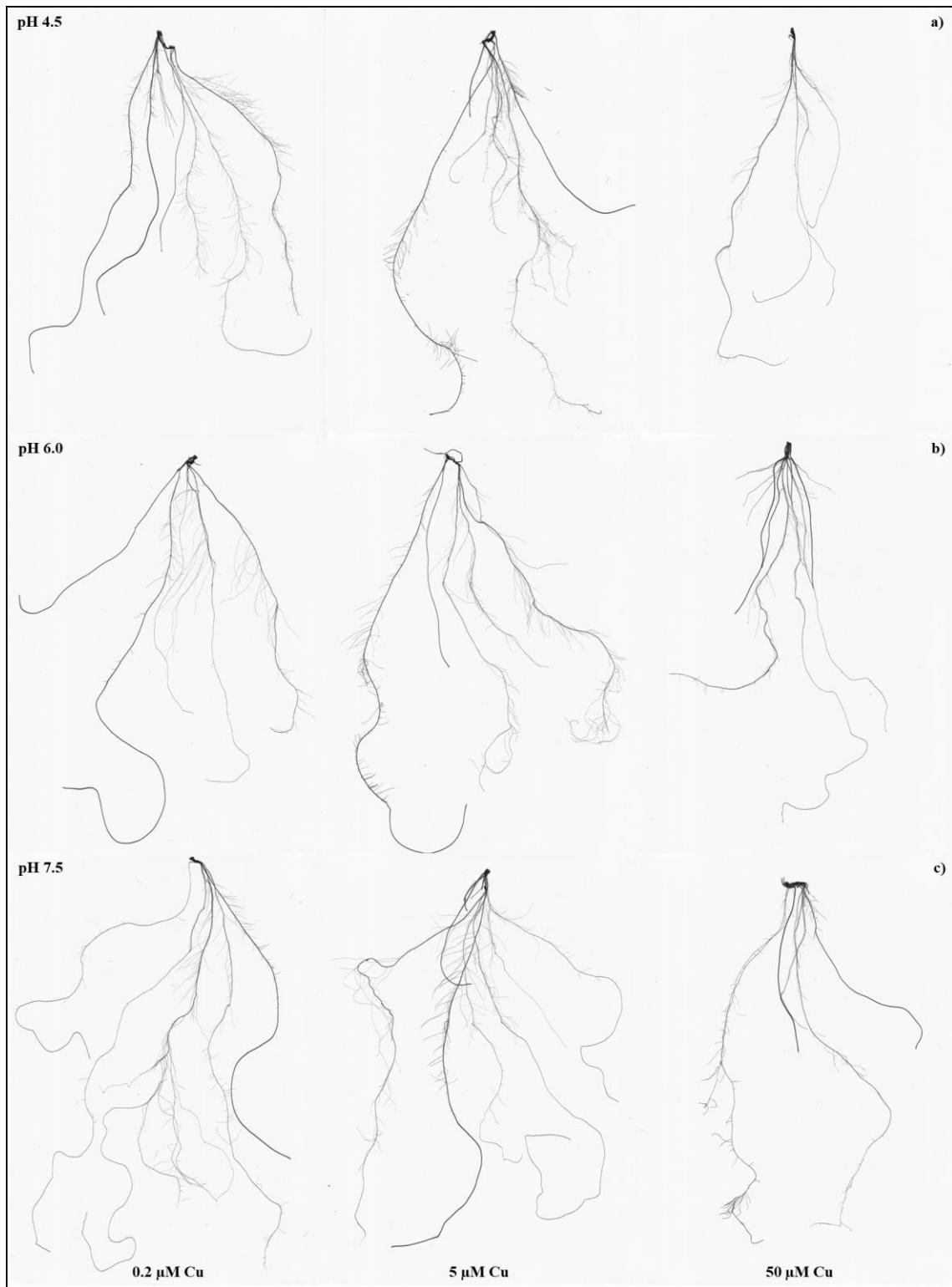


Figure 6. Root system of *Avena sativa* L. cv. Fronteira plants (21 days old plants) grown in nutrient solution at different pH and Cu concentrations.

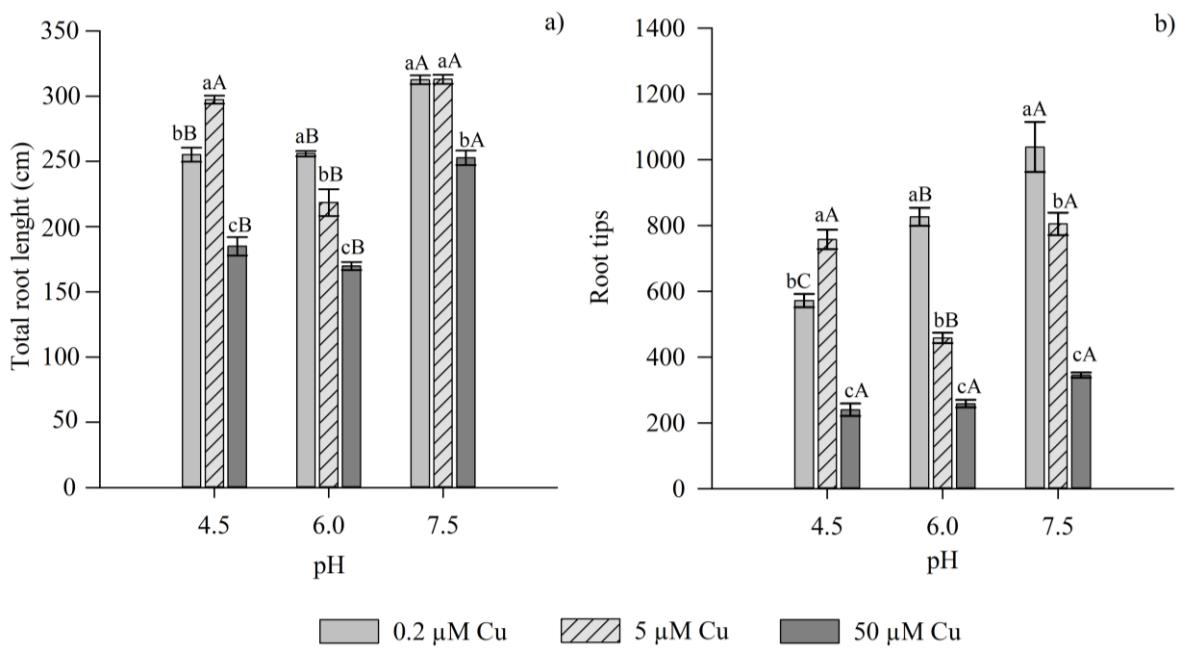


Figure 7. Total root length (a) and root tips (b) of *Avena sativa* L. cv. Fronteira grown in nutrient solution with different pH values and Cu concentrations. Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$); Data are expressed as mean \pm SD, $n = 5$.

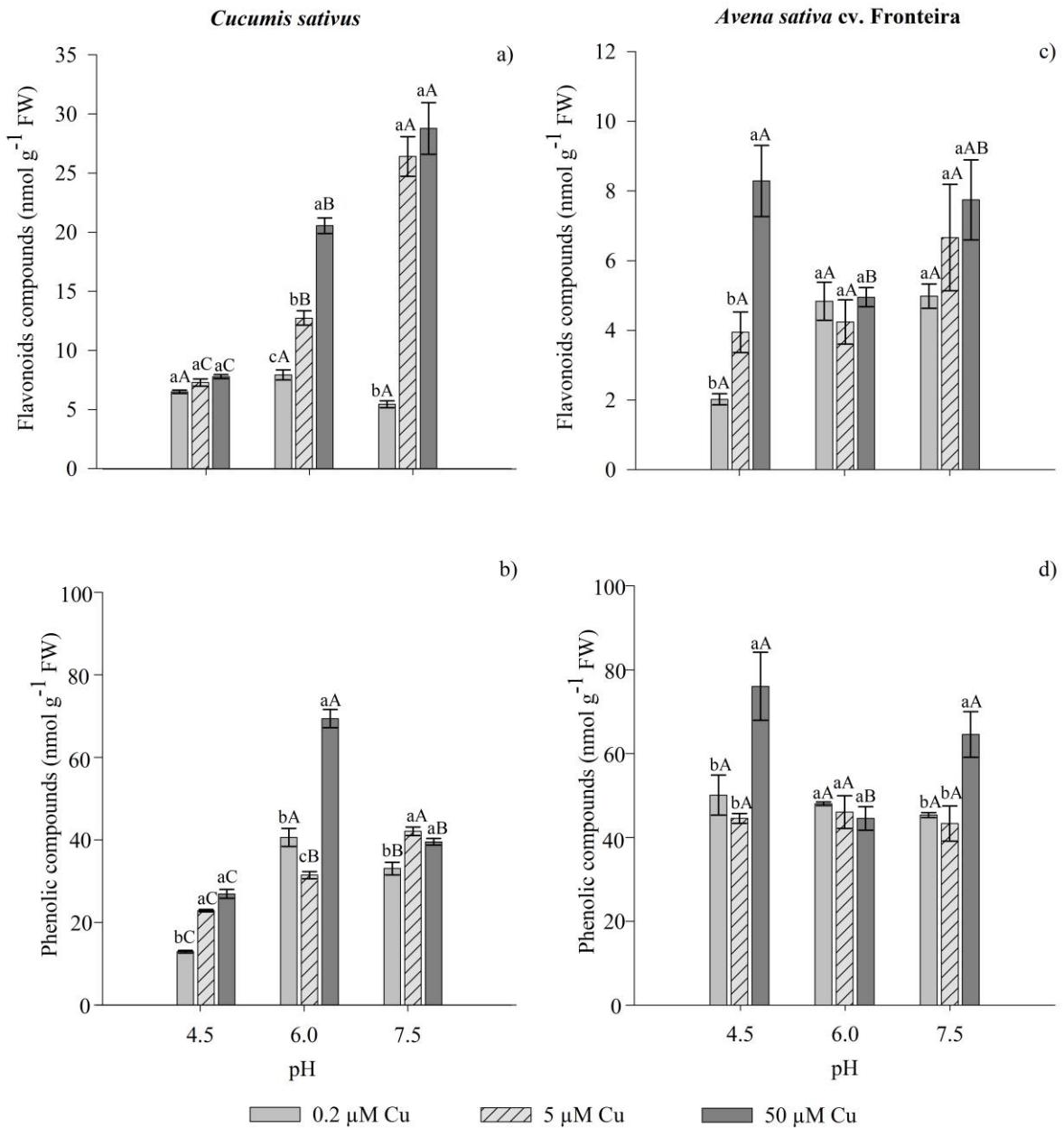


Figure 8. Flavonoids compounds and phenolic compounds in root exudates collected from *Cucumis sativus* (a, b) and *Avena sativa* cv. Fronteira (c, d) grown in nutrient solution with different pH values and Cu concentrations. Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$); Data are expressed as mean \pm SD, $n = 5$.

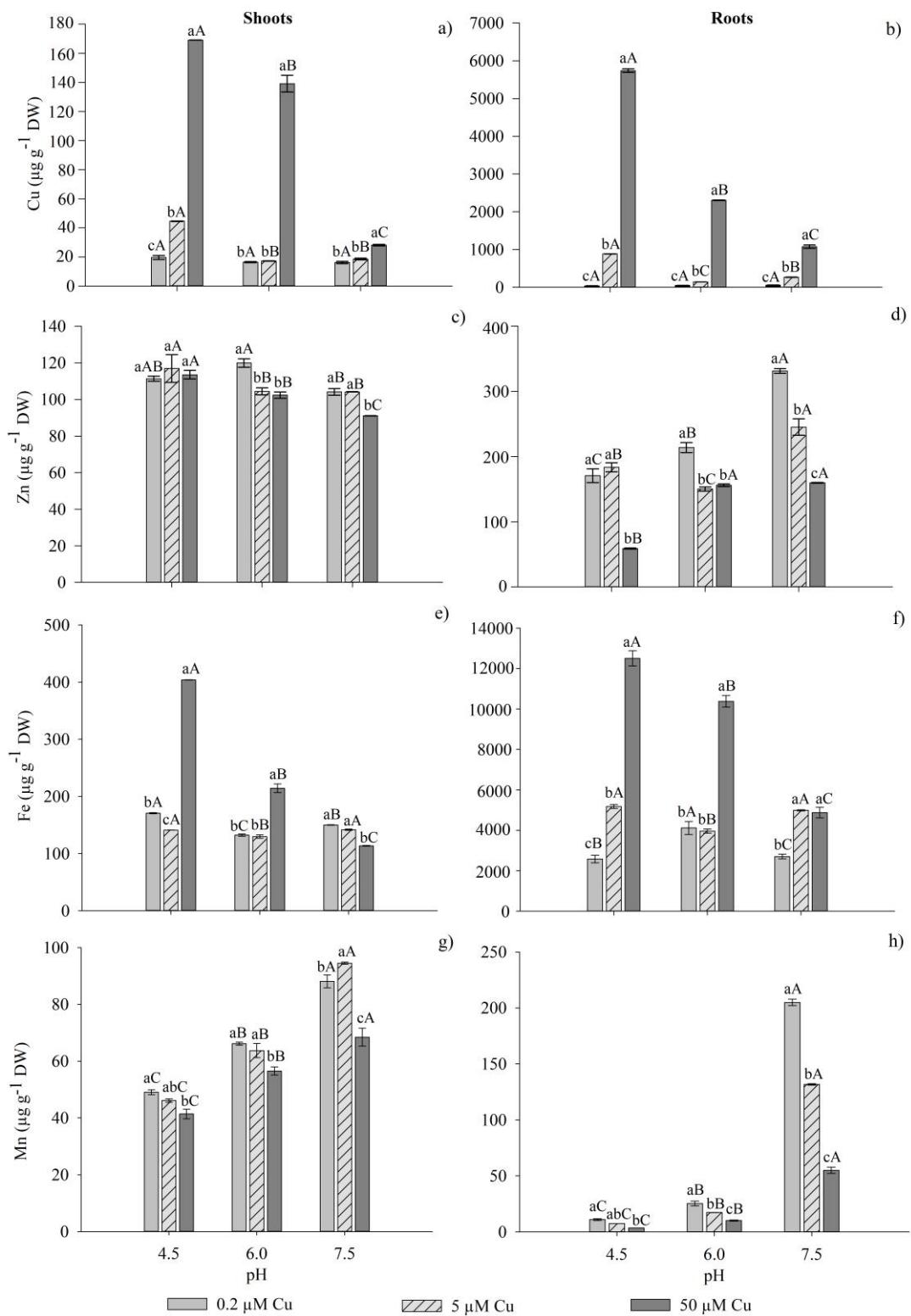


Figure 9. Micronutrient contents in shoots (a, c, e, g) and roots (b, d, f, h) of *Cucumis sativus* grown in nutrient solution with different pH values and Cu concentrations. Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$); Data are expressed as mean \pm SD, $n = 5$.

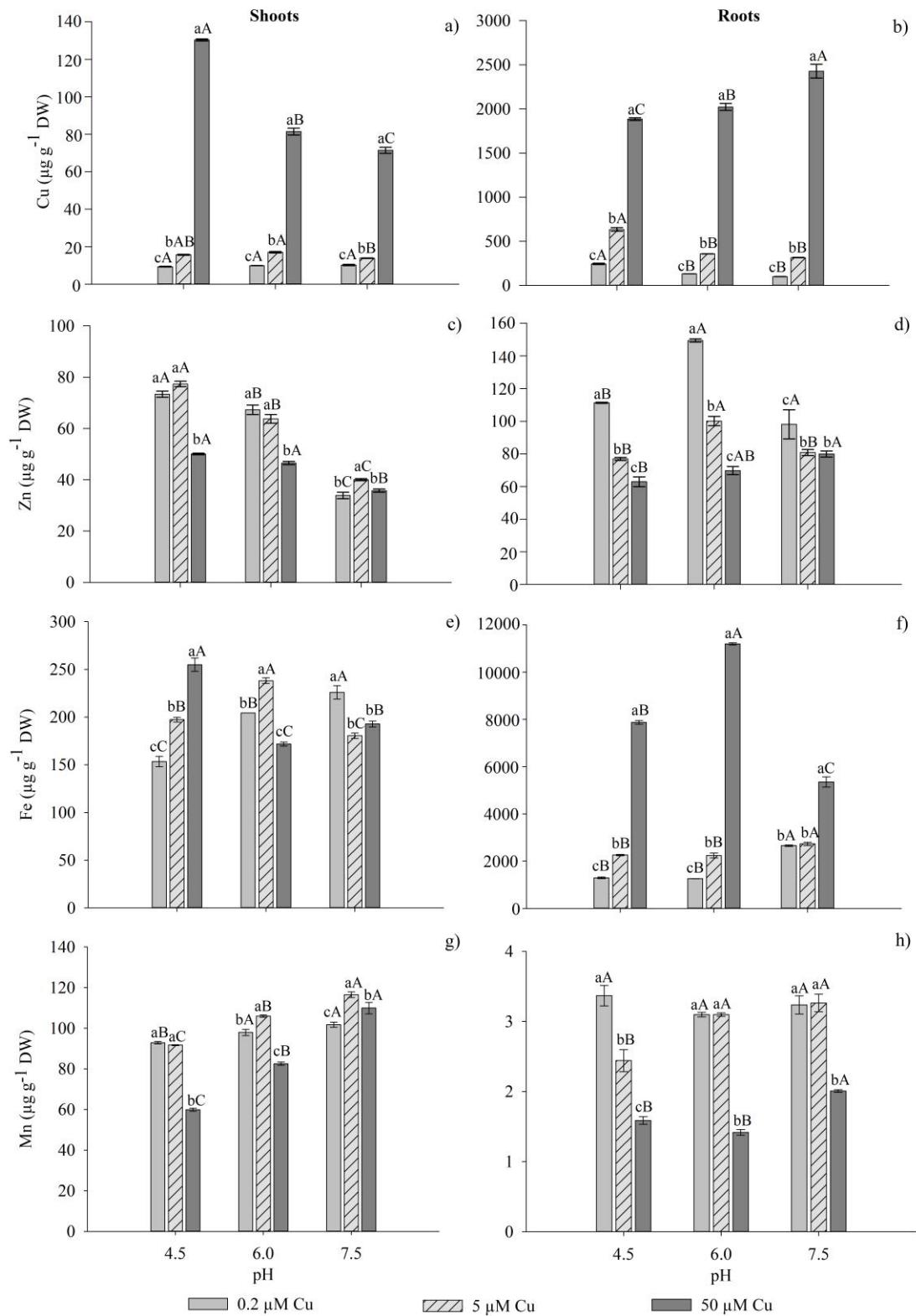


Figure 10. Micronutrient contents in shoots (a, c, e, g) and roots (b, d, f, h) of *Avena sativa* L. cv. Fronteira grown in nutrient solution with different pH values and Cu concentrations. Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$); Data are expressed as mean \pm SD, $n = 5$.

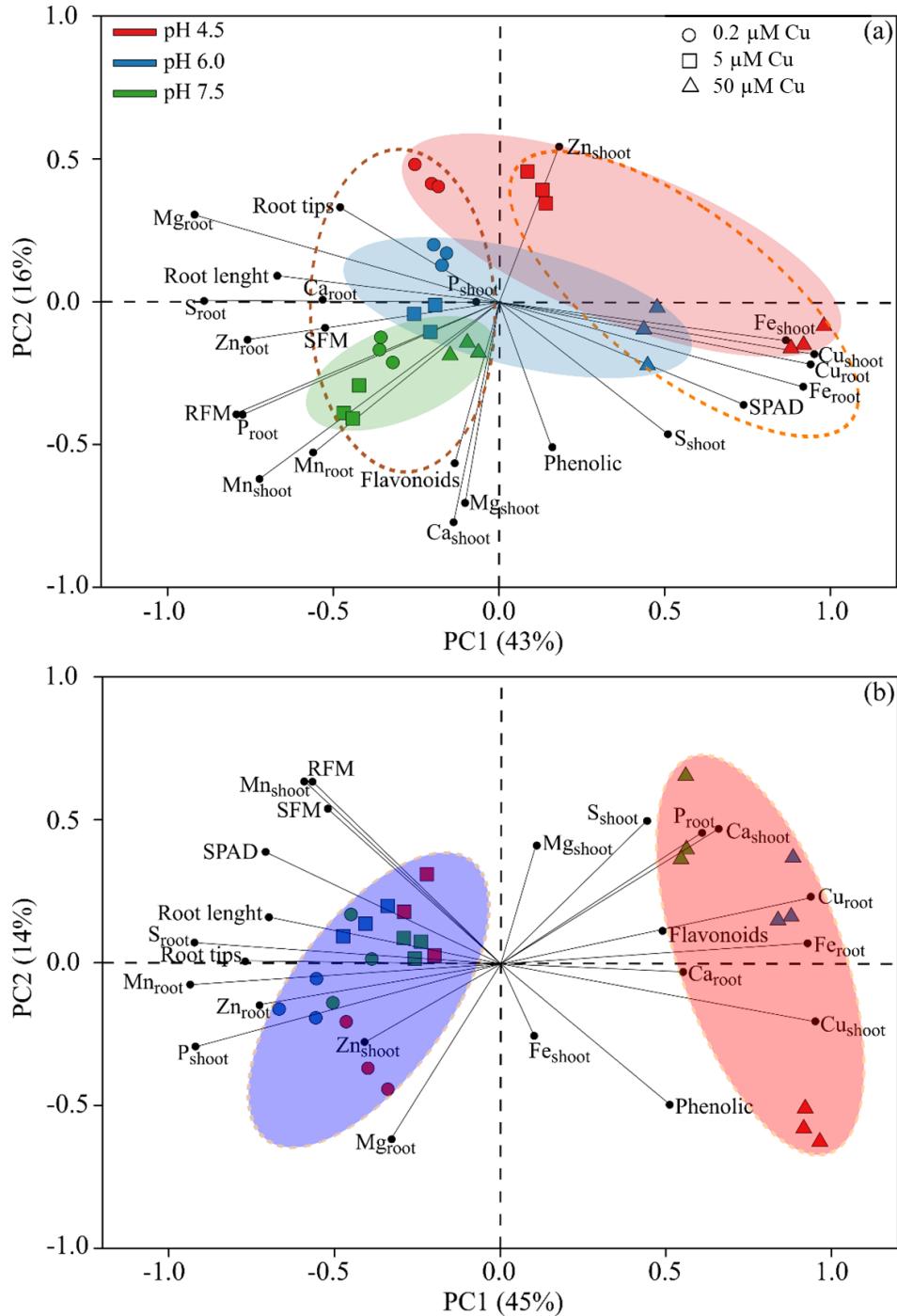


Figure 11. Principal component analysis (PCA) of the nutrients concentration (P, Ca, Mg, S, Cu, Fe, Zn and Mn) in shoots and roots tissue, root exudates (phenolic and flavonoids compounds) root morphological parameters (total root length and root tips) and plants growth parameters (shoot and root fresh matter, and SPAD index) in cucumber (a) and oat plants (b) grown in nutrient solution with different pH values [4.5 (red), 6.0 (blue) and 7.5 (green)] and Cu concentrations [0.2 (circle), 5 (square) and 50 μM Cu (triangle)].

Supplementary Material

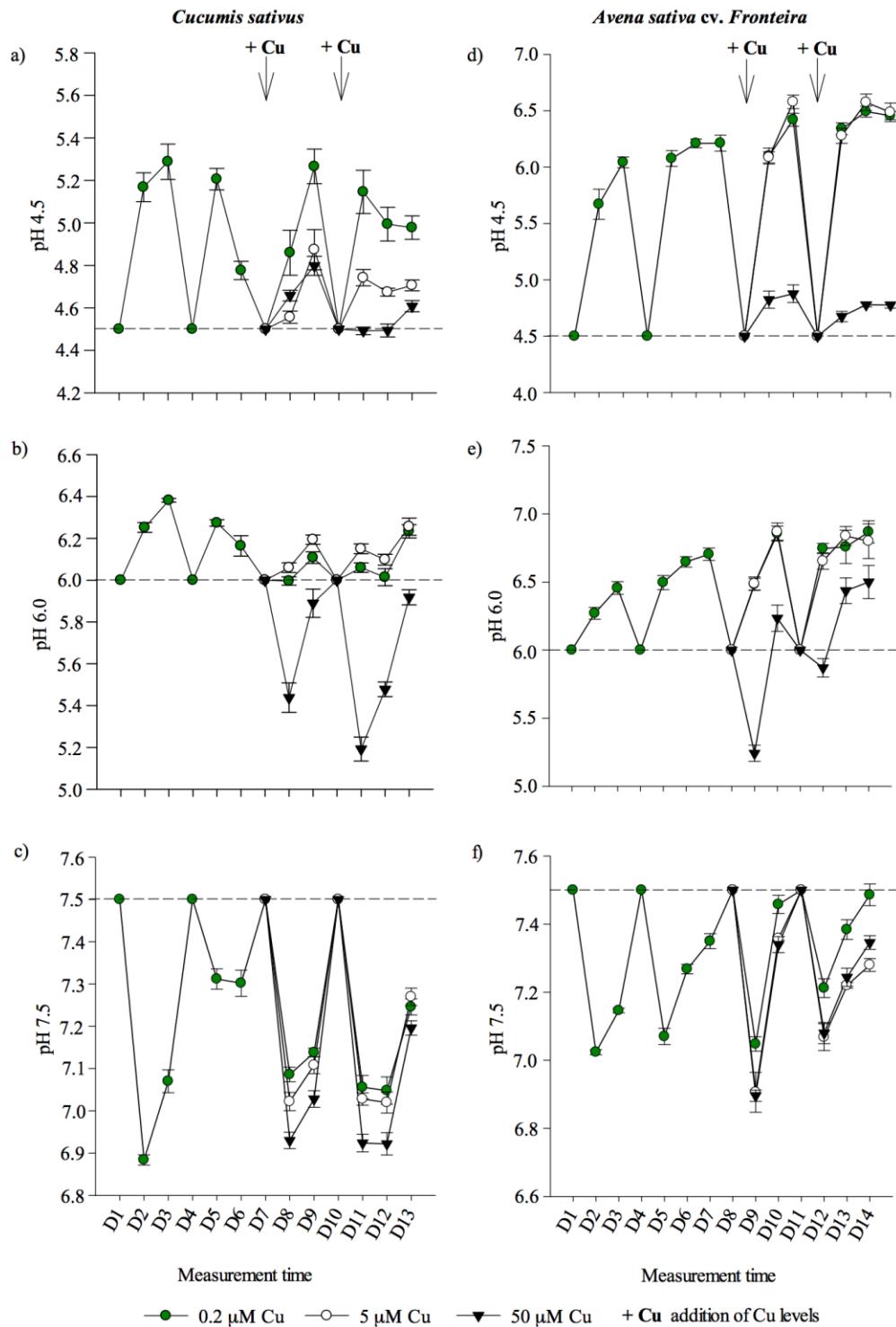


Figure 1. Variation in pH values in the nutrient solution measured every day during the experiment with *Cucumis sativus* (a, b, c), and *Avena sativa* cv. *Fronteira* (d, e, f), at different pH (4.5, 6.0 and 7.5) and Cu concentrations (0.2, 5 and 50 μM Cu).

Table 1. Variation in pH values in the nutrient solution measured every day during the experiment with *Cucumis sativus*, in different pH values (4.5, 6.0 and 7.5) and Cu concentrations (0.2, 5 and 50 µM Cu).

pH	Measurement time	Cu (µM)		
		0.2	5	50
4.5	T7	4.50 a ⁽¹⁾	4.50 a	4.50 a
	T8	4.86 a	4.55 b	4.65 b
	T9	5.26 a	4.87 b	4.79 b
	T10	4.50 a	4.50 a	4.50 a
	T11	5.14 a	4.74 b	4.49 c
	T12	4.99 a	4.67 b	4.49 c
	T13	4.97 a	4.70 b	4.60 b
6.0	T7	6.00 a	6.00 a	6.00 a
	T8	5.99 a	6.06 a	5.43 b
	T9	6.10 a	6.19 a	5.89 b
	T10	6.00 a	6.00 a	6.00 a
	T11	6.06 a	6.15 a	5.19 b
	T12	6.01 a	6.09 a	5.47 b
	T13	6.23 a	6.25 a	5.91 b
7.5	T7	7.50 a	7.50 a	7.50 a
	T8	7.08 a	7.02 b	6.93 c
	T9	7.13 a	7.10 a	7.02 b
	T10	7.50 a	7.50 a	7.50 a
	T11	7.05 a	7.02 a	6.92 b
	T12	7.04 a	7.02 a	6.92 b
	T13	7.24 ab	7.27 a	7.19 b

⁽¹⁾Different lowercase letters indicate difference between Cu doses within the same pH value and time measurement by the Tukey test ($p < 0.05$).

Table 2. Variation in pH in the nutrient solutions measured every day during the conduction of the experiment with *Avena sativa* cv. Fronteira, in different pH values (4.5, 6.0 and 7.5) and Cu concentrations (0.2, 5 and 50 µM Cu).

pH	Measurement time	Cu (µM)		
		0.2	5	50
pH 4.5	T8	4.50 a ⁽¹⁾	4.50 a	4.50 a
	T9	6.09 a	6.08 a	4.82 b
	T10	6.42 a	6.57 a	4.87 b
	T11	4.50 a	4.50 a	4.50 a
	T12	6.34 a	6.27 a	4.67 b
	T13	6.49 a	6.57 a	4.77 b
	T14	6.45 a	6.48 a	4.77 b
pH 6.0	T8	6.00 a	6.00 a	6.00 a
	T9	6.48 a	6.48 a	5.24 b
	T10	6.85 a	6.87 a	6.23 b
	T11	6.00 a	6.00 a	6.00 a
	T12	6.74 a	6.65 a	5.87 b
	T13	6.75 a	6.83 a	6.43 b
	T14	6.86 a	6.80 a	6.50 b
pH 7.5	T8	7.50 a	7.50 a	7.50 a
	T9	7.04 a	6.90 b	6.89 b
	T10	7.45 a	7.35 b	7.34 b
	T11	7.50 a	7.50 a	7.50 a
	T12	7.21 a	7.06 b	7.08 b
	T13	7.38 a	7.22 b	7.24 b
	T14	7.48 a	7.28 b	7.34 b

⁽¹⁾Different lowercase letters indicate difference between Cu doses within the same pH value and time measurement by the Tukey test ($p < 0.05$).

Table 3. Copper speciation in the nutrient solution in function of pH.

Cu (μM)	Cu species	pH		
		4.5	6.0	7.5
0.2	Cu^{2+}	100	98	70
	$\text{Cu}_2(\text{OH})_2^{2+}$	-	-	-
	CuOH^+	-	2	25
	$\text{Cu}(\text{OH})_2$	-	-	5
	$\text{Cu}_3(\text{OH})_4^{2+}$	-	-	-
5	Cu^{2+}	100	98	59
	$\text{Cu}_2(\text{OH})_2^{2+}$	-	-	15
	CuOH^+	-	2	21
	$\text{Cu}(\text{OH})_2$	-	-	3
	$\text{Cu}_3(\text{OH})_4^{2+}$	-	-	2
50	Cu^{2+}	100	98	30
	$\text{Cu}_2(\text{OH})_2^{2+}$	-	-	41
	CuOH^+	-	2	17
	$\text{Cu}(\text{OH})_2$	-	-	10
	$\text{Cu}_3(\text{OH})_4^{2+}$	-	-	2

Table 4. Macronutrient contents in shoots and roots of *Cucumis sativus* L. grown in nutrient solutions at different pH and Cu concentrations.

Element	pH	Cu concentrations		
		0.2 µM Cu	5 µM Cu	50 µM Cu
Shoots				
P (mg g ⁻¹)	4.5	13.23 aA ⁽¹⁾	13.48 aA	14.11 aA
	6.0	14.77 aA	13.06 abA	12.13 bA
	7.5	13.00 aA	14.29 aA	12.96 aA
Ca (mg g ⁻¹)	4.5	35.00 bC	34.15 bB	42.51 aA
	6.0	38.75 bB	45.40 aA	37.18 bB
	7.5	42.42 aA	43.20 aA	41.50 aA
Mg (mg g ⁻¹)	4.5	4.67 aA	5.02 aA	5.30 aA
	6.0	5.07 aA	5.32 aA	5.04 aA
	7.5	5.24 aA	5.65 aA	5.49 aA
S (mg g ⁻¹)	4.5	8.07 bB	6.89 bB	16.75 aA
	6.0	6.23 aB	5.83 aB	7.41 aC
	7.5	15.72 aA	16.80 aA	10.43 bB
Roots				
P (mg g ⁻¹)	4.5	7.74 aB	6.33 bB	6.09 bB
	6.0	9.89 aA	9.51 aA	7.65 bA
	7.5	10.47 aA	10.12 aA	7.91 bA
Ca (mg g ⁻¹)	4.5	27.55 aAB	24.91 aB	19.41 bB
	6.0	24.99 aB	20.72 bC	25.21 aA
	7.5	29.21 aA	31.76 aA	18.77 bB
Mg (mg g ⁻¹)	4.5	2.32 aA	1.73 bA	0.51 cC
	6.0	2.20 aA	1.79 bA	1.07 cB
	7.5	2.12 aA	1.97 abA	1.86 bA
S (mg g ⁻¹)	4.5	5.25 aA	3.50 bC	2.08 cB
	6.0	4.65 aA	4.47 aB	2.10 bB
	7.5	4.78 bA	6.25 aA	3.59 cA

⁽¹⁾Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$).

Table 5. Macronutrient contents in shoots and roots of *Avena sativa* cv. Fronteira grown in nutrient solutions at different pH and Cu concentrations.

Element	pH	Cu concentrations		
		0.2 µM Cu	5 µM Cu	50 µM Cu
Shoots				
P (mg g ⁻¹)	4.5	6.67 aAB ⁽¹⁾	5.93 bC	5.36 cA
	6.0	6.79 aA	6.72 aA	5.05 bB
	7.5	6.56 aB	6.32 bB	4.89 cB
Ca (mg g ⁻¹)	4.5	10.35 bA	12.18 aA	11.48 aB
	6.0	10.12 cA	11.41 bA	12.66 aA
	7.5	9.97 bA	10.22 bB	12.27 aAB
Mg (mg g ⁻¹)	4.5	2.16 bA	2.54 aA	2.37 abA
	6.0	2.30 aA	2.49 aA	2.33 aA
	7.5	2.32 aA	2.26 aA	2.42 aA
S (mg g ⁻¹)	4.5	3.40 bB	4.15 aA	4.04 aB
	6.0	3.62 aAB	3.80 aB	3.75 aB
	7.5	3.74 bA	3.90 bAB	4.40 aA
Roots				
P (mg g ⁻¹)	4.5	5.69 aA	5.41 aB	5.60 aC
	6.0	5.63 bA	5.63 bAB	7.71 aA
	7.5	5.69 bA	5.92 bA	6.53 aB
Ca (mg g ⁻¹)	4.5	11.44 aA	9.83 bB	12.59 aAB
	6.0	10.48 bA	10.10 bB	13.79 aA
	7.5	11.57 bA	13.96 aA	12.06 bB
Mg (mg g ⁻¹)	4.5	15.29 aA	16.07 aA	17.23 aA
	6.0	17.25 aA	16.56 aA	14.04 bB
	7.5	15.49 aA	15.44 aA	12.11 bB
S (mg g ⁻¹)	4.5	3.31 aA	3.26 aA	2.52 bB
	6.0	3.19 aA	3.39 aA	2.39 bB
	7.5	3.14 aA	3.19 aA	2.84 bA

⁽¹⁾Different lowercase letters indicate difference between Cu doses within the same pH value and different capital letters indicate difference between the pH values within the same Cu dose by the Tukey test ($p < 0.05$).

5.2 ESTUDO II

Growth, biochemical and physiological response of grapevine rootstocks to copper excess in nutritive solution²

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Abstract

Frequent applications of leaf cupric fungicides on grapevines increase copper (Cu) content in vineyard soils, and can lead to phytotoxicity, mainly in young and recently transplanted grapevines. Thus, it is necessary to identify rootstocks that are tolerant to Cu excess, as well as understanding the mechanisms against high Cu concentrations in different grapevine rootstocks. The aim of the present study was to assess the growth, biochemical and physiological response of grapevine rootstocks to Cu excess in nutritive solution. Two experiments were carried out: Experiment 1 used *Vitis rotundifolia* cv. Magnolia rootstocks and Experiment 2 used *Vitis vinifera* cv. Paulsen 1103 rootstocks. The rootstocks were acclimated for 10 days in complete Hoagland solution. Subsequently, different Cu concentrations were added to the nutritive solution in the CuSO₄.5H₂O form (in µM): 0.03; 20; 40 and 80. The high Cu concentrations had negative effect on growth of both grapevine rootstocks. The highest Cu concentrations were observed in roots in both rootstocks. Magnolia rootstock showed the greatest tolerance to Cu excess in nutritive solution, with less absorbed Cu as well as less Cu translocation to shoots. Hydrogen peroxide concentrations and lipid peroxidation increased in both shoots and roots of both rootstocks with increased Cu concentrations. The activity of enzyme superoxide dismutase increased in the shoot of both rootstocks due to high Cu concentrations in the solution. The size and rusticity of Magnolia plants may have influenced their response to the excess of this metal available in the solution in comparison to Paulsen plants.

²Artigo elaborado de acordo com as normas de formatação da revista Plant Physiology and Biochemistry

Keywords: Heavy metal, *Vitis rotundifolia* cv. Magnolia, *Vitis vinifera* cv. Paulsen 1103, Phytotoxicity.

1. Introduction

Copper (Cu) is an important micronutrient to physiological processes such as photosynthesis, respiration, carbon and nitrogen (N) metabolism, as well as to plant protection against oxidative stress (Yruela, 2005, 2009; Kabata-Pendias, 2011; Marschner, 2012). However, high Cu concentrations in the soil, such as vineyards subjected to long history of cupric fungicide applications to control leaf fungal diseases, can cause toxicity in grapevines, mainly in young plants recently transplanted to soil of old and eradicated vineyards (Brunetto et al., 2016; Tiecher et al., 2017).

Cu excess can cause anatomical, morphological and physiological changes in plants (Marschner, 2012; Adrees et al., 2015; Cambrollé et al., 2015; Guimarães et al., 2016; Ambrosini et al., 2018; Tiecher et al., 2018). One of the main effects is observed in root system, which shows changes in the cell wall and in cell and tissue arrangement in the root apex, which reduces plant water and nutrient absorption potential and impairs growth (Adrees et al., 2015; Cambrollé et al., 2015; Ambrosini et al., 2015; Guimarães et al., 2016; Ambrosini et al., 2018; Tiecher et al., 2018). Besides, the absorption of high Cu concentrations can cause damages due to oxidative stress given the unbalance between synthesis of antioxidant enzymes and the increased concentration of Reactive Oxygen Species (ROS) such as superoxide (O_2^-), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals (Briat and Lebrunb, 1999; Apel and Hirt, 2004; Girotto et al., 2013; Tiecher et al., 2016). The redox cycle of Cu^{2+} and Cu^+ catalyzes OH^- production after a non-enzymatic chemical reaction between O_2^- and H_2O_2 (Fenton and Haber – Weiss Reaction) (Briat and Lebrunb, 1999). ROS can damage the biomolecules in the cells, and lipid peroxidation in cell membranes is one of the main effects observed during oxidative stress, which results in membrane disruption and cell content leakage (De Vos et al., 1989; Yruela, 2005).

High Cu concentrations can change plant photosynthetic activity due to alterations in biosynthesis of the photosynthetic complex, which is caused by reduced photosynthetic pigment content, as well as by changes in chloroplast structure and in composition of the thylakoid membrane (Cambrollé et al., 2013; Cambrollé et al., 2015). Cu can replace chlorophyll central Mg and jeopardize the process to turn light into chemical energy in the NADPH and ATP form, as well as O_2 release (Yruela, 2005, 2009; Kabata-Pendias, 2011). Plants can trigger mechanisms to keep Cu homeostasis in the tissue and to avoid phytotoxicity

issues in response to Cu excess. Changes in nutritional status, in biochemical and physiological responses, regulation of cell efflux transporters, absorption, Cu compartmentalization in the vacuole, and changes in Cu translocation inside the plants are examples of the herein addressed issue (Yruela 2005; 2009; Pozo et al., 2010; Adrees et al., 2015; Leng et al., 2015). Based on observations of these tolerance mechanisms, it is possible to select genotypes of grapevine rootstocks with greater tolerance to excessive Cu availability. These rootstocks can be a cultivation strategy in soil contaminated with high Cu concentrations. The selection of grapevine rootstocks is important because it allows the proper use and development of a certain genetic material recommended for different environmental conditions. The aim of the present study was to assess the growth, biochemical and physiological response of grapevine rootstocks to Cu excess in nutritive solution.

2. Materials and methods

2.1. Experiment description

Two experiments were carried out in a growth chamber at Federal University of Santa Maria (UFSM). *Vitis rotundifolia* cv. Magnolia rootstock seedlings were used in Experiment 1 and *Vitis vinifera* cv. Paulsen 1103 rootstock seedlings were used in Experiment 2.

Magnolia and Paulsen grapevine rootstocks were produced by *in vitro* multiplication and kept in pots filled with inert substrate. They were stored in greenhouse until the moment of conducting the experiment. The produced seedlings of Magnolia and Paulsen grapevine rootstocks were planted in 3-L pots filled with the nutritive solution by Hoagland & Arnon (1950) at 50% strength, pH of 5.5 and with an aeration system. The nutritive solution presented the following concentrations (in mg L⁻¹): N = 85.31; P = 7.515; K = 104.75; Ca = 97.64; Mg = 23.68; S = 11.54; Fe = 2.68; Cu = 0.03; Zn = 0.13; Mn = 0.11; B = 0.27; Mo = 0.05; Ni = 0.01. Following 10 days cultivation in complete nutritive solution for plants acclimatization, nutritive solution was supplemented with different Cu concentrations, added as CuSO₄.5H₂O form (in µM): 0.2; 20; 40 and 80. One Magnolia plant and two Paulsen plants were inserted in each pot. Experiments 1 and 2 followed a completely randomized design, with three and four repetitions per treatment, respectively. Seedlings of grapevine rootstocks were grown under 16-h light photoperiod at 25°C, for 20 days in Experiment 1 and for 7 days in Experiment 2.

2.2. Height, dry matter production and nutrient concentrations in grapevine rootstock tissue

Plant shoot height was measured with measuring tape at the end of each experiment. Plants were harvested and separated into shoot and roots. Shoot and root samples were washed in distilled water and stored in forced air-circulation oven at 65°C until reaching constant weight. Dry matter was determined with precision balance. Tissue was ground in Wiley type mill and subjected to nitro-perchloric digestion (Embrapa, 2009). The concentrations of calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), zinc (Zn) and iron (Fe) extracts were measured in atomic absorption spectrophotometer (EAA; Varian SpectrAA-600, Australia). Potassium (K) concentration was determined in flame photometer (DM62, Digimed, Brazil). Phosphorus (P) concentration was determined through colorimetry, based on the methodology described by Murphy & Riley (1962) in spectrophotometer (SF325NM, Bel Engineering, Italy). Nitrogen (N) was extracted using sulfuric acid digestion (Tedesco et al., 1995) and its determination followed the Kjeldahl method carried out in steam drag distiller (TE-0364, Tecnal, Brazil) (Bremner and Mulvaney, 1982).

2.3. Chlorophyll-a fluorescence

The chlorophyll-a fluorescence analysis was carried out in pulse-modulated fluorometer JUNIOR-PAM (Walz, Germany). Chlorophyll-a fluorescence measurements were performed at the end of Experiments 1 and 2 on completely expanded leaves from plants' upper third. Fluorescence parameters were measured between 08:00 and 10:00 am (Ferreira et al., 2015). Leaves were left to acclimate to darkness in aluminum foil envelops for 30 minutes before the measurements in order to allow initial fluorescence determination (F_0). Subsequently, plants were subjected to saturating pulse of light ($10.000 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 0.6s for maximal fluorescence (F_m) determination. Maximum quantum yield of PSII (F_v / F_m), effective quantum efficiency of PSII (Y (II)) and non-photochemical quenching (NPQ) were calculated based on the parameters set for fluorescence (Maxwell e Johnson, 2000).

The maximal electron transport rate (ETRmax) was determined in the dark period before dawn (05:00- 06:00 am). ETR was assessed by light curve emission (photosynthetically active radiation, PAR) at nine intensity levels (0; 125; 190; 285; 420; 625; 820; 1,150 and $1,500 \mu\text{mol electrons m}^{-2} \text{s}^{-1}$) for 10 s in each sample. The following equation was used to adapt the measurements: $\text{ETR} = \text{ETRmax} [1 - e^{kQ}]$; wherein, k is an adequate constant and Q is light intensity (PAR) (Rascher et al., 2000).

2.4. Biochemical analyses

Shoot and root from each plant were separated at plant harvesting and stored in liquid N₂, soon after. They were then stored at -80°C in ultrafreezer. Leaf lipid peroxidation was calculated by determination of malondialdehyde (MDA) concentration, as lipid peroxidation product from the reaction with thiobarbituric acid (TBA) was assessed based on the method by Moshaty et al. (1993). Hydrogen peroxide concentration (H₂O₂) in the shoot and roots was carried out based on Loreto and Velikova (2001). The activity of enzyme superoxide dismutase (SOD) was assessed based on the spectrophotometric method described by Giannopolitis and Ries (1977). Each SOD unit was defined as the amount of enzyme inhibiting nitro-blue tetrazolium (NBT) in one 50% photo-reduction (Beauchamp and Fridovich, 1971).

2.5. Statistical analysis

The recorded results were subjected to variance analysis in Sisvar software, version 4.0 (Ferreira, 2008). The means of the assessed parameters were compared to each other through Tukey test at 5% error probability, whenever they were significant.

3. Results

3.1. Height and yield of shoot and root dry matter

Plants from *Vitis rotundifolia* cv. Magnolia grapevine rootstock (hereafter referred as “Magnolia”) in Experiment 1 showed decreased height and shoot dry matter yield under Cu excess compared to control plants (Figure 1a and 1b). However, roots of Magnolia rootstocks cultivated in solution presenting concentrations of 40 and 80 µM Cu recorded the best yield (Figure 1c). Experiment 2 did not show differences in the height of *Vitis vinifera* cv. Paulsen 1103 grapevines (hereafter referred to as “Paulsen”) under Cu excess compared to control plants (Figure 2a). Shoot dry matter was decreased, mainly at concentrations 20 and 40 µM Cu (Figure 2b). Root dry matter in Paulsen plants also decreased due to Cu concentration increase, and the lowest values of it were observed at concentrations of 40 and 80 µM Cu (Figure 2c).

3.2. Nutrient concentrations in the tissue

The highest Cu concentrations were observed in the shoot of grapevines grown in the 80 µM Cu treatment (Table 1). The highest N, K, Ca, Mn and Fe concentrations were recorded for the shoot of grapevine rootstocks grown in control solution in comparison to the

treatment based on Cu addition (Table 1). The highest Cu concentrations in the root system were observed in treatments added with 40 and 80 µM Cu. The highest Fe, Zn and Mn concentrations were shown by the roots of grapevines grown in control solution in comparison to plants cultivated under solution added with Cu (Table 1).

The highest Cu concentration in Experiment 2 was observed in the shoots and roots of grapevines grown in solution added with 80 µM Cu (Table 2). N and P recorded the highest concentration in the shoot of grapevines cultivated in control solution added with 40 µM Cu (Table 2). The highest Cu and Zn concentrations were observed in the shoot of grapevines grown in control solution (Table 2). The highest P, K, Ca, Mg, Zn and Mn concentrations were recorded for plants grown in control solution. The highest Fe concentration was observed in the roots of grapevines cultivated in control solution added with Cu (Table 2).

3.3. Chlorophyll-a fluorescence

Maximum quantum yield of PSII (F_v/F_m) and effective quantum efficiency of PSII ($Y(II)$) in Experiment 1 showed no difference between treatments (Figure 3b, c). Initial fluorescence values (F_0) decreased in treatments added with Cu in comparison to grapevines grown in control solution (Figure 3a). The highest non-photochemical quenching (NPQ) values were observed in grapevines under 40 and 80 µM Cu treatments (Figure 3d).

The highest $Y(II)$ values in Experiment 2 were recorded for grapevines grown in control solution and solution added with 20 µM Cu (Figure 4c). The highest F_0 values were observed in seedlings of grapevine rootstock grown under 40 and 80 µM Cu treatments (Figure 4a). NPQ was higher in grapevines treated with 40 µM Cu (Figure 4d).

Electron transport rate (ETR) decreased due to addition of high Cu concentrations (40 and 80 µM Cu) in comparison to the control treatment and with 20 µM Cu treated plants in both experiments (Figure 5a, b). The ETR rate decreased in the treatment with 80 µM Cu - such decrease was more significant in the Paulsen rootstock than in Magnolia (Figure 5a, b).

3.4. Biochemical analysis

Leaf lipid peroxidation in Experiment 1 accessed by malondialdehyde (MDA) concentration was higher in grapevines cultivated in solution with 20 µM Cu (Figure 6a). The concentration of hydrogen peroxide (H_2O_2) was higher in the shoot of rootstocks from grapevines grown in solutions added with Cu (Figure 6c). The highest values recorded for the activity of enzyme superoxide dismutase (SOD) in the shoot were observed in grapevines grown in solution with 80 µM Cu (Figure 6c). The highest lipid peroxidation (MDA) and

H_2O_2 values were observed in grapevine roots grown in solution with 80 μM Cu (Figure 6d, e). The highest SOD activity was observed in the roots of grapevines cultivated in solution with 40 μM Cu (Figure 6f).

Leaf lipid peroxidation (MDA) in Experiment 2 was higher in grapevines cultivated in control solutions and in the ones with 80 μM Cu (Figure 7a). The concentration of H_2O_2 was higher in the leaves of grapevine rootstocks cultivated in solutions with 20 and 80 μM Cu (Figure 7b). The highest SOD activity was observed in the shoot of grapevines grown in solution with 80 μM Cu (Figure 7c). The highest lipid peroxidation (MDA) and H_2O_2 values were observed in the roots of grapevines grown in solution with 80 μM Cu (Figure 7d, e). The lowest SOD activity was observed in roots of grapevines cultivated in solution with 40 μM Cu (Figure 7f).

4. Discussion

The decreased Magnolia and Paulsen rootstocks growth due to Cu addition, mainly in the treatment added with 80 μM Cu in comparison to treatment added with 0.12 μM , resulted from the phytotoxic effect of this metal. High Cu concentrations in the environment can change root morphology and plant biochemical and physiological functions (Lequeux et al., 2010; Cambrollé et al., 2015). Copper (Cu) excess in the root system can lead to root apex darkening and thickening, root growth reduction, root length reduction, mean root diameter reduction and abnormal ramification (Ambrosini et al., 2015; Guimarães et al., 2016), a fact that can decrease water and nutrient absorption (Toselli et al., 2009). Overall, nutrient concentration and accumulation in the present study, mainly in the shoot, was higher in Magnolia and Paulsen rootstocks grown in control solution than in plants cultivated under Cu excess. The excess of copper (Cu) availability can influence the homeostasis of ions, in different ways, in different plants, due to changes in ion absorption and translocation, such as Mn, Fe, Zn, Ca and P (Kopittke and Menzies, 2006; Kopittke et al., 2011; Marastoni et al., 2018).

However, the highest root dry matter yield in Magnolia plants were observed in the ones grown under high Cu concentrations. This outcome can be explained by the greater tolerance shown by these plants to the Cu concentrations used in the current study. Magnolia plants presented greater rusticity, bigger size and significant root system than Paulsen plants. There was lower Cu absorption and translocation by Magnolia plants and, consequently, their root system may have been stimulated to grow as a strategy to trigger their defense system. This outcome goes against observations on other experiments that have used smaller plants,

which showed reduced amount of root dry matter due to increased Cu concentration (Juang et al., 2012; Chen et al., 2013; Ambrosini et al., 2015).

The highest Cu concentrations observed in the shoot and root system of grapevines grown in solutions added with 40 and 80 μM Cu are related to higher Cu availability. On the other hand, Cu amount accumulated in the shoot of Magnolia rootstocks decreased in comparison to the control. Cu accumulation in the shoot of Paulsen rootstocks was higher in plants cultivated in solution added with 80 μM Cu. Copper (Cu) absorption by the root system of Magnolia rootstocks was reduced in order to trigger the defense system in plants subjected to higher Cu concentrations (80 μM Cu) in comparison to plants grown in solution with 40 μM Cu, which showed similar Cu concentrations in the root system. Therefore, Magnolia plants may keep the Cu accumulated in the root system in order to avoid its translocation to the shoot and, consequently, to avoid the phytotoxic effect. The lowest Cu absorption and accumulation in the root system could be a defense mechanism driven by plants subjected to high Cu availability (Juang et al., 2012; Ambrosini et al., 2015; Cambrollé et al., 2013). Cu can remain in the apoplast, likely linked to cell wall, or compartmentalized in the vacuole of root cells, a fact that diminishes Cu transportation to the root and, consequently, reduces the phytotoxic effect in plants (Cambrollé et al., 2013; Ambrosini et al., 2015; Tiecher et al., 2017; Ricachenevsky et al., 2018).

The exudation of organic acids by the root system in Magnolia plants may have acted as a defense mechanism against the excess of Cu available in the solution. When such process is triggered, part of Cu in the solution can be complexed by organic acids, such as phenolic compounds, carboxylic acids and phytosiderophores. This process decreased the percentage of Cu^{2+} (free) species, which can be absorbed and cause toxicity in plants (Brunetto et al., 2016; De Conti et al., 2018; Marastoni et al., 2018). Hydroxyl and carboxyl groups of phenolic compounds can set stronger bonds to Cu and Fe than many low molecular weight organic acids (Jung et al., 2003; Martell and Smith, 1989). Besides, OH^- efflux through the root system can make the rhizosphere pH more alkaline and diminish Cu bioavailability for plant absorption (Bravin et al., 2009a; 2009b; Bravin et al., 2012). Plant genotype and size may also have influenced the behavior and response to the excess of this metal in the solution. Although Paulsen plants presented reduced Cu translocation factor in the roots in comparison to the shoot - due to Cu concentration increase in the solution - absorbed Cu depending on its concentration in the solution and transportation to the shoot, a fact that could potentiate phytotoxicity (Miotto et al., 2014). This finding justifies the phytotoxic effect observed in

Paulsen rootstocks cultivated in high Cu concentration solution, right after treatment application (3 or 4 days later).

The highest F0 rates in Paulsen grapevines and the lowest Y(II) values in treatments added with 40 and 80 µM Cu pointed towards lower rates of energy being assimilated through the photochemical process, and well as to higher radiation emission in the fluorescence or heat form (Maxwell and Johnson, 2000). The highest NPQ rates, mainly in the rootstock of Magnolia grapevines grown in solutions added with 40 and 80 µM Cu, have shown damage in the photosynthetic apparatus, since the absorbed radiation – which was not used in the photosynthesis process – was emitted in the form of heat and protected the leaves from damages induced by light, such as in ROS formation (Maxwell and Johnson, 2000; Cambrollé et al., 2011). The ETR was lower in leaves of Magnolia and Paulsen rootstocks grown in solutions with 40 and 80 µM Cu, likely due to changes caused by high Cu concentrations in the photosynthetic apparatus of these plants (Maxwell and Johnson, 2000; Cambrollé et al., 2012; Tiecher et al., 2016; 2017).

High Cu concentrations have favored ROS formation in the cells. ROS react with the biomolecules in the cells and cause lipid peroxidation in cell membranes, which leads to lower membrane selectivity and to disruption and leak of cell content (De Vos et al., 1989; Yruela, 2005). Plants activate defense systems, such as the synthesis of antioxidant enzymes, in response to increased ROS formation (Gill and Tuteja, 2010). The highest H₂O₂ and MDA concentrations either in the shoot or in the root system in the rootstock of Paulsen grapevines grown in solution with 80 µM Cu indicate oxidative stress due to Cu excess in the tissue. Although plants also presented higher SOD activity in the shoot, our data suggest that it was not possible to avoid damages caused by Cu excess. SOD acts as the first defense line against ROS, it catalyzes O₂⁻ decrease and triggers H₂O₂ and O₂ formation, as well as diminishes the possibility of OH⁻ formation from O₂⁻ (Mittler, 2002). The H₂O₂ formed from the SOD activity can be further eliminated by peroxidase enzymes (PODs), catalase (CAT) and ascorbate peroxidase (APX) (Hegedüs et al., 2001). The lipid peroxidation estimated based on MDA concentration is an important tool to evaluate cell damage caused by Cu excess (Yang et al. 2011). Although the highest H₂O₂ and SOD were observed in the shoot of Magnolia rootstocks, MDA values were high in treatments based on high Cu concentrations – a fact that can represent a balance trend between ROS formation and cell antioxidant defense system. On the other hand, the root system of Magnolia grapevines rootstocks showed the highest H₂O₂ and MDA concentrations in comparison to the SOD activity; however, it was similar to the

proportion in the shoot. Besides, the root system is a less sensitive organ to damage caused by oxidative stress in comparison to the shoot (Yang et al. 2011; Ambrosini et al., 2015).

5. Conclusion

High Cu concentrations in the solution resulted in lower dry matter accumulation in the rootstocks of Magnolia and Paulsen grapevines. There was higher Cu accumulation in plants' root system, and it acted as defense strategy against the excess of Cu available in the solution. Magnolia rootstocks recorded lower Cu absorption and translocation to the shoot. The higher size, root system structure and Magnolia plant rusticity have influenced the response to the excess of material available in the solution in comparison to Paulsen plants, which were smaller. The use of rootstocks that are tolerant to the excess of Cu available can be an alternative to manage vineyard sites contaminated with excess of heavy metals.

6. Contributions

Edicarla Trentin: study design, accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Letícia Morsch, Daniela Basso Facco and Rodrigo Knevitz Hammerschmitt: accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Camila Peligrinotti Tarouco and Fernando T. Nicoloso: study design, biochemical analysis in plants, photosynthetic analysis, accomplishment of the experiment and writing of the article. Maristela Machado Araujo and Álvaro Luís Pasquetti Berghetti: chlorophyll a fluorescence analysis, accomplishment of the experiment and writing of the article. Paulo Ademar Avelar Ferreira, Felipe Klein Ricachenevsky and Gustavo Brunetto: study design, interpretation of results and writing of the article. George Wellington Bastos de Melo: interpretation of results and writing of the article.

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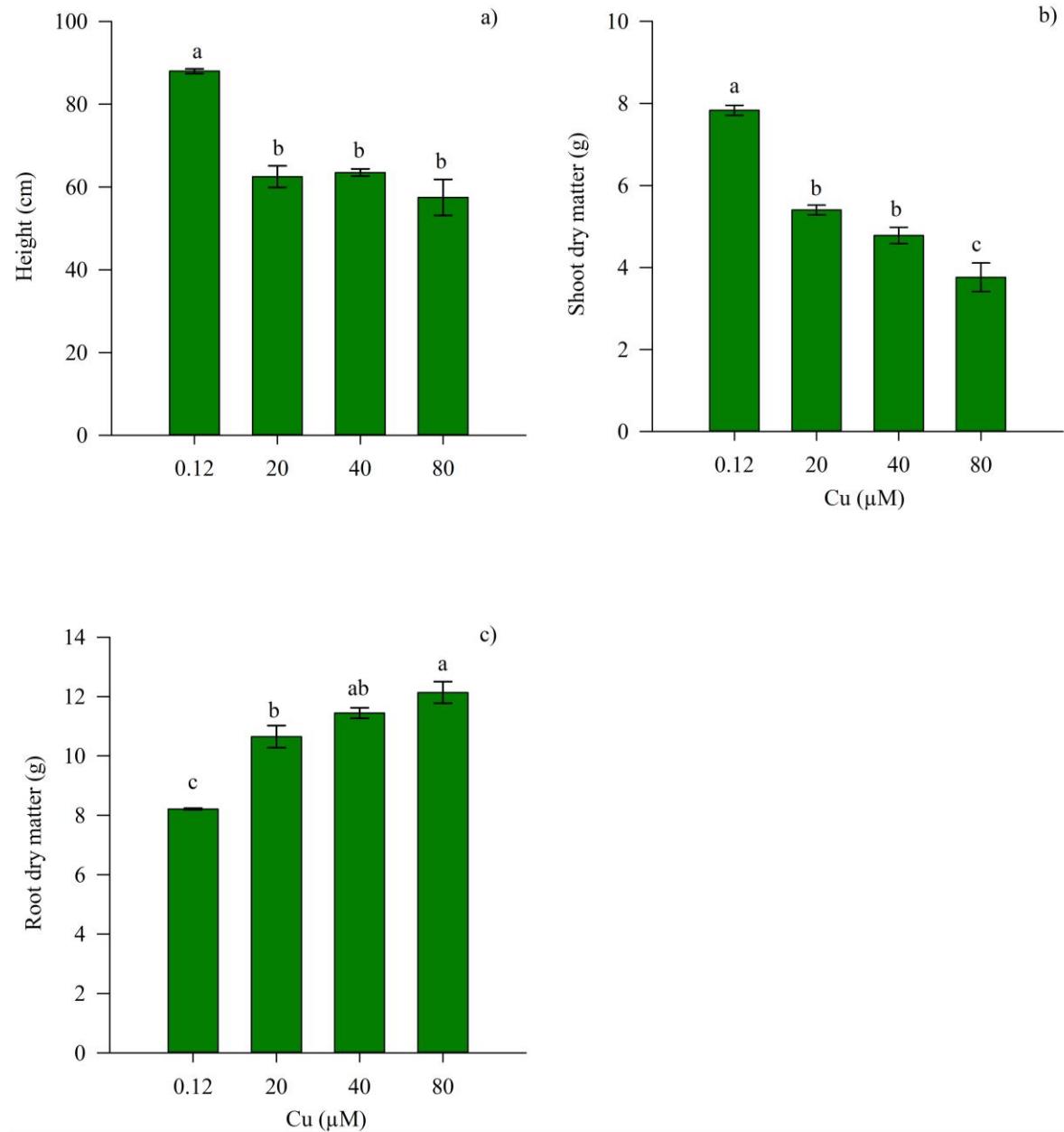


Figure 1. Height (a), shoot (b) and root (c) dry matter yield of grapevines (*Magnolia* rootstock) grown in nutritive solution under high Cu content. Histograms presenting the same lowercase letter were not statistically different in the Tukey test at 5%.

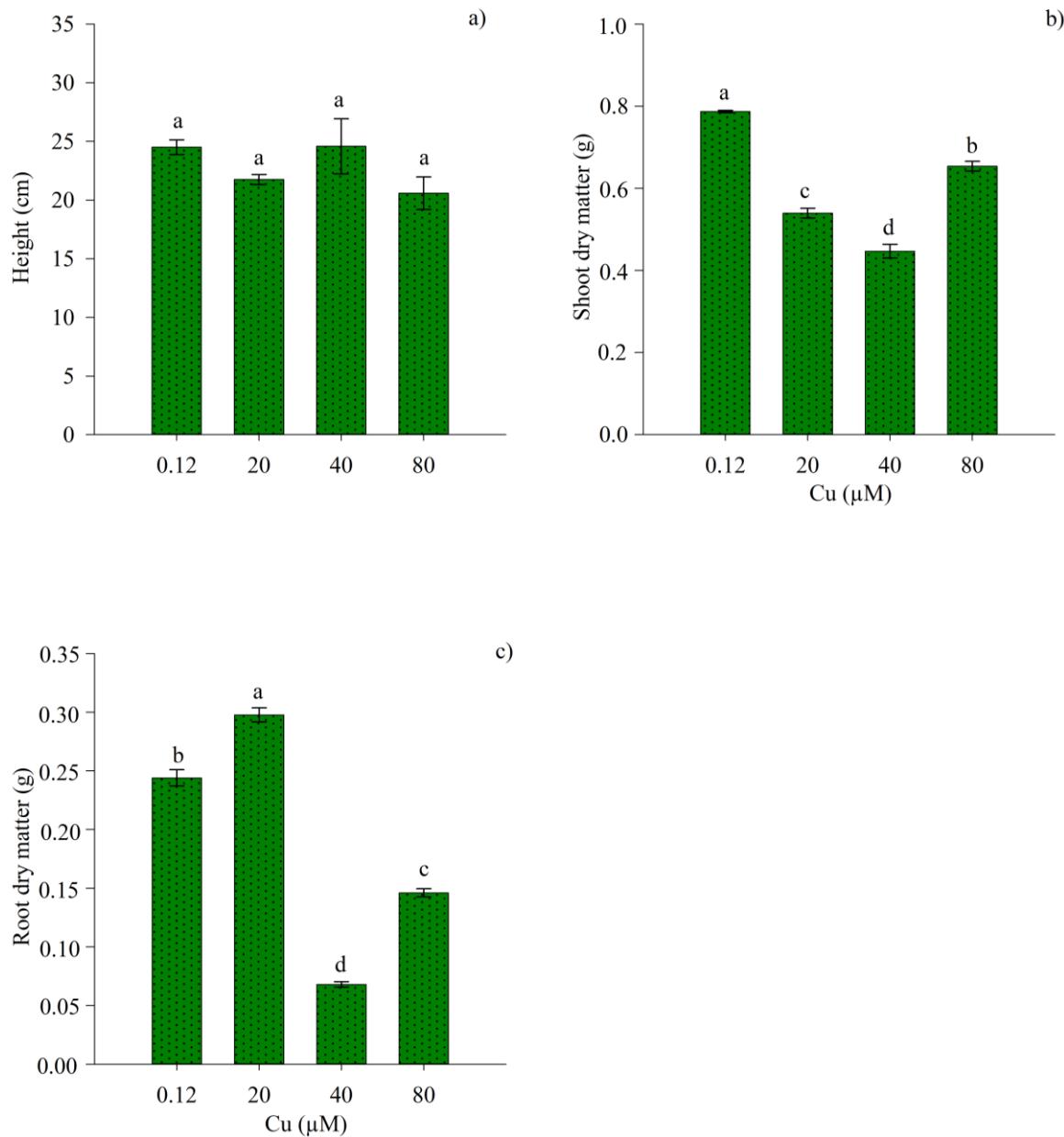


Figure 2. Height (a), shoot (b) and root (c) dry matter yield of grapevines (Paulsen rootstock) grown in nutritive solution under high Cu content. Histograms presenting the same lowercase letter were not statistically different in the Tukey test at 5%.

Table 1. Nutrient concentration in shoots and roots of grapevines (Magnolia rootstock) grown in nutritive solution under high Cu content.

Nutrients	Shoots				
	Control	20 µM Cu	40 µM Cu	80 µM Cu	CV%
N (g kg ⁻¹)	15.45 a ⁽¹⁾	13.26 bc	12.48 c	12.28 c	6.73
P (g kg ⁻¹)	2.43 a	2.11 a	1.74 a	1.78 a	14.80
K (g kg ⁻¹)	13.44 a	10.92 b	10.01 bc	9.55 c	3.32
Ca (g kg ⁻¹)	19.79 a	12.83 b	12.05 b	11.87 b	7.11
Mg (g kg ⁻¹)	4.90 a	5.29 a	5.35 a	4.38 a	7.63
Cu (mg kg ⁻¹)	22.61 b	23.63 b	23.58 b	30.91 a	10.00
Zn (mg kg ⁻¹)	42.05 a	44.45 a	32.19 b	26.09 b	9.48
Fe (mg kg ⁻¹)	101.43 a	67.89 b	57.84 b	76.56 b	12.34
Mn (mg kg ⁻¹)	199.95 a	99.15 b	71.85 c	92.40 b	4.50
Roots					
N (g kg ⁻¹)	6.57 b	7.06 ab	7.39 a	6.63 ab	4.25
P (g kg ⁻¹)	1.26 a	1.22 a	1.35 a	1.42 a	7.18
K (g kg ⁻¹)	5.32 a	5.18 a	4.41 a	4.34 a	9.76
Ca (g kg ⁻¹)	16.27 a	15.15 ab	15.71 a	12.76 b	6.91
Mg (g kg ⁻¹)	6.69 a	6.80 a	5.42 a	5.17 a	13.55
Cu (mg kg ⁻¹)	66.08 d	1914.68 b	4227.77 a	4435.08 a	3.22
Zn (mg kg ⁻¹)	256.64 a	142.44 b	85.09 c	66.22 d	5.24
Fe (mg kg ⁻¹)	18235.34 a	6180.29 b	4489.79 c	4387.94 c	4.78
Mn (mg kg ⁻¹)	428.72 a	151.00 bc	115.55 c	169.52 b	8.62

⁽¹⁾ Means followed by the same lowercase letter in rows (treatments) do not differ from each other by the Tukey test at 5% (P<0.05).

Table 2. Nutrient concentration in shoots and roots of grapevines (Paulsen rootstock) grown in nutritive solution under high Cu content.

Nutrients	Shoots				
	Controle	20 µM Cu	40 µM Cu	80 µM Cu	CV%
N (g kg ⁻¹)	22.13 a ⁽¹⁾	18.15 b	20.02 ab	17.55 b	5.63
P (g kg ⁻¹)	9.66 ab	9.09 b	10.85 a	6.52 c	5.80
K (g kg ⁻¹)	22.83 a	17.35 c	18.55 bc	21.26 ab	5.22
Ca (g kg ⁻¹)	24.35 a	18.26 bc	19.60 b	17.19 c	4.44
Mg (g kg ⁻¹)	12.63 b	14.15 b	16.05 a	13.71 b	4.79
Cu (mg kg ⁻¹)	14.23 c	15.61 c	24.61 b	32.61 a	13.30
Zn (mg kg ⁻¹)	98.62 a	54.62 c	68.58 b	54.75 c	7.58
Fe (mg kg ⁻¹)	250.04 a	236.71 a	236.33 a	231.63 a	3.77
Mn (mg kg ⁻¹)	755.12 b	930.12 a	447.87 d	582.62 c	5.44
Roots					
N (g kg ⁻¹)	26.84 a	17.62 b	25.68 a	22.98 a	8.04
P (g kg ⁻¹)	5.92 a	3.20 b	2.68 b	3.56 b	10.03
K (g kg ⁻¹)	31.23 a	13.38 b	11.02 b	12.60 b	9.29
Ca (g kg ⁻¹)	18.69 a	12.67 b	12.36 b	12.02 b	8.14
Mg (g kg ⁻¹)	10.09 a	4.02 c	6.62 b	2.94 c	14.71
Cu (mg kg ⁻¹)	34.73 d	1398.98 c	2630.23 b	5221.11 a	4.17
Zn (mg kg ⁻¹)	326.75 a	108.74 b	109.24 b	112.61 b	5.67
Fe (mg kg ⁻¹)	5544.72 a	4316.94 b	4230.35 b	5897.19 a	4.37
Mn (mg kg ⁻¹)	246.25 a	127.19 b	126.44 b	86.82 c	8.01

⁽¹⁾ Means followed by the same lowercase letter in rows (treatments) do not differ from each other by the Tukey test at 5% (P<0.05).

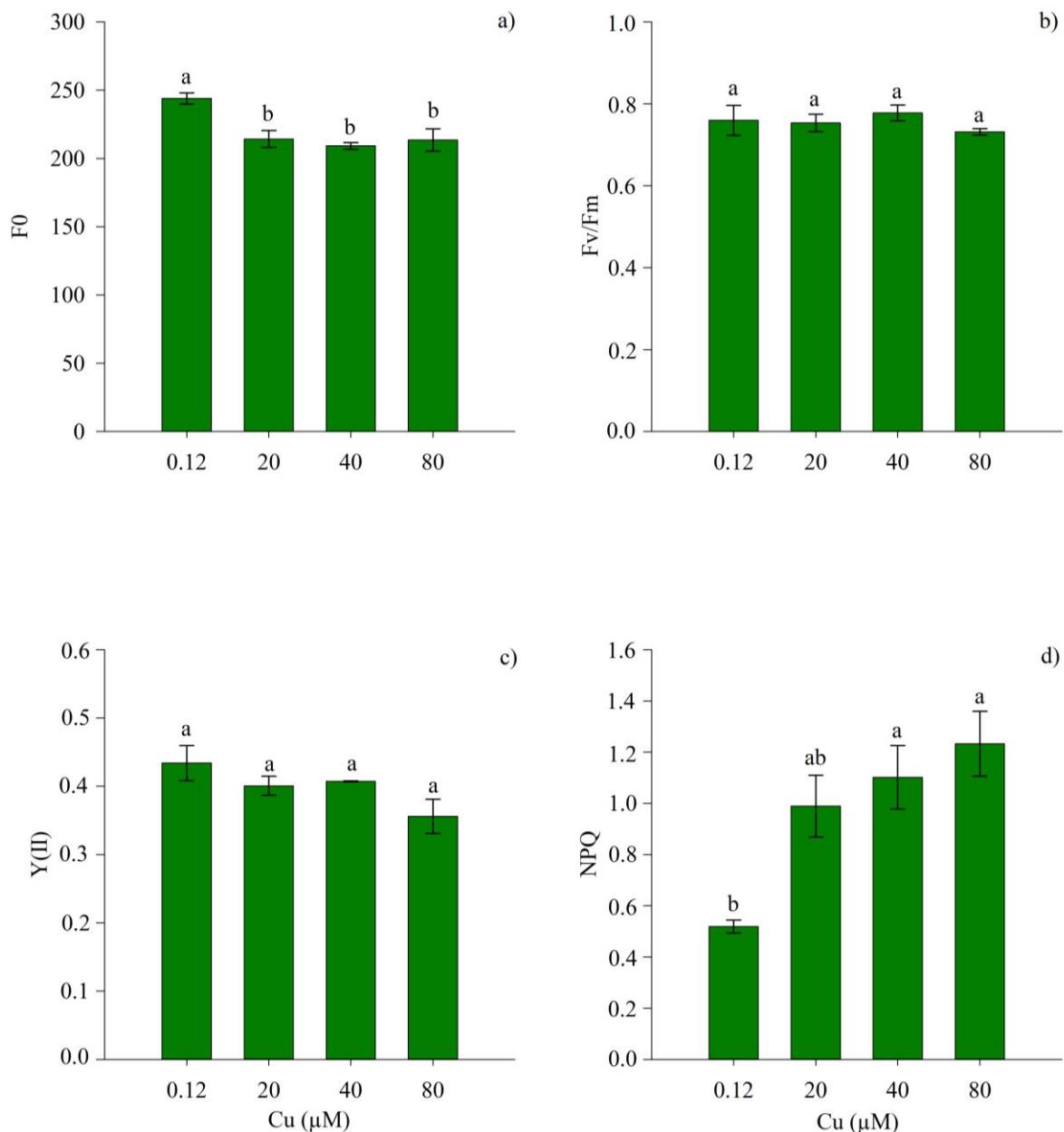


Figure 3. Initial fluorescence (F0) (a), maximum PSII quantum yield (Fv/Fm) (b), effective quantum efficiency of PSII (Y(II)) (c) and non-photochemical quenching (NPQ) (d) in the leaves of grapevines (Magnolia rootstock) grown in nutritive solution under high Cu content. Histograms presenting the same lowercase letter were not statistically different in the Tukey test at 5%.

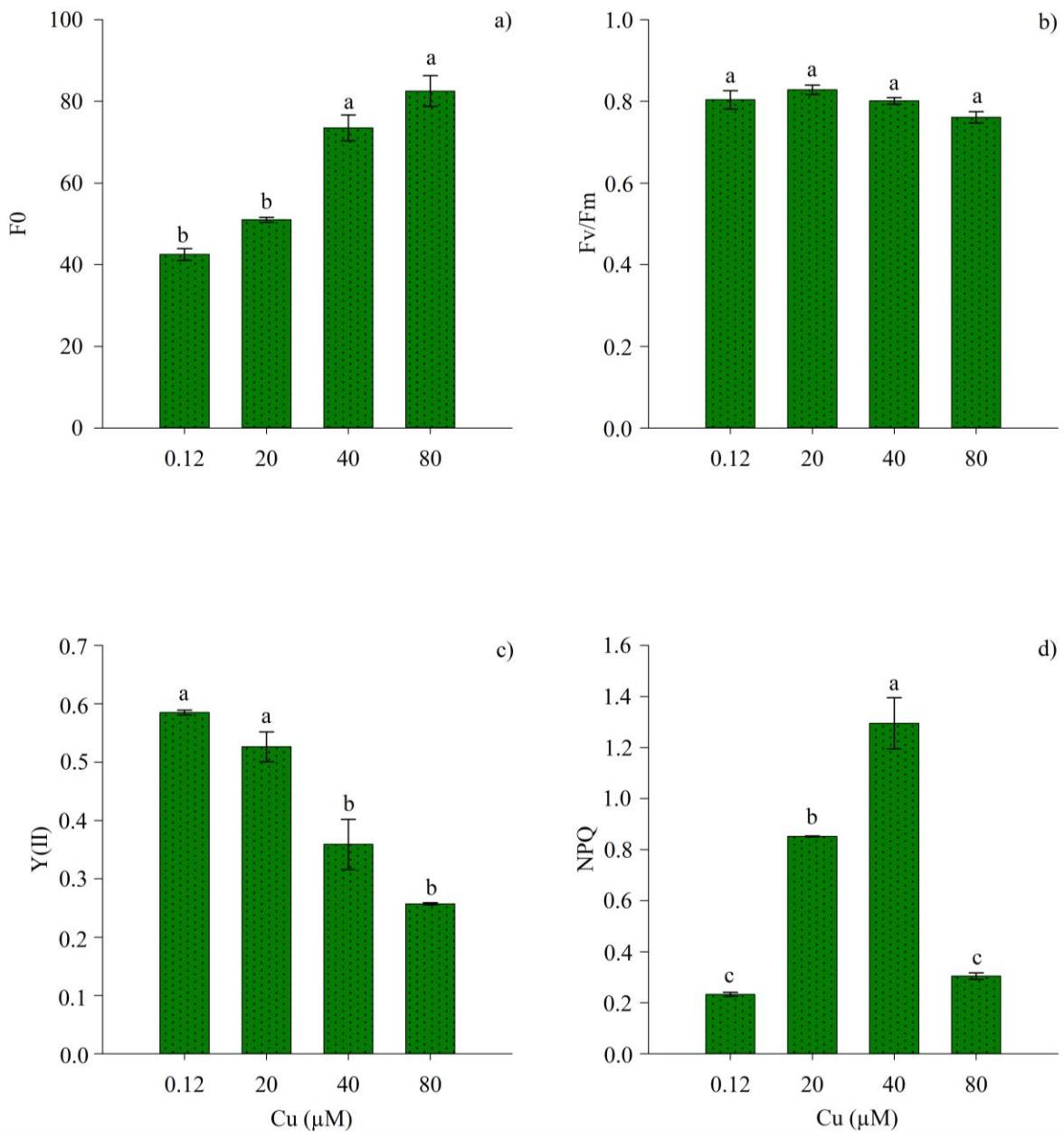


Figure 4. Initial fluorescence (F_0) (a), maximum PSII quantum yield (F_v/F_m) (b), effective quantum efficiency of PSII ($Y(\text{II})$) (c) and non-photochemical quenching (NPQ) (d) in leaves of grapevines (Paulsen rootstock) grown in nutritive solution under high Cu content. Histograms with the same lowercase letter were not statistically different in the Tukey test at 5%.

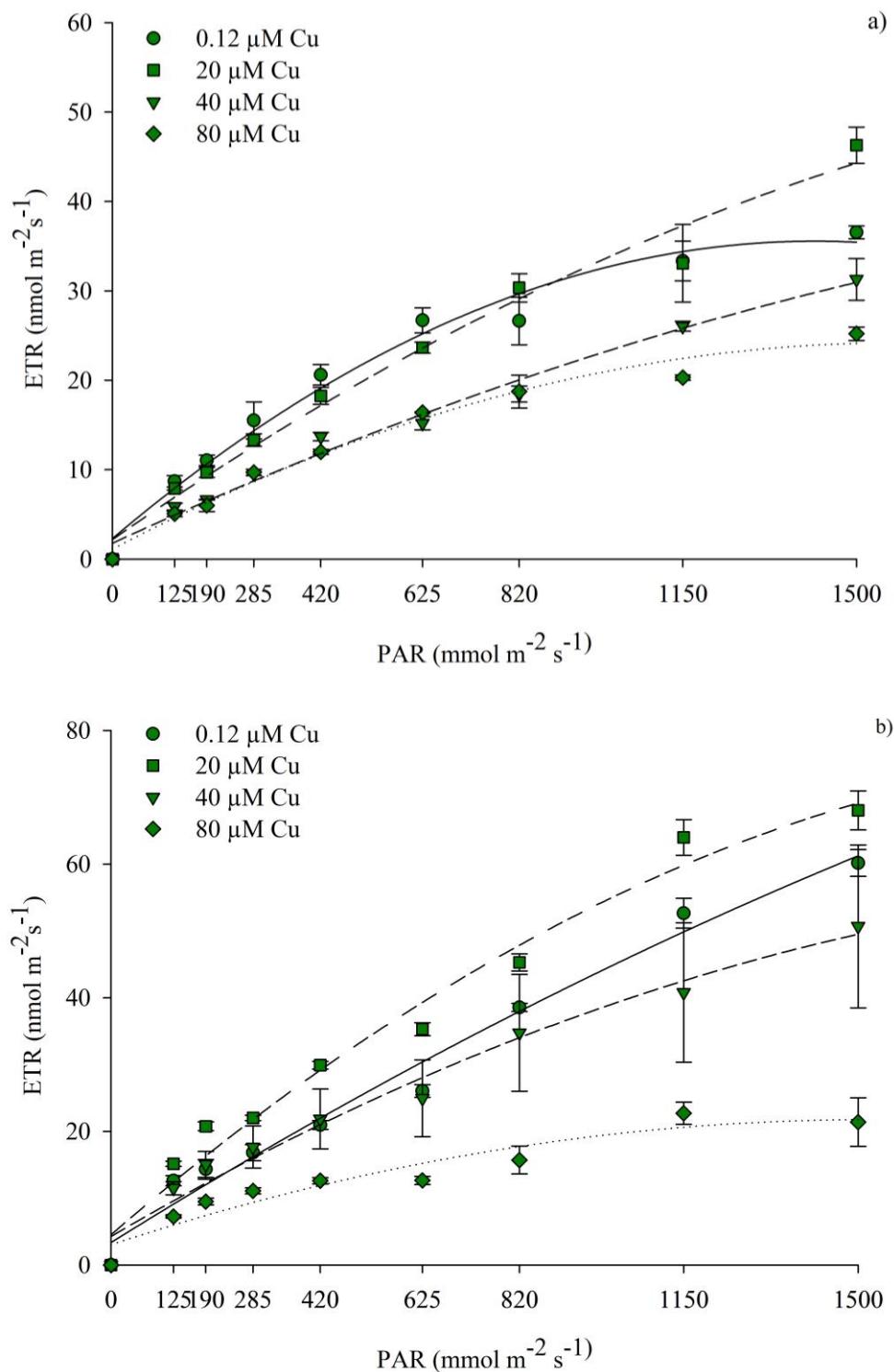


Figure 5. Electron transportation rate (ETR) and photosynthetic active radiation (PAR) in leaves of Magnolia grapevine rootstock (a) and Paulsen rootstock (b) grown in nutritive solution under high Cu content.

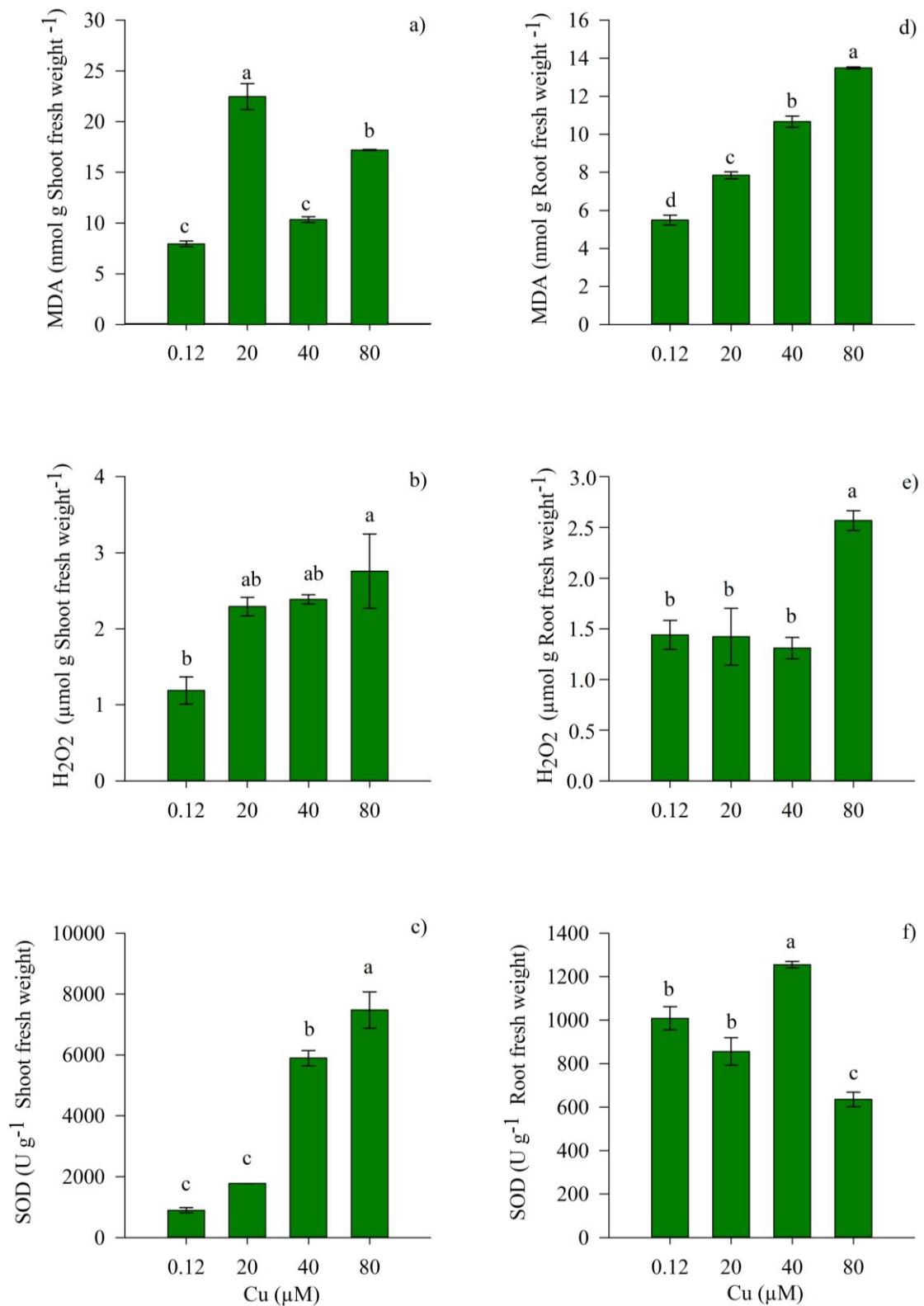


Figure 6. Lipid peroxidation (MDA) level (a), H_2O_2 concentration (b) SOD activity (c) in leaves and roots (d, e, f), respectively, of grapevines (Magnolia rootstock) grown in nutritive solution under high Cu content. Histograms presenting the same lowercase letter were not statistically different in the Tukey test at 5%.

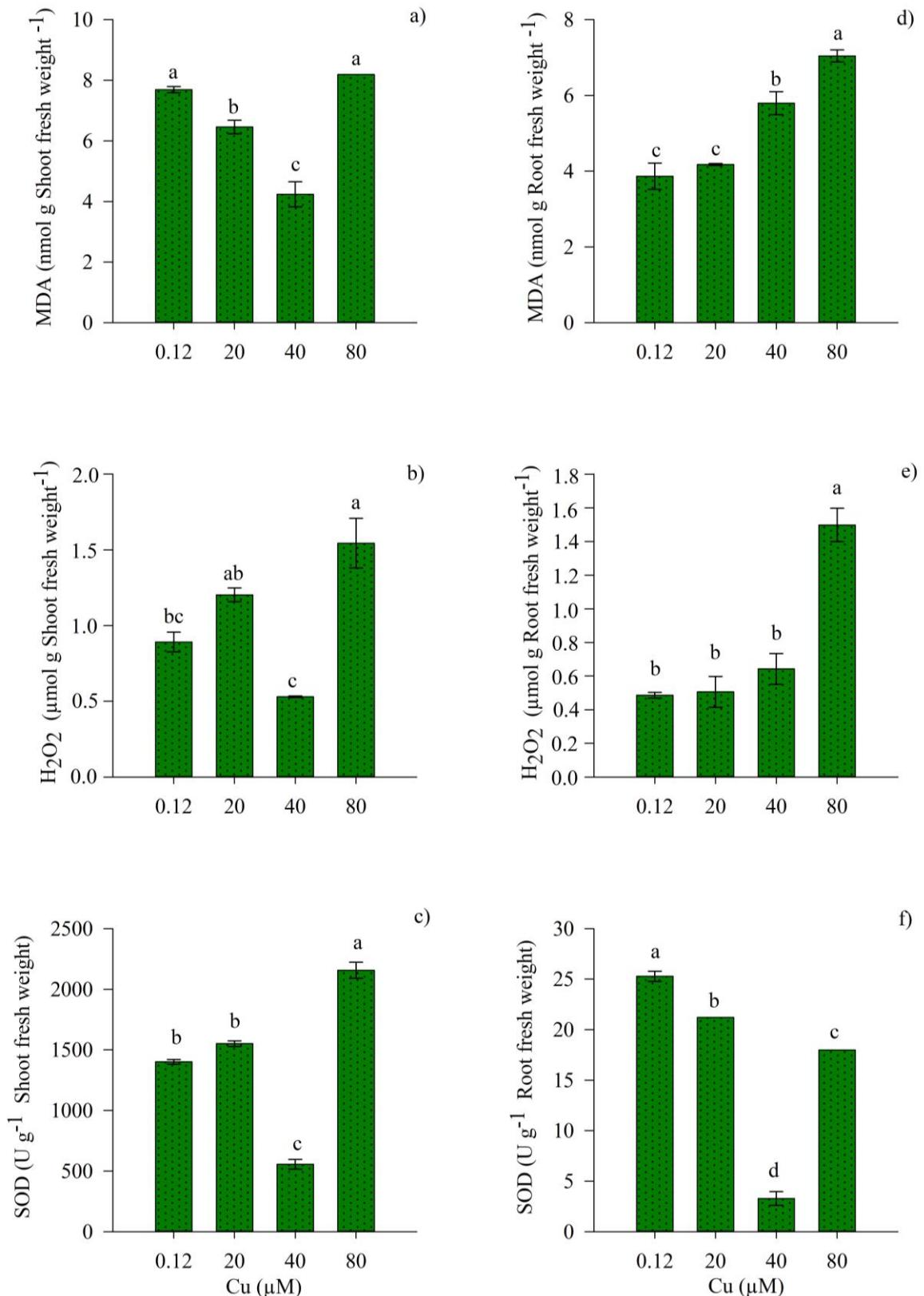


Figure 7. Lipid peroxidation (MDA) level (a), H_2O_2 concentration (b) SOD activity (c) in leaves and roots (d, e, f), respectively, of grapevines (Paulsen rootstock) grown in nutritive solution under high Cu content. Histograms presenting the same lowercase letter were not statistically different in the Tukey test at 5%.

5.3 ESTUDO III

Identifying grapevine rootstocks tolerant to copper excess³

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Abstract

Selection and use of grapevine rootstocks with potential to tolerate Cu excess can be a strategy for contaminated sites. Nonetheless, Cu tolerant rootstocks are not yet sufficiently known. Therefore, the aim of the current study is to identify grapevine rootstocks with potential to tolerate excessive Cu concentrations. Four grapevine rootstock genotypes were tested, namely: Paulsen 1103, IAC 572, SO4 and Isabel. Plants were cultivated in nutrition solution added with the following treatments: 0.3 µM Cu (control) and 80 µM Cu (Cu excess). Growth, nutrient concentration in tissue (shoot and root), physiological and biochemical parameters were assessed. Rootstocks showed different growth responses to Cu excess in solution. SO4, IAC 572 and Isabel markedly reducing growth under Cu excess compared to plants in control solution, whereas genotype Paulsen 1103 showing a less pronounced effect. All rootstocks showed decreases in macronutrients concentration in shoots and roots of plants subjected to Cu excess. The root system of all genotypes presented Cu increase under high Cu concentration, as well as higher POD activity and H₂O₂ concentration than the control. Isabel presented the greatest sensitivity to Cu excess, this outcome was clearly observed through leaf wilting and yellowing. Paulsen 1103 rootstock presented smaller changes in the observed parameters in high Cu concentration solution than in control solution. Our results indicate that Paulsen 1103 is the most tolerant to Cu excess, whereas Isabel is the most sensitive. There are natural genetic variations in tolerance to this abiotic stress that typically affects grapevine plants.

Keywords: Abiotic stress. Antioxidant enzymes. Copper. Grapevine. Natural genetic variation. Photosynthetic activity.

³Artigo elaborado de acordo com as normas de formatação da revista Plant Physiology and Biochemistry

1. Introduction

Constant application of chemicals to the phytosanitary control of fungal diseases in vineyards increases the content of heavy metals such as copper (Cu) in the soil (Giroto et al., 2014; Miotto et al., 2014; Brunetto et al., 2016; Tiecher et al., 2017, 2018; Baldi et al., 2018). Cu excess can cause toxicity in plants by decreasing photosynthesis, as it interferes in the synthesis of photosynthetic pigments and in photosynthetic apparatus functioning, and resulting in decreased growth (Yruela et al., 2009; Marschner, 2012; Adrees et al., 2015). High Cu concentrations can cause anatomic and morphological changes in roots, hindering water and nutrient absorption (Yruela et al., 2009; Marschner, 2012; Adrees et al., 2015). Plants increase the synthesis or activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD) - which act in ROS detoxification (Yruela et al., 2009; Marschner, 2012; Adrees et al., 2015) – in order to defend themselves from such an unbalance. However, excessive Cu presence in cells often impairs their ability to reverse oxidative stress effects, since Cu stimulate ROS (reactive oxygen species) formation by Fenton chemistry, resulting in damage to macromolecules.

The adoption of strategies based on genetic variation, such as selection of rootstock genotypes tolerant to Cu excess, might help keeping the yield rates of old vineyards. Most of the cultivated grapevines are grafted due to the introduction of phylloxera *Daktulosphaira vitifoliae* (Fitch) from the late 19th century (Ollat et al. 2016a). In addition to phylloxera, rootstocks can contribute to the control of other biotic and abiotic constraints (Ollat et al. 2016a). Rootstock selection takes into account the compatibility among genetic materials (rootstock and production variety); plant vigor; propagation easiness; effects on fruits' physical-chemical features; resistance to insects, diseases and nematodes; adjustment to soil features (pH, texture, depth, fertility, salinity, humidity) and climate (temperature and rainfall) (Warschefsky et al., 2016; Ollat et al. 2016a).

Most grapevine rootstocks are interspecific hybrids of American species, such as *Vitis riparia*, *Vitis rupestris*, and *Vitis berlandieri* which are adapted to specific cultivation conditions, resulting in hybrids with a huge variety of traits (Zhang et al., 2016; Ollat et al. 2016b). *Vitis riparia* is adapted to relatively wet environments with a shallow root system, *V. rupestris* grow better in gravel and sandy soils with a deep rooting growth habit, while *V. berlandieri* is adapted to calcareous high pH soils (Zhang et al., 2016; Ollat et al. 2016b). The herein used grapevine rootstocks were Paulsen 1103 (*Vitis berlandieri* x *Vitis rupestris*), IAC 572 ((*Vitis Riparia* x *Vitis rupestris*) x *Vitis caribaea*), SO4 (*Vitis berlandieri* x *Vitis riparia*) and Isabel (*Vitis labrusca*). Paulsen 1103 is adapted to dry and clayey soils, provides high

canopy vigor, high rooting rate, resistance to downy mildew (*Plasmopara viticola*), fusariosis (*Fusarium oxysporum* f. sp. *herbemontis*) and phylloxera; SO4 provides high canopy vigor, high rooting rate, is resistant to mildew and moderately adapted to acid and saline soils; IAC 572 is adapted to clayey, sandy and acid soils, provides high canopy vigor, high rooting rate, is resistant to mildew, furasiosis, phylloxera and nematodes; Isabel is commonly used as a scion grafted onto rootstocks, but can also be planted directly in soil, provides lower canopy vigor, and is resistant to anthracnose (*Elsinoe ampelina*) (EMBRAPA, 2018).

Grapevine rootstocks can present natural variation, which can result in distinct responses to biotic and abiotic stress (Warschefsky et al., 2016; Ollat et al. 2016a, 2016b). Plants can present different defense mechanisms against environmental abiotic stress caused by excess of metals available in crop sites (Yruela, 2005; 2009; Adrees et al., 2015). Under Cu excess, plants with increase vigor may dilute Cu, resulting in decreased toxicity. Plants may also reduce Cu uptake; decrease Cu translocation to shoots to protect the photosynthetic apparatus; or detoxify Cu in root cell wall and/or vacuoles (Yruela 2005; 2009; Adrees et al., 2015). Plants can activate different defense mechanisms depending on genotype, Cu concentration and plants' exposure time to metals (Yruela, 2003; 2009; Fidalgo et al., 2013; Marastoni et al., 2019a). However, there is little knowledge about the tolerance of grapevine rootstocks to Cu excess in the soil. Thus, the aim of this study was to identify grapevine rootstocks with potential to tolerate high Cu concentrations.

2. Materials and Methods

2.1 Plant material and growth conditions

The experiment was conducted in a greenhouse at the Department of Soil Science at Federal University of Santa Maria (UFSM), Santa Maria City, Brazil. First, grapevine rootstocks were cultivated from stakes to induce budding and seedling formation within a 3-month period-of-time. The stakes used were derived from Paulsen 1103 (*Vitis berlandieri* x *Vitis rupestris*), IAC 572 ((*Vitis Riparia* x *Vitis rupestris*) x *Vitis caribaea*), SO4 (*Vitis berlandieri* x *Vitis riparia*) and Isabel (*Vitis labrusca*) plants. Plantlets were transferred to pots (3-L capacity, Hoagland & Arnon (1950)) with 0.2 % of nutrient solution at pH 5.5. The solution comprised (mg L⁻¹) N = 85.31, P = 7.515, K = 104.75, Ca = 97.64, Mg = 23.68, S = 11.54, Fe = 2.68, Cu = 0.03, Zn = 0.13, Mn = 0.11, B = 0.27, Mo = 0.05 and Ni = 0.01. The solution was continuously aerated and replaced every three days. Each pot had one plant, which was fixed with Styrofoam covers to decrease water loss due to evaporation.

After the acclimation period for 10 days, plants were cultivated for another 15 days under different Cu concentrations. The following treatments were applied: 0.3 µM Cu (Control) and 80 µM Cu. Copper (Cu) was supplemented with CuSO₄.5H₂O. The experiment followed a completely randomized design, with four repetitions per treatment.

2.2 Height, dry matter yield and tissue nutrient concentration

Shoot height was measured at the end of the experiment with the aid of measuring tape. Plants were collected after 15 days of cultivation. Shoots and roots were separated, washed in running water and, subsequently, in distilled water. Samples were dried in forced air-circulation oven at ± 65°C until constant mass was reached. Next, root and shoot dry mass was measured on precision scale. Samples were ground in Wiley mill and prepared for chemical analysis.

Part of the tissue (shoots and roots) was prepared and subjected to nitric-perchloric digestion (Embrapa, 2009). Calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), zinc (Zn) and iron (Fe) concentrations in the extract were determined in atomic absorption spectrophotometer (AAS, Varian SpectrAA-600, Australia); potassium (K), in flame photometer (DM62, Digimed, Brazil) and phosphorus (P) concentration was determined through the methodology by Murphy & Riley (1962) by means of colorimetry carried out in spectrophotometer (SF325NM, Bel Engineering, Italy). The remaining tissue was subjected to sulfur digestion (Tedesco et al., 1995) to find nitrogen (N) concentration, based on the Kjeldahl method conducted in steam distiller (TE-0364, Tecnal, Brazil) (Bremner and Mulvaney, 1982).

Copper (Cu) translocation factor (TF) from the root system to the shoot was calculated through the follow equation: TF= C_{shoot}/C_{root}; wherein, C_{shoot} represents Cu concentration (mg kg⁻¹ dry matter) in the shoot and C_{root} is Cu concentration in the roots (Chopin et al., 2008; Busuioc et al., 2011).

2.3 Photosynthetic activity

The photosynthetic activity was measured 15 days after treatment application. Measurements of leaves located in the upper middle third of plant shoot were taken with the aid of infra-red gas analyzer (Li-6400, Li-COR Inc., Neb., USA). Net photosynthetic rate (A), internal CO₂ concentration (Ci), transpiration rate (E), CO₂ stomatal conductance (Gs), water use efficiency (WUE) and instantaneous carboxylation efficiency (A/Ci) were determined in leaf chamber, at CO₂ concentration of 400 µmol mol⁻¹, temperature of 20/25°C, relative

humidity of $50 \pm 5\%$ and photon flux density of $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Net photosynthetic rate (A), internal CO₂ concentration (Ci), transpiration rate (E) and CO₂ stomatal conductance (Gs) were calculated through the equations by Von Caemmerer and Farquhar (1981).

2.4 Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence analysis was carried out 15 days after treatment application with the aid of JUNIOR-PAM fluorometer (Walz, Germany). Fluorescence measurements were taken between 8:00 am and 10:00 am, under radiation of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, on average, in leaves located in the upper third of the shoot (Ferreira et al., 2015). Leaves were allowed to acclimate to the dark for 30 min before the readings; leaf blade was covered with aluminum paper to determine the minimal initial fluorescence level (F0) ($<0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$ per $1.8 \mu\text{s}$). Subsequently, they were subjected to saturating light pulse ($10,000 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 0.6 s to determine maximum fluorescence (Fm). Maximum quantum yield of PSII (Fv/Fm), effective quantum efficiency of PSII (Y(II)) and non-photochemical quenching (NPQ) were calculated based on fluorescence parameters (Maxwell and Johnson, 2000).

Maximum electron transport rate (ETRmax) was determined in the dark period - before dawn (5:00-6:00 am) (Ferreira et al., 2015). The ETR of each sample was evaluated through light curve emission (photosynthetically active radiation, PAR) at 9 different intensity levels (0; 125; 190; 285; 420; 625; 820; 1,150; and $1,500 \mu\text{mol electrons m}^{-2} \text{s}^{-1}$) for 10 seconds. Measurements were calculated through equation: $ETR = ETRmax [1-e^{kQ}]$, wherein, k is the constant and Q is light intensity (PAR) (Rascher et al., 2000).

2.5 Enzymatic activity

Root samples were collected at the end of the experiment, immediately placed in liquid N₂ and stored in ultrafreezer at -80°C until the time to carry out the enzyme analysis.

Hydrogen peroxide (H₂O₂) concentration in the tissue (shoots and roots) was determined based on Loreto and Velikova (2001). The aliquot of approximately 0.1 g of sample was homogenized in 1.5 mL of 0.1% TCA (w/v). The homogenate was centrifuged at 10,000g for 15 minutes at 4°C. H₂O₂ concentration was determined by comparing its absorbance to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml K 1 mol L⁻¹ at 390 nm, at standard calibration curve.

The aliquot of 1 g of tissue (shoots and roots) was homogenized in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8), added with 1 mM EDTA and 1% Triton X-100, for the enzymatic analysis. The homogenate was centrifuged at 13,000 g for 20 minutes at 4 °C. The

supernatant was used to determine the protein content (Zhu et al., 2004). Superoxide dismutase (SOD) activity was determined through the colorimetric method described by Giannopolitis and Ries (1977); peroxidase activity (POD) in the extract was determined based on Zeraik et al. (2008).

2.6 Statistical analysis

Results were subjected to analysis of variance in Sisvar software, version 4.0 (Ferreira, 2008). When the effect of treatments was significant, means recorded for grapevine rootstocks were separated through Tukey test at 5% ($p<0.05$), and means recorded for the treatments (control and with high Cu addition) were separated by t-test (LSD) at 5% ($p<0.05$).

The multivariate principal component analysis (PCA) was carried out in Canoco software, version 4.5 (Ter Braak and Smilauer, 2002) to complete the variance analysis. Principal component analysis (PCA) was carried out based on the following photosynthetic parameters: net photosynthetic rate, stomatal conductance, intercellular CO₂ concentration, transpiration rate, water use efficiency and instantaneous efficiency of carboxylation; as well as on chlorophyll *a* fluorescence parameters such as initial fluorescence, effective quantum efficiency of PSII, maximum PSII quantum yield and non-photochemical quenching; on N, P, K, Ca, Mg, Cu, Zn, Fe and Mn concentration in the shoot and root; on oxidation stress parameters (POD and H₂O₂ in the roots); and on plant growth parameters such as plant height, root and shoot dry mass. Principal component analysis (PCA) was conducted based on a set of principal components (in this case we used components 1 and 2) that reflect a set of orthogonal standardized linear combinations; together these combinations explained the observed data original variability.

3. Results

3.1 Growth of different grapevine rootstock genotypes

Rootstocks SO4, IAC 572 and Isabel grown in high Cu concentration solution presented reduction by 41%, 35% and 13% in plant height in comparison to the control solution, respectively (Figure 1a). Also, IAC 572 and Isabel showed reduction by 51% and 26% in plant dry mass yield grown in high Cu concentration solution in comparison to the control, respectively (Figure 1b). On the other hand, Paulsen 1103 and SO4 presented higher shoot dry mass yield in high Cu concentration solution than plants cultivated in control solution (Figure 1b). Root system development of rootstocks IAC 572 and SO4 cultivated in excess Cu solution presented root dry mass yield 1.67 and 1.26 times higher than seedlings

cultivated in control solution, respectively (Figure 1c). While Paulsen showed root dry mass 2.17 times lower in solution with high Cu concentration than in control solution (Figure 1c).

3.2 Nutrient homeostasis affected by Cu excess in rootstocks' shoot and roots

The concentration of N, P, K, Ca, Mg and Mn was higher in the shoot of rootstocks grown in control solution than in Cu excess solution (Table 1, 2). The highest Cu concentrations in the shoot were observed for Isabel rootstock grown in control solution; however, higher Cu concentration in the shoot was observed for rootstocks Paulsen 1103 and SO4 grown in high Cu concentration solution in comparison to control solution (Table 2).

The highest P, K, Ca and Mg concentrations in the root were observed for all the rootstocks grown in control solution (Table 1). The highest Cu concentrations in the roots were seen in rootstocks grown in Cu excess solution (Table 2). Rootstocks Paulsen 1103 and SO4 presented the highest Fe concentrations in the roots when they were grown in high Cu concentration solution, and the highest Mn concentrations in the roots were observed in plants cultivated in control solution. The highest Fe concentrations in rootstocks IAC 572 and Isabel were observed in plants grown in control solution and the highest Mn concentration in these rootstocks was recorded for plants cultivated in high Cu concentration solution (Table 2). The highest Zn concentrations were observed in roots of most rootstocks cultivated in control solution, except for Paulsen 1103, which presented the highest Zn concentration in the roots of plants grown in Cu excess solution (Table 2).

Copper (Cu) translocation factor (TF) values from the root system to the shoot were higher in rootstocks cultivated in control solution than in the ones cultivated in high Cu concentration solution. Rootstocks SO4 and Isabel presented the highest TF values (Table 3).

3.3 Based on the photosynthetic activity, Paulsen 1103 is the genotype most tolerant to Cu excess

Rootstocks cultivated under higher Cu concentrations have significantly reduced liquid photosynthesis rates (A), stomatal conductance (Gs), CO₂ intercellular concentration (Ci) and transpiration rate (E) in comparison to the ones cultivated in control solution (Figure 2a, b, c, d). The photosynthetic rate ranged from 3.94 to 13.26 µmol CO₂ m⁻² s⁻¹, which were the highest values observed for rootstocks Paulsen 1103 and Isabel (Figure 2a). Stomatal conductance in rootstocks SO4 and Isabel was 60.47 and 14.62 times higher in control solution than that recorded in high Cu concentration solution (Figure 2b). The highest values of intracellular CO₂ concentration and transpiration rate were found in plants subjected to the

control treatment (Figure 2c). The instantaneous efficiency of carboxylation (A/C_i) was 1.93 times higher in rootstock Paulsen 1103 cultivated in control solution than in the ones cultivated in high Cu concentration solution (Figure 2e). Water use efficiency (WUE) was higher in rootstock SO4 than in the other rootstocks; it was 11.72 times higher in high Cu concentration solution than in plants grown in control solution (Figure 2f). Therefore, Paulsen 1103 was shown to be capable of keeping photosynthesis under Cu excess conditions, whereas the other genotypes were more affected by Cu.

3.4 Based on Chlorophyll *a* fluorescence, Isabel rootstock was the genotype most sensitive to Cu excess

The highest initial fluorescence values (F_0) were observed for SO4 grown under both culture conditions (Figure 3a). Rootstocks SO4, IAC 572 and Isabel did not present differences between Cu treatments in F_0 , whereas rootstock Paulsen 1103 showed F_0 27% higher, when it was grown in high Cu concentration solution than plants cultivated in control solution (Figure 3a). Rootstocks Paulsen 1103 and SO4 evidenced lower values of effective quantum efficiency of Photosystem II ($Y(II)$), which were 1.24 and 2.43 times lower in plants cultivated in high Cu concentration solution than in control solution, respectively (Figure 3b). The highest values of PSII maximum quantum yield (F_v/F_m) were observed for Paulsen 1103 and Isabel grown under both culture conditions (Figure 3c). Rootstock IAC 572 presented higher F_v/F_m value, when it was cultivated in Cu excess solution than plants cultivated in control solution. Rootstocks Paulsen 1103 and SO4 showed higher values of fluorescence loss - in the form of heat (NPQ) -, which were 1.33 and 1.62 times lower, when they were cultivated in Cu excess solution, than that of plants subjected to the control treatment, respectively (Figure 3d). On the other hand, IAC 572 presented 1.26 times NPQ reduction in the Cu excess treatment, in comparison to the control (Figure 3d).

Electron Transport rate (ETR) increased according to the photosynthetic active radiation intensity emitted in all rootstocks, in both treatments (Figure 4 a, b). Lower ETR values were observed for all rootstocks grown in high Cu concentration solution than for plants cultivated in control solution. Rootstock IAC 572 presented the highest ETR in the control treatment, approximately $80 \text{ nmol m}^{-2} \text{ s}^{-1}$ under the higher emitted radiation ($1,500 \text{ nmol m}^{-2} \text{ s}^{-1}$). The lowest ETR value was recorded for rootstock Isabel, approximately $50 \text{ nmol m}^{-2} \text{ s}^{-1}$ under the highest emitted radiation value (Figure 4a).

The highest ETR values were observed for SO4 subjected to the treatment with high Cu concentration; it showed values close to $40 \text{ nmol m}^{-2} \text{ s}^{-1}$ under the maximal emitted

radiation (Figure 6b). On the other hand, Isabel presented the lowest ETR value, which reached approximately $20 \text{ nmol m}^{-2} \text{ s}^{-1}$ under maximal emitted radiation (Figure 4b). Therefore, these data suggest that rootstock Isabel records the greatest photosynthesis loss when exposed to Cu excess, indicating this is the most sensitive genotype.

3.5 Enzymatic activity

SOD and POD activities and H_2O_2 concentration were assessed in the shoot and root of different rootstocks (Figure 5). Rootstocks IAC 572, Isabel and SO4 did not produce biomass enough to enzymatic and H_2O_2 concentration analyses in shoots when they were cultivated in high Cu concentration solution (Figure 5 a, c and e). SOD activity in shoots was 25.04 and 2.66 times higher in rootstocks Paulsen 1103 and SO4 cultivated in Cu excess solution, respectively, than in plants subjected to the control treatment (Figure 5a). POD activity did not present statistic difference in these two rootstocks (Figure 5c). Shoot H_2O_2 concentration in rootstock Paulsen 1103 was 1.83 times higher in Cu excess solution than in plants grown in control solution (Figure 5e).

POD activity and H_2O_2 concentration were higher in the roots of all the rootstocks cultivated in high Cu concentration solution than in plants cultivated under the control treatment (Figures 5d, f). On the other hand, SOD concentration in roots of all the rootstocks showed the lowest activity when they were cultivated in high Cu concentration solution (Figures 5b). SO4 showed the highest SOD concentration in roots grown in control solution, 1.46 times higher than plants cultivated in Cu excess solution (Figure 5 b). IAC 572 cultivated in high Cu concentration presented the highest POD concentration in roots, 4.09 times higher than plants cultivated in control solution (Figure 5 d). H_2O_2 concentration in the roots of Paulsen cultivated in high Cu concentration solution was the highest, 7.71 times than plants grown in control solution (Figure 5f).

3.6 Principal component analysis (PCA)

Principal component analysis (PCA) was carried out by only extracting the two first components (PC1 and PC2), which, together, explained 62.33% original data variability (Figure 6). Principal component 1 explained 42.31% variability and recorded the strongest influence on N, K and Ca concentration in the shoot; as well as on P, K, Mg and Cu in the roots and photosynthetic rate, on stomatal conductance, internal CO_2 concentration and transpiration rate. Principal component 1 (PC1) was efficient in separating treatments into two great groups: the group to the right (smaller ellipse), which held treatments with rootstocks

subjected to high Cu concentration solution; and the group to the left, which encompassed the same rootstocks under control conditions, i.e., low Cu concentration solution (bigger ellipse) (Figure 6). Principal component 2 (PC2) explained approximately 20% data variation; it encompassed response variables that have mostly influenced N concentration in the roots, as well as the association between photosynthetic rate and internal CO₂ concentration. Principal component 2 (PC2) was efficient in separating grapevine rootstocks IAC 572 and Isabel from the genetic materials of Paulsen 1103 and SO4 when they were subjected to control conditions. The separation of grapevine rootstocks was subtler in the highest Cu concentration solution; therefore, it separated grapevine rootstock Paulsen 1103 from the other ones.

4. Discussion

Different responses between rootstock genotypes cultivated in control solution and in high Cu concentration solution can be attributed to genetic variability intrinsic to each genotype (Pommer et al., 2003). IAC 572 plants presented the highest height and shoot dry mass values, mainly in the control solution, because they are vigorous grapevine rootstocks (Figure 1) (Embrapa, 2018). Responses of rootstocks cultivated in Cu excess solution can be related to mechanisms developed to adapt to Cu toxicity (Marastoni et al., 2019b). According to these authors, grape rootstocks' mechanisms to adapt to Cu excess can vary between different genetic materials; for example, rootstock Fercal induces root system development as a mechanism to tolerate Cu excess, since it enables root to increase and, consequently, allows greater Cu accumulation in the roots. On the other hand, rootstock 196.17 induces responses to molecular level by increasing the regulation of divalent cation transporters in response to Cu-induced Mn deficit.

The lowest height and shoot dry mass values recorded for rootstocks IAC 572 and Isabel cultivated in high Cu concentration solution (Figure 1 a, b) can be explained by the effect of Cu excess, which impairs plant growth (Tiecher et al., 2017; 2018; Marastoni et al., 2019a; 2019b). On the other hand, Paulsen 1103 plants, although presenting lower shoot growth values than other rootstocks, presented increased shoot dry mass in the Cu excess solution. Shoot growth stimulus and root system reduction in Paulsen 1103 plants under high Cu availability condition can be related to plant response mechanism to Cu excess. The highest MSR values recorded for SO4 and IAC 572 in high Cu concentration solution (Figure 1 c) can be associated with anatomic and morphological changes caused by Cu excess in the roots and/or, yet, they can be a defense strategy of plants against the excess of metal availability (Marastoni et al., 2019b). High Cu concentration availability in solution can

change the cell differentiation process and thicken the roots, mainly in the apical region, as well as induce growth in secondary lateral roots (Ambrosini et al., 2015; Guimarães et al., 2016; Somavilla et al., 2018). On the other hand, root system growth induction in high Cu concentration solution can be a plant defense mechanism against the excess of metals; this process is expressed by the greatest Cu absorption in functional groups deprotonated in the cell wall (Guimarães et al., 2016; Marastoni et al., 2019a). Part of Cu can be stored in cell vacuoles in the root system, and act as cell detoxification mechanism and Cu concentration control in cells (Ricachenevsky et al., 2018); besides, the root surface area increase potentiates water and nutrient absorption by plants (Ambrosini et al., 2015; Guimarães et al., 2016; Marastoni et al., 2019a).

Nutrient concentration reduction in the shoot and roots of rootstocks grown in Cu excess solution can be related to changes in plants' mineral composition due to high Cu concentration in solution (Kabata-Pendias, 2011; De Freitas et al., 2015; Marastoni et al., 2019a) and to changes caused by Cu excess in plants' root system. These changes can hinder water and nutrient absorption, in comparison to plants subjected to the control treatment (Tables 1, 2) (Adrees et al., 2015; Ambrosini et al., 2015). Copper (Cu) is a divalent cation that can have negative influence on the absorption and translocation of other nutrients - at high concentrations - (Reichman, 2002; Zanin et al., 2015; Marastoni et al., 2019a) depending on plant species and growth conditions (Pii et al., 2015). The highest Cu concentration was observed for the root system of all rootstocks analyzed, mainly in Isabel and IAC 572 (Table 2). Cu translocation rate from the root system to the shoot was lower in these plants than in the ones subjected to the control, which, in its turn, has favored Cu accumulation in the root system. The highest Cu concentration in plants' root system, as previously detailed, can act as defense mechanism through the excessive accumulation of metals in cell wall and their storage in vacuoles, since such an accumulation reduces the translocation of high Cu concentrations to plant shoots, that is more sensitive to toxic effects due to Cu excess and, consequently, it can reduce toxicity (Ambrosini et al., 2015; 2018; Tiecher et al., 2018).

Parameters related to plant photosynthetic activity were higher in all rootstocks cultivated in control solution than in the ones grown in Cu excess solution (Figure 3). This outcome can be attributed to the phytotoxic effect of Cu excess solution on the integrity and functions of the photosynthetic apparatus, as well as on the synthesis of photosynthetic pigments (Cambrollé et al., 2015; Ambrosini et al., 2018). Rootstocks SO4, IAC 572 and Isabel presented similar behavior to the photosynthetic variables, mainly when it comes to the Gs, Ci and E parameters in the control solution in comparison to Paulsen 1103, given its

higher plant vigor. Paulsen 1103 presented the highest A/Ci values in control solution and in high Cu concentrations; this outcome evidences high carboxylation efficiency, mainly in control solution. The highest WUE values were observed for rootstocks SO4, IAC 572 and Isabel grown in high Cu concentration solution due to the lower stomatal conductance presented by these plants and, consequently, to their lower water loss (Schwalbert et al., 2019). Changes in parameters related to plants' photosynthetic activity caused by high Cu concentrations were also observed in other studies (Cambrollé et al., 2015; Ambrosini et al., 2018; De Conti et al., 2018; Schwalbert et al., 2019; Trentin et al., 2019). But, negative effects on photosynthetic activity are observed in different Cu concentrations in the soil, solution or tissue, which is associated with the species and rootstock.

The initial chlorophyll *a* fluorescence was higher in rootstock Paulsen 1103 cultivated in high Cu concentration solution (Figure 4); it can be explained by the reduced content of photosynthetic pigments and by damages to plant photosynthetic apparatus resulting from metal excess - this process leads to the emission of more radiation in the form of fluorescence (Cambrollé et al., 2015). The values of Y(II) significantly reduced in rootstocks Paulsen 1103 and SO4 grown in Cu excess solution in comparison to plants grown in control solution; this outcome can be related to their higher sensitivity to Cu excess for this variable, that influences the photosynthetic apparatus of these plants (Cambrollé et al., 2015; Ambrosini et al., 2018). The highest NPQ values observed for rootstocks SO4 and Paulsen 1103 cultivated in Cu excess solution can be explained by the effect of Cu excess on the photosynthetic apparatus, it reduces the amount of radiation emitted for the photosynthetic and fluorescence activity, as well as increases the amount of radiation lost in the form of heat (Cambrollé et al., 2015; Ambrosini et al., 2018). Electron transportation rate is closely correlated to plant photosynthetic activity (Maxwell and Johnson, 2000); in this case, rootstocks under all cultivation conditions recorded increased ETR due to increased emitted radiation. However, ETR values in the rootstocks cultivated in control solution are two times higher than those observed for rootstocks grown in high Cu concentration solution (Figure 4). This result can be related to the effect of Cu excess on photosynthetic pigment reduction and to damages caused to the photosynthetic apparatus of plants (Cambrollé et al., 2013; 2015; Tiecher et al., 2016; 2017). Rootstock Isabel presented the lowest ETR values and was the first to present clear toxicity symptoms due to Cu excess in the solution (Figure 4), such as leaf wilting and yellowing. This outcome pointed out Isabel its higher sensitivity to Cu excess in comparison to the other assessed plants.

High Cu concentrations stimulate ROS (reactive oxygen species) formation, which can be harmful for plants and cause lipid peroxidation in cell membranes (Marschner, 2012; Adrees et al., 2015). Plants synthesize enzymes accountable for ROS detoxification in response to its formation (Marschner, 2012); the formation of H₂O₂ takes place through the dismutation of superoxide (O₂⁻) by superoxide enzyme dismutase (SOD). H₂O₂ level in roots was higher in all rootstocks cultivated in Cu excess solution, probably due to ROS formation (Marschner, 2012; Miotto et al., 2014; Adrees et al., 2015). Plants also increase the synthesis of peroxidases (POD), and it inactivates H₂O₂ molecules in response to H₂O₂ concentration increase, which, consequently, reduces the damages caused by oxidative stress in plants. Copper (Cu) concentration in the root of rootstocks was related to POD in the roots (Figure 5), due to the activation of antioxidant defense mechanism developed to fight ROS formed by Cu excess in the tissue. ROS production increase resulted from the excess of Cu available, as well as the activation of antioxidant enzymatic mechanisms, as observed by Miotto et al. (2014) in grapevine leaves of variety Cabernet Sauvignon (*Vitis vinifera*) grafted in rootstock SO4 in three different vineyards that were implanted in 2004, 1998 and 1977. Tiecher et al. (2016) - in oat plants (*Avena strigosa* Schreb.) - and Tiecher et al. (2017) observed increased synthesis of antioxidant enzymes in young grapevines cultivated in soil with high Cu and Zn content, due to the excess of metal availability.

Principal component analysis (Figure 6) separated the two culture conditions in control and high Cu concentration, based on the negative effects caused by Cu excess on the assessed variables. Rootstocks cultivated in high Cu concentration solution formed the groups based on Cu concentration in root system, on antioxidant enzyme (POD) response and on photosynthetic variables (NPQ and WUE). Rootstocks subjected to control solution were also separated into two groups based on features intrinsic to each genetic material and to their similarities; IAC 572 and Isabel were separated from genetic materials Paulsen 1103 and SO4. On the other hand, rootstock Paulsen 1103 grown in high Cu concentration solution was separated from the other rootstocks, due to its different behavior in response to the exposure to stress conditions caused by Cu excess.

5. Conclusions

The assessed rootstocks presented different responses to Cu excess in solution depending on features intrinsic to each genetic material. All rootstocks showed negative effects on their growth, photosynthetic and biochemical activity due to the excess of Cu availability. Rootstock Isabel presented the highest sensitivity to Cu excess, which was clearly

detected based on leaf wilting and yellowing. Rootstocks SO4 and IAC 572 presented the more robust growth and physiological data in control conditions, likely because they were the most vigorous genotypes; however, when they were exposed to high Cu concentrations, they presented considerable reduction in comparison to plants cultivated in control solution. Rootstock Paulsen 1103 presented smaller changes in the observed parameters when it was cultivated in high Cu concentration solution than plants subjected to the control solution, and this outcome can be related to its greater ability to tolerate Cu excess. Thus, our results pointed out that Paulsen 1103 seems to be the most tolerant genotype, whereas Isabel is the most sensitive to Cu excess.

6. Contributions

Edicarla Trentin: study design, accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Letícia Morsch, Simoni Weide Belles and Jacson Hindersmann: accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Camila Peligrinotti Tarouco and Fernando T. Nicoloso: study design, biochemical analysis in plants, photosynthetic analysis, accomplishment of the experiment and writing of the article. Álvaro Luís Pasquetti Berghetti: chlorophyll a fluorescence analysis, accomplishment of the experiment and writing of the article. Paulo Ademar Avelar Ferreira, Felipe Klein Ricachenevsky and Gustavo Brunetto: study design, interpretation of results and writing of the article. Henrique Pessoa dos Santos and George Wellington Bastos de Melo: interpretation of results and writing of the article.

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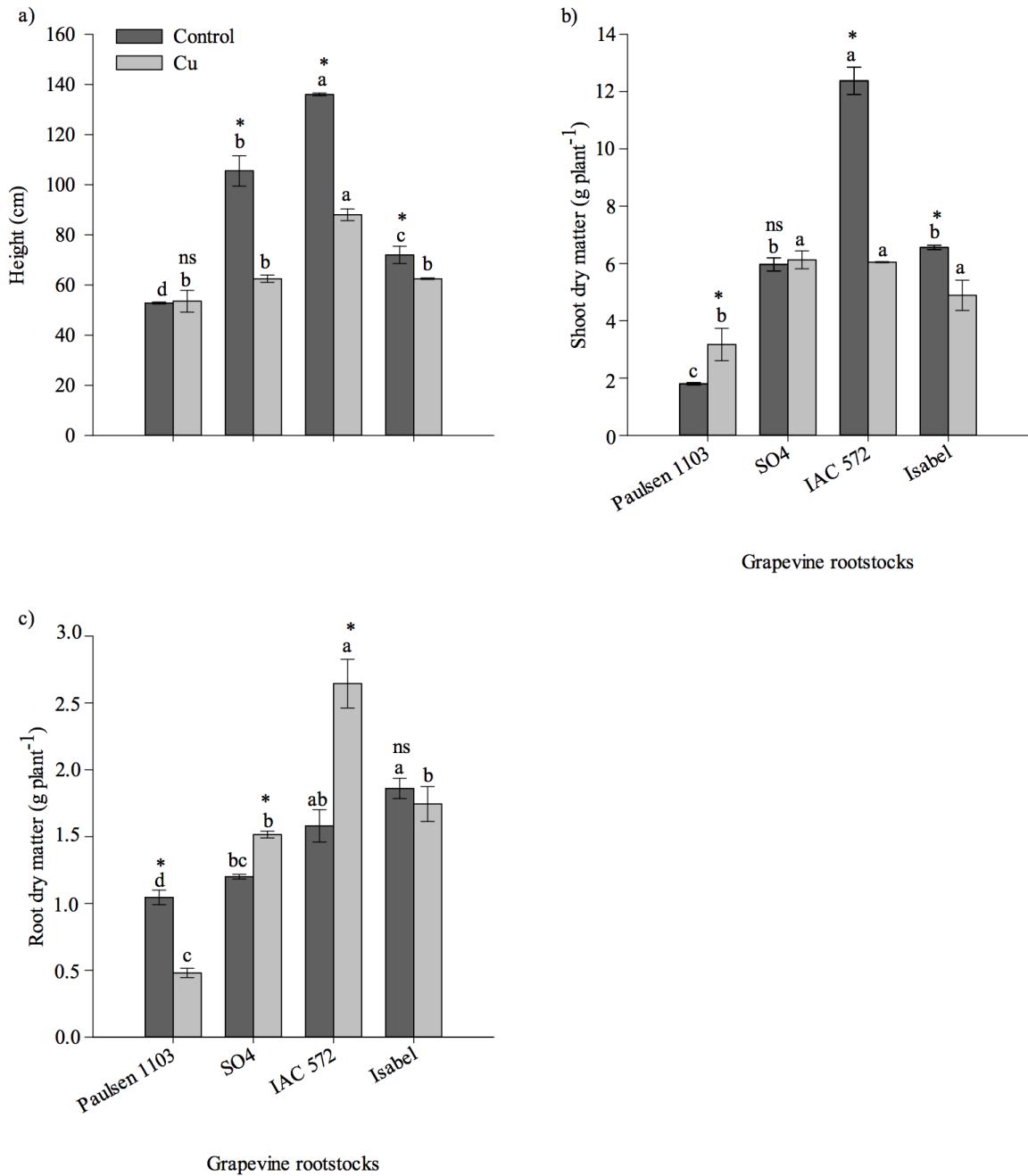


Figure 1. Height (a), shoot (b) and root (c) dry mass yield of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition (control) and high Cu content solution. Means followed by the same letter represent the comparison to rootstocks in each treatment through Tukey test at 5% ($P<0.05$). nsnon-significant; *significant (F test $p <0.05$) difference between Cu levels in the same rootstock.

Table 1. Macronutrient concentration (g kg^{-1}) in the shoot and roots of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition (control) and high Cu content solution.

Rootstocks	Treatment	N	P	K	Ca	Mg
		Shoots (g kg^{-1})				
Paulsen 1103	Control	25.94 a ^{ns(1)}	11.98 a*	19.52 a*	24.31 ab*	6.30 a*
	Cu	23.64 a	6.83 b	14.29 a	18.37 b	4.90 ab
SO4	Control	27.75 a*	12.48 a*	20.05 a*	25.60 a*	6.31 a*
	Cu	24.08 a	9.55 a	12.31 b	19.92 a	5.08 a
IAC 572	Control	28.07 a*	8.23 c*	19.79 a*	24.95 ab*	5.08 b*
	Cu	22.88 a	6.18 b	14.77 a	14.82 c	4.28 b
Isabel	Control	29.25 a*	10.36 b*	17.76 b*	23.96 b*	4.85 b*
	Cu	25.36 a	6.94 b	14.77 a	14.83 c	3.52 c
Roots (g kg^{-1})						
Paulsen 1103	Control	14.35 b	6.09 a*	18.83 c*	11.42 c ^{ns}	5.11 b*
	Cu	20.50 b*	2.49 b	9.10 a	11.40 b	1.44 a
SO4	Control	25.78 ab	4.97 b*	28.62 a*	14.95 b ^{ns}	6.48 a*
	Cu	28.40 a*	3.61 a	9.09 a	14.11 a	2.36 a
IAC 572	Control	28.40 a*	4.61 b*	29.64 a*	16.94 a*	6.29 a*
	Cu	23.24 b	3.11 ab	9.70 a	12.07 b	2.28 a
Isabel	Control	23.54 b ^{ns}	4.73 b*	23.42 b*	13.20 bc ^{ns}	6.02 ab*
	Cu	22.97 b	2.90 b	6.45 a	11.83 b	1.98 a

(1) Means followed by the same letter represent the comparison to rootstocks in each treatment through Tukey test at 5% ($P<0.05$). nsnon-significant; *significant (F test $p <0.05$) difference between Cu levels in the same rootstock.

Table 2. Micronutrient concentration in the shoot and roots of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition (control) and high Cu content solution.

Rootstocks	Treatment	Cu	Zn	Fe	Mn
		Shoots (mg kg ⁻¹)			
Paulsen 1103	Control	5.50 b ^{ns(1)}	27.70 a*	174.83 b ^{ns}	267.16 a*
	Cu	6.16 a	16.28 b	188.73 ab	70.37 ab
SO4	Control	4.66 b ^{ns}	23.70 b*	246.93 a*	198.88 b*
	Cu	6.84 a	11.84 c	163.33 bc	87.04 a
IAC 572	Control	6.02 b ^{ns}	24.34 ab*	178.84 b ^{ns}	121.74 d*
	Cu	5.46 a	21.24 a	199.92 a	51.70 b
Isabel	Control	8.38 a*	22.00 b ^{ns}	132.48 c ^{ns}	153.46 c*
	Cu	5.54 a	22.29 a	145.81 c	78.86 a
Roots (mg kg ⁻¹)					
Paulsen 1103	Control	51.46 a	19.54 c ^{ns}	625.12 b	232.20 a ^{ns}
	Cu	4937.52 c*	24.40 b	792.07 b*	200.08 b
SO4	Control	24.66 a	50.26 a*	664.80 ab	260.50 a*
	Cu	6377.28 b*	25.42 b	931.87 a*	196.90 b
IAC 572	Control	35.62 a	37.04 b ^{ns}	725.52 a*	250.78 a
	Cu	8540.28 a*	35.90 a	244.40 c	344.20 a*
Isabel	Control	31.28 a	38.98 b*	677.36 ab*	231.04 a ^{ns}
	Cu	8933.40 a*	30.04 ab	294.32 c	236.54 b

⁽¹⁾Means followed by the same letter represent the comparison to rootstocks in each treatment through Tukey test at 5% ($P<0.05$). nsnon-significant; *significant (F test $p <0.05$) difference between Cu levels in the same rootstock.

Table 3. The translocation factor (TF) of Cu from the root system to the shoot of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition (control) and high Cu content solution.

Rootstocks	Treatment	TF
Paulsen 1103	Control	0.095 d*
	Cu	0.001 a
SO4	Control	0.207 b*
	Cu	0.001 a
IAC 572	Control	0.175 c*
	Cu	0.000 a
Isabel	Control	0.226 a*
	Cu	0.001 a

⁽¹⁾Means followed by the same letter represent the comparison to rootstocks in each treatment through Tukey test at 5% ($P<0.05$). nsnon-significant; *significant (F test $p <0.05$) difference between Cu levels in the same rootstock.

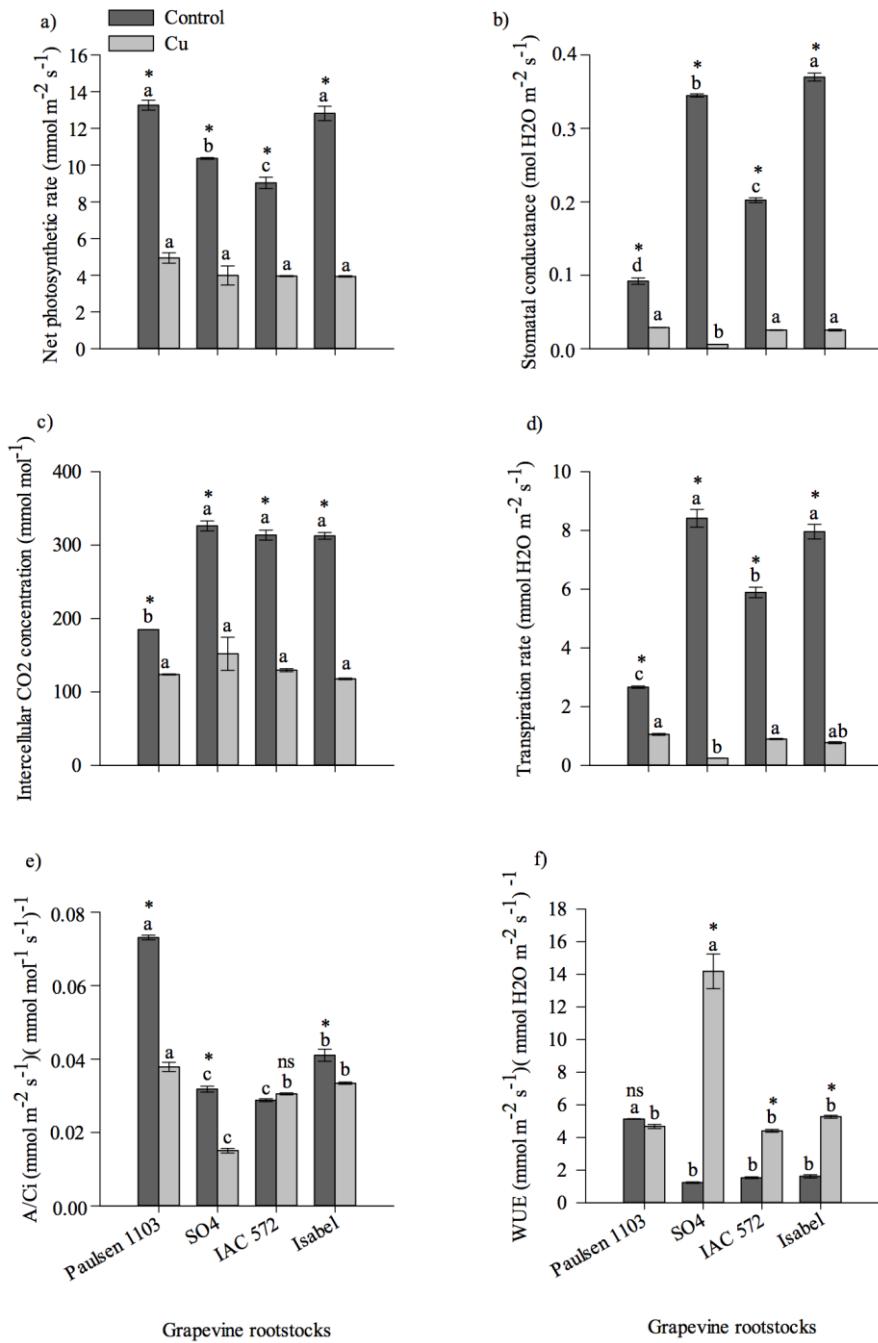


Figure 2. Net photosynthetic rate (A) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (a), stomatal conductance (Gs) ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (b), intercellular CO₂ concentration (Ci) ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) (c), transpiration rate (E) ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (d), water use efficiency (WUE) [$(\mu\text{mol m}^{-2} \text{ s}^{-1})(\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1})^{-1}$] (e) and instantaneous efficiency of carboxylation (A/Ci) [$(\mu\text{mol m}^{-2} \text{ s}^{-1})(\text{mmol mol}^{-1} \text{ s}^{-1})^{-1}$] (f), in leaves of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition (control) and high Cu content solution. Means followed by the same letter represent the comparison to rootstocks in each treatment through Tukey test at 5% ($P<0.05$). ns non-significant; *significant (F test $p <0.05$) difference between Cu levels in the same rootstock.

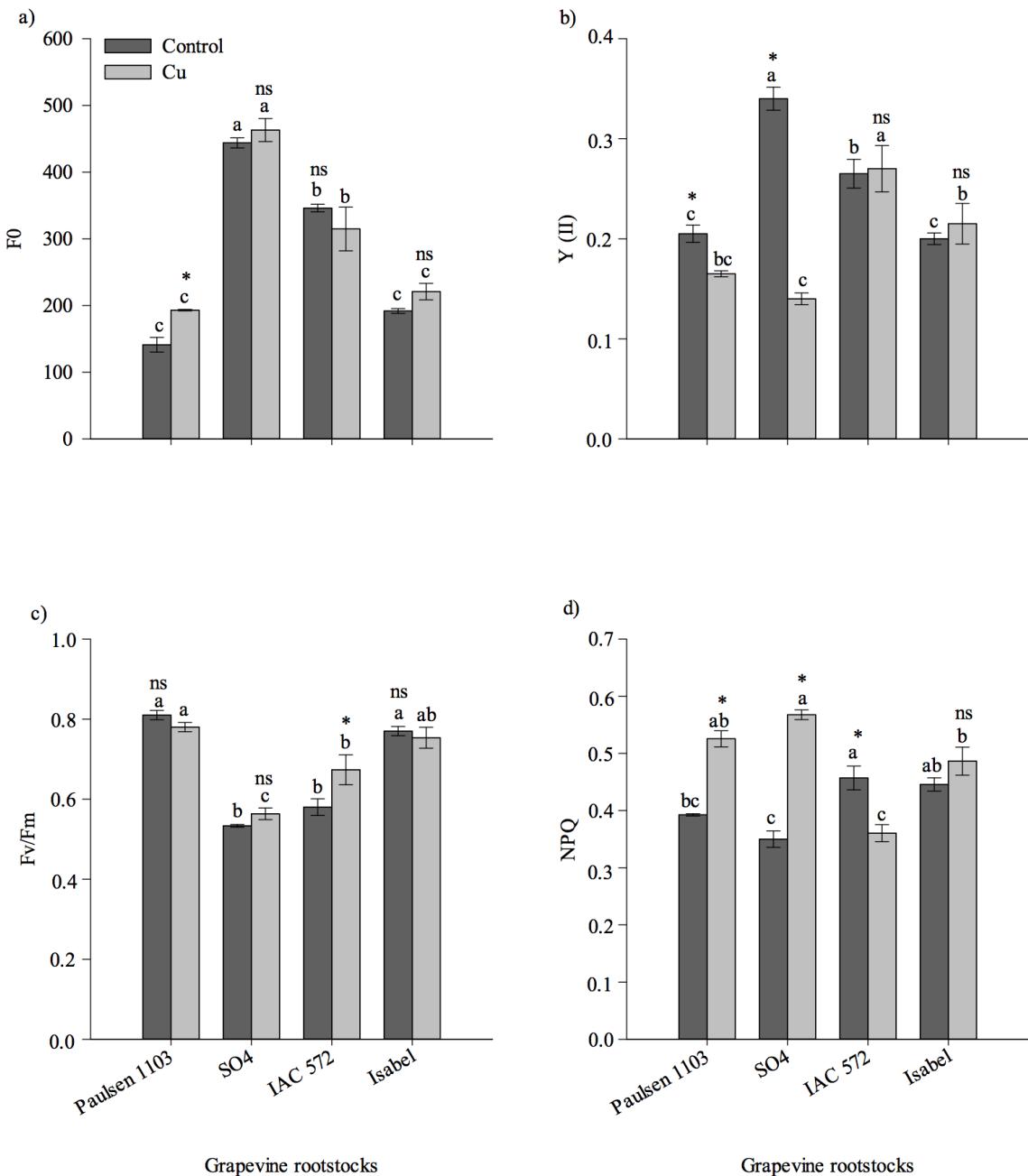


Figure 3. Initial fluorescence (F_0), effective quantum efficiency of PSII ($Y(II)$) (b), maximum PSII quantum yield (F_v/F_m) (c), and non-photochemical quenching (NPQ) (d), in leaves of grapevine rootstocks (Paulsen 1103, Magnolia, SO4, IAC 572 and Isabel) grown in standard nutrition (control) and high Cu content solution. Means followed by the same letter represent the comparison to rootstocks in each treatment through Tukey test at 5% ($P<0.05$). ns non-significant; *significant (F test $p <0.05$) difference between Cu levels in the same rootstock.

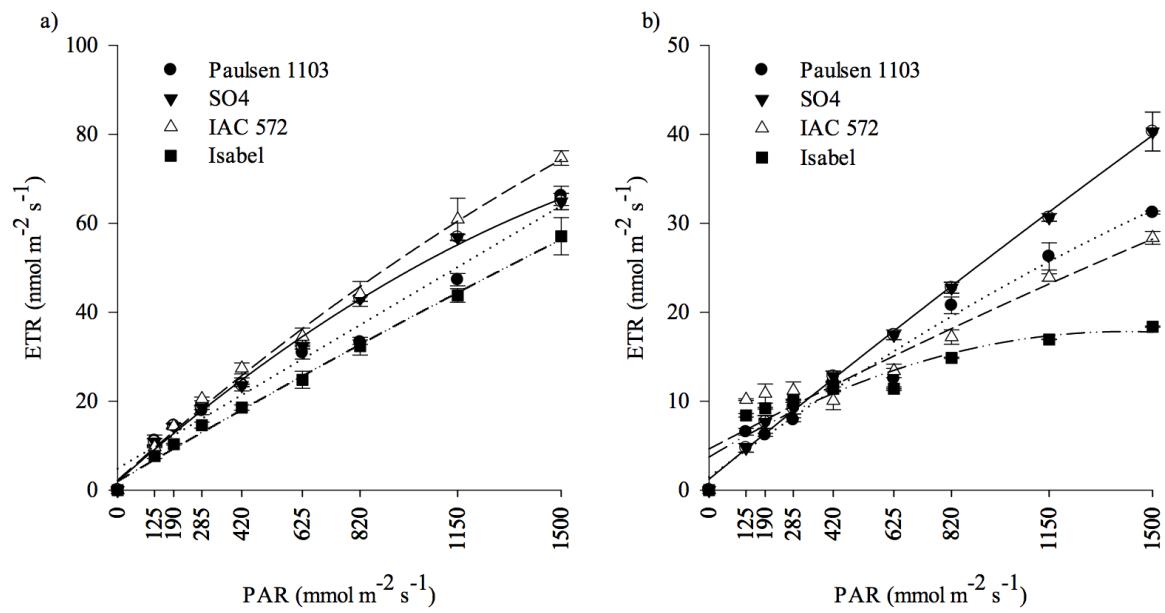


Figure 4. Electron transport rate (ETR) in leaves of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition (control) (a) and high Cu content solution (b).

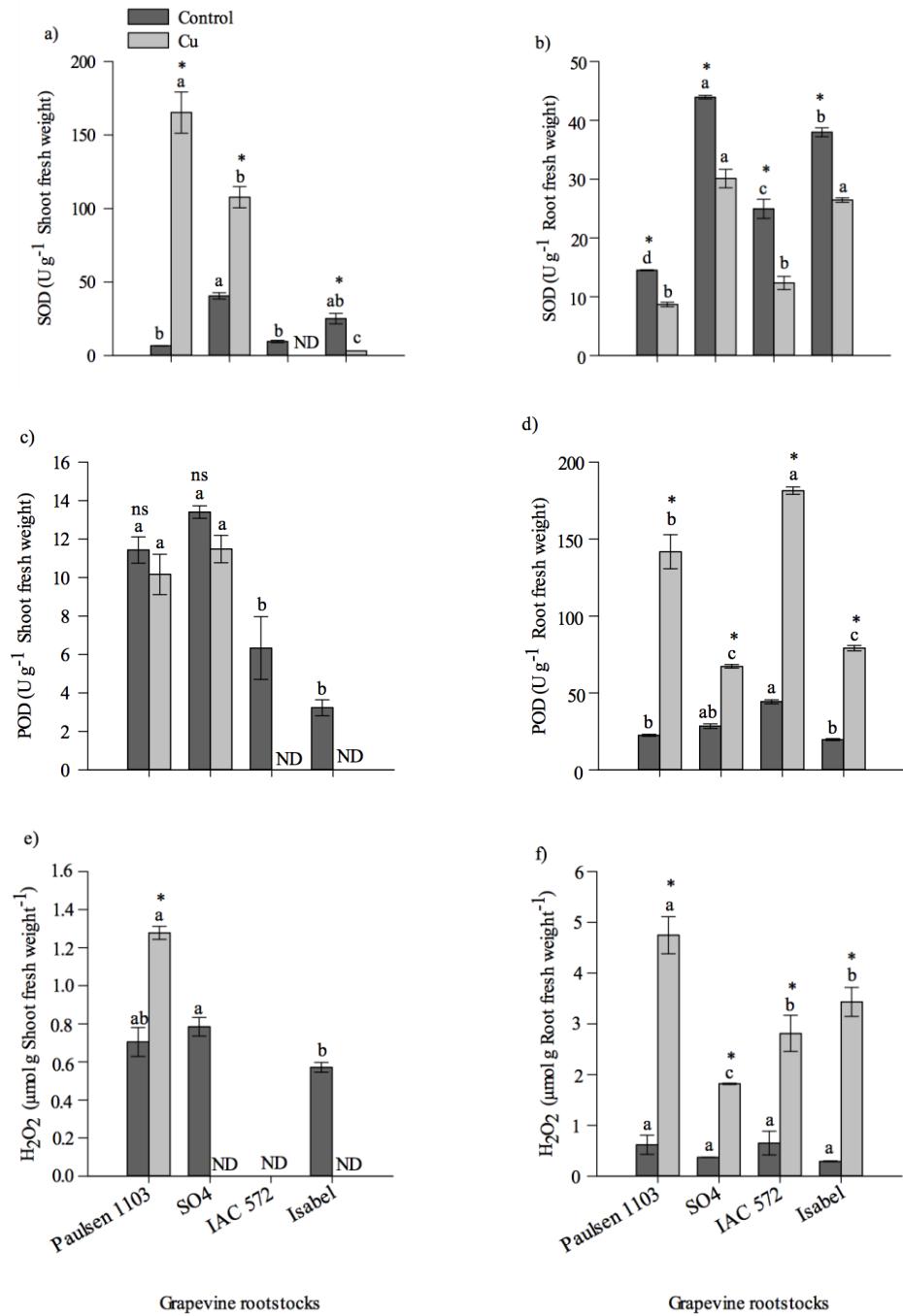


Figure 5. Activity of the enzyme superoxide dismutase (SOD) in the shoot (a) and roots (b); peroxidase (POD) in the shoot (c) and roots (d); and H₂O₂ concentration in the shoot (e) and roots (f) of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition and high Cu content solution. Means followed by the same letter between grapevine rootstocks subjected to each treatment did not differ from each other in the Tukey test at 5% (P<0.05). *non-significant; * significant (t test p < 0.05) difference between treatments, with and without Cu addition, in the same grapevine rootstock. ND: data is not available.

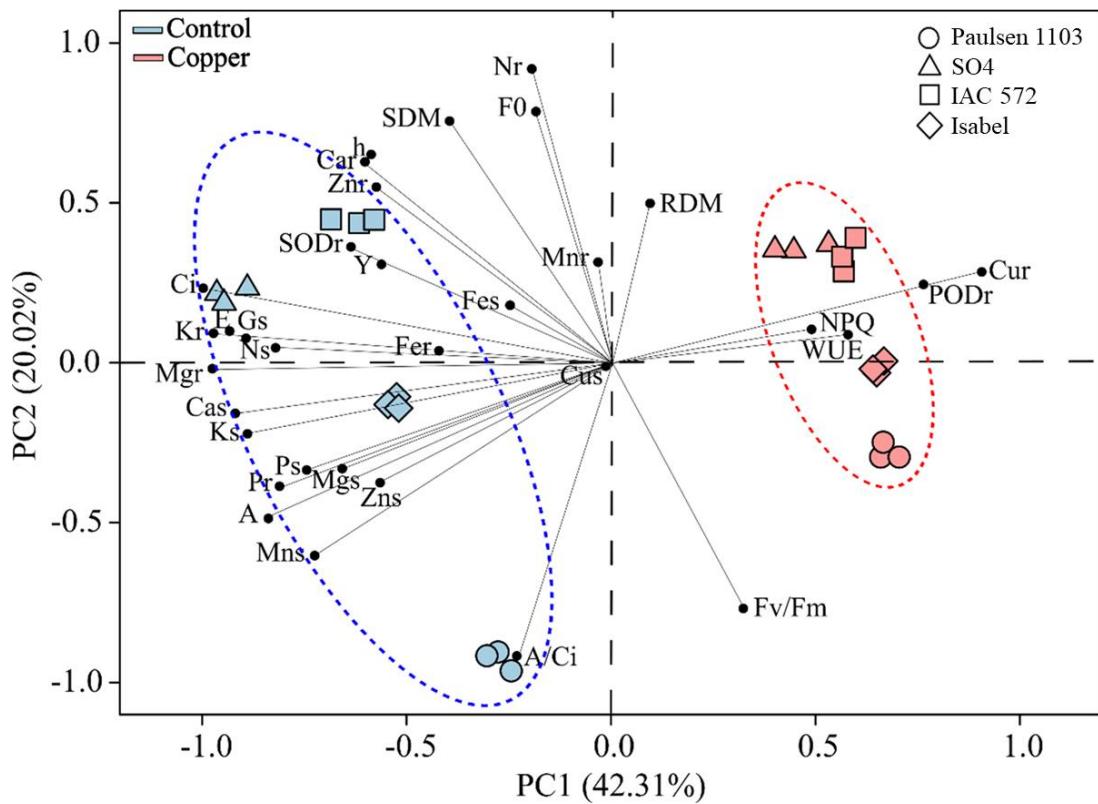


Figure 6. Principal component analysis (PCA), based on photosynthetic parameters [net photosynthetic rate (A), stomatal conductance (Gs), intercellular CO₂ concentration (Ci), transpiration rate (E), water use efficiency (WUE) and instantaneous efficiency of carboxylation (A/Ci)], on chlorophyll *a* fluorescence parameters [Initial fluorescence (F₀), effective quantum efficiency of PSII (Y), maximum PSII quantum yield (F_v/F_m) and non-photochemical quenching (NPQ)], on N, P, K, Ca, Mg, Cu, Zn, Fe and Mn concentration in tissues [shoot(s) and root(s)], oxidative stress parameters (SOD and PDI in roots), on plant growth parameters [root dry mass (RDM) and shoot (SDM); and plant height (h)] in four grapevine rootstocks [Paulsen 1103 (circle), SO4 (triangle), IAC 572 (square) and Isabel (rhombus)] grown in standard nutrition (control) (blue) and high Cu content solution (red).

5.4 ESTUDO IV

The tolerance of grapevine rootstocks to copper excess and to the use of calcium and phosphorus to mitigate its phytotoxicity⁴

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Abstract

High soil copper (Cu) concentrations in vineyards can cause phytotoxicity to grapevine rootstocks. In order to mitigate toxicity, the use of grapevine rootstock genetic variation and the application of amendments such as phosphorus (P) and calcium (Ca) are possible strategies. The aim of the present study is to assess the tolerance of grapevine rootstocks to Cu excess and whether P and/or Ca can reduce phytotoxicity caused by Cu. The experiment was conducted in greenhouse, with plants exposed to high Cu concentration (60 µM Cu) based on its occurrence and phytotoxicity in the field. Grapevine rootstock seedlings were produced from selected stakes: Paulsen 1103 (*Vitis berlandieri* x *Vitis rupestris*); SO4 (*Vitis berlandieri* x *Vitis riparia*); IAC 572 ((*Vitis Riparia* x *Vitis rupestris*) x *Vitis caribaea*); and Isabel (*Vitis labrusca*). Seedlings were grown in nutrition solution added with the following treatments: 0.3 µM Cu (Control); 60 µM Cu; 60 µM Cu and 62 mg L⁻¹ P; 60 µM Cu and 400 mg L⁻¹ Ca, for 21 days. High Cu concentration caused phytotoxicity in all rootstocks, impairing their growth and decreasing both nutrient concentration and photosynthetic activity. Among the tested rootstocks, Isabel was the most sensitive to Cu, presenting leaf wilting and yellowing. Roots accumulated the highest Cu concentration, even when P and Ca were applied as Cu amendment. Nevertheless, P and Ca addition had positive effect on the photosynthetic activity of all rootstocks 21 days after cultivation, although it was not enough to revert growth to levels comparable with controls. Ca application also favored root system growth in SO4 and IAC 572, as well as photosynthetic pigments in Paulsen 1103, IAC 572 and Isabel. Overall, based on the results, rootstocks Paulsen 1103 and SO4 can be more tolerant to phytotoxicity caused by Cu, whereas Isabel was the most sensitive. The

⁴Artigo elaborado de acordo com as normas de formatação da revista Chemosphere

application of P and Ca was not efficient in mitigating Cu phytotoxicity in grapevine plants grown in solution.

Keywords: Heavy metal. Amendments. Photosynthetic activity. Culture strategy. Contaminated vineyards.

1. Introduction

Copper (Cu) is an important micronutrient for plant growth and development (Kabata-Pendias, 2011); however, accumulation in plant tissue can cause phytotoxicity (Kabata-Pendias, 2011; Marschner et al., 2012; Miotto et al., 2014; Tiecher et al., 2016; 2017; 2018; De Conti et al., 2018). Plants can trigger mechanisms to control Cu homeostasis when they are exposed to Cu excess, such as lowering Cu absorption by modulating expression of membrane transporters; accumulation of excessive Cu in the root system linked to the cell wall or storage in vacuoles, reducing metal translocation to plants' shoots (Yruela, 2005; 2009; Adrees et al., 2015). Whenever plants are exposed to Cu excess, they activate the antioxidant defense system (Adrees et al., 2015; Marschner et al., 2012); however, response to Cu excess can vary depending on genotype and/or species, Cu concentration and exposure time (Yruela, 2003; 2009; Fidalgo et al., 2013; Marastoni et al., 2019a).

Thus, the use of grapevine rootstocks more tolerant to high Cu concentration in the soil can be a strategy in contaminated vineyards. Rootstocks are often selected based on the match between genetic materials (rootstock and production variety), on propagation ability, plant vigor, effect on fruits' photochemical features, tolerance to pests and diseases, adjustment to soil and climate features (Ollat et al., 2016; Warschefsky et al., 2016). Because rootstocks are selected from species *Vitis* and hybrids, they can present different growth, rooting, tolerance to environmental stress behaviors, as well as other physiological features (Pongrácz 1983, Christensen 2003; Holland et al., 2018). However, information about grapevine rootstocks' genetic variation for tolerance to Cu excess remains scarce.

In addition, the use of chemicals that have amendment effect, i.e., that mitigate Cu availability in the soil and that favor plant growth and development, can help culture management in contaminated sites. Some strategies have been assessed in order to find ways to reduce toxicity caused by Cu, including the use of chemical substances that bind to Cu to reduce its availability for plant absorption (Pietrzak and Uren, 2011; Mackie et al., 2012; Ferreira et al., 2018; Baldi et al., 2018a; Trentin et al., 2019; De Conti et al., 2019). Among them, one finds phosphorus (P) (Baldi et al., 2018; 2018b; 2018c) and calcium (Ca) (Chen et

al., 2013; Min et al., 2013; Hippler et al., 2018). However, the positive effect of P and Ca on the mitigation of phytotoxicity caused by Cu in different grapevine rootstocks remains poorly understood.

Phosphorus (P) immobilizes heavy metals in the soil due to its ability to form stable molecules (Cao et al., 2002; Miretzky and Fernandez-Cirelli, 2008; Baldi et al., 2018b); its action can take place through ion exchange, sorption and surface complexation and precipitation (Theodoratos et al., 2002; Cao et al., 2004; Liu and Zhao, 2007; Baldi et al., 2018b). Besides, P addition helps nurturing plants and favors their growth (Marschner et al., 2012); moreover, it is essential for enzyme activation, synthesis and membrane stability, photosynthesis, respiration and for the metabolism of carbohydrates (Taiz and Zeiger, 2013; Marschner et al., 2012). Phosphorus (P) addition also minimizes the toxicity effect caused by Cu in plants by improving plant nutrition status and favoring its growth, and the preservation of photosynthetic apparatus efficiency (Baldi et al., 2018; 2018b; 2018c).

Calcium (Ca) addition can also decrease phytotoxicity caused by Cu and favor plant growth (Chen et al., 2013). This macronutrient is part of the cell wall middle lamella, and is important for cell stability and integrity cell elongation and division, as well as osmoregulation, as secondary cell messenger in metabolic regulation, as enzymatic cofactor in the hydrolysis of ATP and phospholipids (Taiz and Zeiger, 2013; Marschner et al., 2012). Higher Ca level can act as amendment by increasing cell wall thickness, reducing thickening effects and shortening the root system due to toxicity caused by Cu under high availability; moreover, it can lead to greater Cu accumulation in the root system and to lower translocation to the shoot, which reduces the toxicity symptoms (Chen et al., 2013).

Thus, the use of rootstocks with the potential to tolerate Cu excess, as well as P and Ca application to mitigate high Cu concentration, can have positive effect on the reduction of phototoxicity symptoms and, consequently, it can be a strategy in sites presenting Cu excess. The aim of the present study was to assess the tolerance of grapevine rootstocks used in Southern Brazil to Cu excess and the effectiveness of P and Ca application to mitigate phytotoxicity caused by it.

2. Materials and Methods

2.1 Plant material and growth conditions

The experiment was conducted in a greenhouse at the Department of Soil Science of Federal University of Santa Maria (UFSM). Grapevine rootstock stakes were placed in substrate for 3 months to induce budding and seedling formation. The selected stakes were:

Paulsen 1103 (*Vitis berlandieri* x *Vitis rupestris*); SO4 (*Vitis berlandieri* x *Vitis riparia*); IAC 572 ((*Vitis Riparia* x *Vitis rupestris*) x *Vitis caribaea*) and Isabel (*Vitis labrusca*). Isabel is commonly used as a canopy grafted onto rootstocks, but can also be planted directly in soil. Plants were transferred to 1-L pots added with Hoagland & Arnon (1950) nutrition solution presenting 50% force, at pH 5.5. Standard nutrition solution presented the follow composition (mg L⁻¹): N = 85.31, P = 7.515, K = 104.75, Ca = 97.64, Mg = 23.68, S = 11.54, Fe = 2.68, Cu = 0.03, Zn = 0.13, Mn = 0.11, B = 0.27, Mo = 0.05, Ni = 0.01. The nutrition solution was continuously aerated and replaced every 3 days. Each pot had one plant, which was fixed with Styrofoam. The cultivated plants were allowed to acclimate in standard nutrient solution for 10 days.

Plants were grown for 21 days under different solution conditions after the acclimation period was over. The adopted treatments were 0.3 µM Cu (Control), 60 µM Cu, 60 µM Cu and 62 mg L⁻¹ P, 60 µM Cu and 400 mg L⁻¹ Ca. Phosphorus (Cu) concentrations were chosen based on previous experiments (data not shown) by Soares et al. (2006), who used P to soften phytotoxicity caused by Zn. The selected Ca dose met that used by Chen et al. (2013): applying the double of the used concentration due to the higher Cu concentration adopted in the present study. Phosphorus was added with CuSO₄.5H₂O; P, with NH₄H₂PO₄; and Ca, with Ca(NO₃)₂.4H₂O. The experiment followed a completely randomized design, with four replicates per treatment.

2.2 Height, dry mass yield and nutrient concentration in tissue

Shoot height was measured at the beginning and at the end of the experiment, with the aid of measuring tape. Relative growth rate (RGR) was calculated based on the ratio between plant total fresh mass weight after (at the end of the experiment) and before treatment addition to the nutrition solution, at sampling period (21 days after cultivation). The following equation was used to calculate RGR (Equation 1):

$$RGR = (\text{Final plant fresh weight} / \text{Initial plant fresh weight})/\text{time} \quad \text{Equation 1}$$

Plant Cu absorption was calculated based on the bioaccumulation factor (BAF) (Equation 2), which is defined as the concentration of an element in a plant due to its concentration in solution. Cu translocation in plants was calculated based on the translocation index (TI) (Equation 3), which took in consideration Cu concentration in plant shoots in comparison to its concentration in the roots.

$$\text{BAF} = \text{Metal shoot} / \text{Metal soil solution} \quad \text{Equation 2}$$

$$\text{TI} = \text{Metal shoot} / \text{Metal root} \quad \text{Equation 3}$$

Plants were collected 21 days after cultivation; shoot and roots were separated and samples were dried in forced air-circulation oven at 65°C until constant weight. Subsequently, root and shoot dry masses were measured, samples were ground in Wiley mill and prepared for chemical analysis.

Samples were prepared and subjected to nitric-perchloric digestion (Embrapa, 2009). Calcium (Ca), magnesium (Mg), manganese (Mn), Cu, zinc (Zn) and iron (Fe) concentrations in the extract were determined in atomic absorption spectrophotometer (AAS, Varian SpectrAA-600, Australia). Phosphorus (P) concentration was determined based on the methodology by Murphy & Riley (1962) through colorimetry carried out in spectrophotometer (SF325NM, Bel Engineering, Italy). Potassium (K) concentration was determined in flame photometer (DM62, Digimed, Brazil).

2.3 Photosynthetic activity

The photosynthetic activity was measured in infra-red gas analyzer (Li-6400, Li-COR Inc., Neb., USA) 7 and 21 days after treatment addition; the analysis was applied to leaves located in the upper middle third of the shoot. The following parameters were determined: net photosynthetic rate (A), internal CO₂ concentration (Ci), transpiration rate (E), stomatal conductance to CO₂ (Gs), water use efficiency (WUE) and instantaneous carboxylation efficiency (A/Ci) through Ribulose-1,5-bisphosphate carboxylase/oxygenase. Measurements were taken in leaf chamber, at CO₂ concentration of 400 µmol mol⁻¹, temperature of 20/25 °C, relative humidity of 50 ± 5% and photon flux density of 1,000 µmol m² s⁻¹. Net photosynthetic rate (A), instantaneous carboxylation efficiency (Ci), transpiration rate (E) and stomatal conductance to CO₂ (Gs) were calculated based on Von Caemmerer and Farquhar (1981).

2.4 Photosynthetic pigment content

Four leaves were collected in the upper third of the shoot and immediately placed in liquid N₂ and stored in -80°C, at harvest. Samples were macerated in liquid N₂, and 0.05 g of tissue were weighed and incubated in dimethylsulfoxide (DMSO) at 65°C until the samples were completely bleached (Hiscox and Israelstam, 1979). Absorbance in supernatant extract was determined at 470, 645 and 663 nm in spectrophotometer. Chlorophyll a, b and total (a + b), and carotenoid concentrations were estimated based on the equation by Lichtenthaler (Lichtenthaler, 1987).

2.5 Statistical analysis

Results were subjected to analysis of variance in Sisvar software, version 4.0 (Ferreira, 2008). Means were separated through Scott-Knot test at 5% when the effect of the treatment was significant.

The multivariate principal component analysis was carried out to complete the analysis of variance; it was carried out in Canoco Software, version 4.5 (Ter Braak and Smilauer, 2002). The principal component analysis (PCA) was performed based on data about the concentration of elements P, K, Ca, Mg, Cu, Mn, and Zn in the roots and shoot, on photosynthetic parameters (net photosynthetic rate, internal CO₂ concentration, transpiration rate, stomatal conductance to CO₂, water use efficiency and instantaneous carboxylation efficiency), photosynthetic pigments (chlorophyll *a* and *b*, and carotenoids) and on plant growth parameters (root and shoot dry mass, plant height, and relative growth rate). The analysis (PCA) was carried out based on a set of principal components (1 and 2), which reflect a set of standardized orthogonal linear combinations that together explain data original variability.

3. Results

3.1 Plant growth parameters

All grapevine rootstocks grown in high Cu concentration solution, with and without amendments addition, presented lower height, shoot dry mass and relative growth rate (RGR) values than plants grown in control solution (Figure 1; 2 a, b, d). Figure 1 shows that all rootstocks presented considerable shoot and root system growth reduction due to Cu excess, as well as root darkening. Isabel clearly presented the highest sensitivity to Cu excess, which was expressed by leaf wilting and yellowing (Figure 1). The addition of Ca and P, as Cu amendments, did not present visual effect on the shoot growth of rootstocks Paulsen and SO4; on the other hand, Ca addition has favored the shoot growth of IAC and Isabel rootstocks (Figure 1). The SO4 rootstock showed the highest plant height and dry matter values of the shoot in control solution, with an increase of approximately 50% compared to plants grown in high Cu concentration (Figure 2a, b). Rootstocks Paulsen 1103 and IAC 572 presented greater reductions in plant height and shoot dry mass values when they were grown in high Cu concentration solution than the control plants: 66% and 68% plant height reduction and 75% and 69% shoot dry mass reduction, respectively (Figure 2a, b). Isabel showed the highest RGR values in control plants, but with reduction by 62% in plants grown in high Cu concentration solution (Figure 2d). The highest root dry mass values were observed for

rootstock Paulsen 1103 grown in control solution and for SO4 grown in control and Cu + Ca treatment, in comparison with the other rootstocks and treatments (Figure 2c). Rootstocks IAC 572 and Isabel did not present significant difference between treatments for variable root system dry mass (Figure 2c).

All rootstocks grown in solution added with Cu, with and without amendments, presented the lowest values for plant height and leaf number increase in comparison to plants grown in control solution (Table 1). Rootstocks Paulsen 1103, IAC 572 and Isabel grown in high Cu concentration solution did not present increased plant height value, and it was also observed for rootstocks SO4 and IAC 572 grown in Cu + P solution (Table 1). Rootstock IAC 572 grown in all treatments added with high Cu concentration, Paulsen 1103 grown in Cu + Ca solution and SO4 in Cu + P solution did not present an increased number of leaves per plant (Table 1).

3.2 Nutrient concentration in plant tissue

All rootstocks grown in solution with Cu excess presented higher Cu concentration in the shoot and roots when compared to the control (Figure 3e, f). Isabel plants grown in solution added with Cu + P recorded the highest Cu concentration in the shoot (Figure 3e). The highest Cu concentrations were observed in the root system of all rootstocks (approximately 99% of the total Cu in plants) (Table 2), mainly in solution presenting high Cu concentration without the addition of amendment substances (Figure 3f). Isabel presented the highest concentration of Cu in the roots when cultivated in solution with high Cu concentration, which may indicate that this genotype is more sensitive. On the other hand, rootstocks SO4, IAC 572 and Isabel showed lower Cu values in the root system of plants grown in solution added with Cu + Ca and Cu + P than plants grown in solution only added with high Cu concentration. There was slight increase in P concentration in the shoot of rootstocks SO4, IAC 572 and Isabel grown in solution added with Cu + P (Figure 3). Isabel recorded the highest P concentration in the shoots and roots when it was grown in solution added with Cu + P (Figures 3a, b). However, Paulsen 1103, SO4 and IAC 572 presented the highest P concentration in roots grown in control solution (Figure 3b). The highest Ca concentration was observed in the shoot of Paulsen 1103 grown in solution added with Cu + Ca (Figure 3c). All rootstocks presented the highest Ca concentration in the root system when they were grown in solution added with Cu + Ca (Figure 3d). P and Ca concentration partitioning between shoot and root ranged from 40% to 60% (Table 2).

Copper translocation index in plants was extremely low, mainly in rootstocks grown in solution with high Cu concentration, with and without amendment addition (Table 3). Bioaccumulation rate (BAF) was high in all rootstocks grown in control solution because of the low Cu concentration in the nutrition solution (Table 3). Lower BAF values were observed in all rootstocks grown in high Cu concentration solution than in plants subjected to treatments with amendments (Table 3).

3.3 Photosynthetic activity

Photosynthetic parameter analyses were carried out 7 and 21 days after the addition of the treatments in solution. There was significant reduction in the liquid photosynthetic rate (A) and efficient instantaneous carboxylation (A/Ci) in all rootstocks subjected to high Cu concentration when compared to the control, 7 days after treatment addition to the solution (Figure 4a, e). Rootstocks grown in control solution presented the highest A and A/Ci values, they were followed by the ones grown in Cu + Ca solution (Figures 4a, e). Paulsen 1103 plants showed the highest A and A/Ci values, mainly in control solution (Figure 4a). Higher stomatal conductance (Gs) and transpiration rate (E) values were observed in rootstocks SO4, Paulsen 1103 and Isabel grown in control solution, mainly in SO4, which presented the highest Gs and E values (Figure 4b, d). The highest intracellular CO₂ concentrations were observed for rootstock SO4 in control solution; in Isabel grown in high Cu concentration solution and Cu + Ca solution; and, in SO4, IAC 572 and Isabel grown in Cu + P solution (Figure 4c). The highest water use efficiency values (WUE) were observed for rootstock Paulsen 1103 grown in control solution and in high Cu concentration solution (Figure 4f). Rootstocks Paulsen 1103 and IAC 572 presented the highest WUE values in Cu + Ca solution (Figure 4f).

At 21 days after experiment installation, the highest A values were observed for rootstock Paulsen 1103 grown in control solution and for IAC 572 grown in Cu + Ca solution (Figure 5a). Rootstocks Paulsen 1103 and SO4 presented the highest A values in control solution, whereas IAC 572 and Isabel recorded the highest A values in Cu + Ca solution (Figure 5a). The highest Gs values were observed in rootstock SO4 grown in control solution; it was followed by Paulsen 1103 and Isabel, also in control solution (Figure 5b). Rootstocks Paulsen 1103, SO4 and IAC 572 recorded the highest internal CO₂ concentration value in control solution; on the other hand, Isabel showed the highest values of this variable in plants grown in Cu + P solution (Figure 5c). The highest E values were observed for rootstocks

Paulsen 1103 and SO4 in control solution (Figure 5d). Rootstock IAC 572 showed the highest A/Ci and WUE values; they were followed by Isabel, both in Cu + Ca solution (Figure 5e, f).

3.4 Photosynthetic pigment content

The highest chlorophyll *a* values were observed for Isabel plants grown in solution added with high Cu concentration, for Isabel and SO4 grown in Cu + P solution and for Isabel and Paulsen 1103 plants grown in Cu + Ca solution (Figure 6a). Isabel presented the highest chlorophyll *b* values in high Cu concentration solution and with Cu + P; IAC 572 and SO4, in control solution; Paulsen 1103, SO4 and Isabel, in Cu + Ca solution (Figure 6b). The highest total chlorophyll values were observed for Isabel grown in high Cu concentration solution, for Isabel and SO4 grown in Cu + P solution; for Paulsen 1103, SO4 and Isabel grown in Cu + Ca solution (Figure 6c). Isabel presented the highest values of carotenoids in plant tissue when it was grown in high Cu concentration solution added with Cu + P and with Cu + Ca (Figure 6d).

3.5 Principal component analysis

The principal component analysis (PCA) was carried out by only extracting the two first components (PC1 and PC2), which together explained almost 50% original data variability (Figure 7). Principal component 1 (PC1) explained 32% variability and recorded the strongest influence on plant height, shoot dry mass, K, Mg, and Cu concentration in the roots, and on the photosynthetic rate. The PC1 was efficient in separating treatments into two great groups: the group to the right (opaque green ellipse), where the control treatments applied to all rootstocks were grouped in, besides grapevines SO4 grown in high Cu content and Ca (as amendment) (Cu + Ca); and the group to the left (opaque red ellipse), which comprised the combinations of all rootstocks subjected to Cu, Cu + P, and to Cu + Ca, except for SO4. Principal component 2 (PC2) explained approximately 16% data variation; response variables that most influenced it were chlorophyll *b* and carotenoid concentrations. The PC2 was efficient in comparing rootstocks; Paulsen 1103 prevailed in the group placed above the PC2 axis and Isabel prevailed in the group below it. Rootstocks SO4 and IAC572 occupied different portions on the PC2 axis depending on Cu availability, which has featured the similarity among materials. Copper (Cu) concentration in the roots and shoot were autocorrelated to each other. These variables were negatively correlated to the photosynthetic rate, which was positively correlated to the instantaneous efficiency of Rubisco carboxylation. The Zn concentration was correlated with Ci. The concentration of carotenoids and

chlorophyll b correlated positively with each other. The concentrations of Ca, Zn, and P in roots were autocorrelated to each other. The Gs and E were autocorrelated. Shoot dry mass was positively correlated to plant height. The relative growth rate was positively correlated to K, Mg, and Mn concentration in the roots, as well as to root dry mass. Stomatal conductance was positively correlated to the transpiration rate.

4. Discussion

The reduction observed in shoot and root dry mass, plant height, leaf number per plant and relative growth rate (RGR) of all rootstocks in solution with high Cu concentration and amendments, when compared to the control can be explained by the phytotoxic effect caused by Cu excess in plants, hindering plants growth. This effect was also observed by Baldi et al. (2018a; 2018b) in young grapevines grown in soil with Cu concentration higher than 200 mg kg⁻¹, which showed growth reduction. High Cu concentration in plant tissue can cause morphological changes in shoot and roots; thus, it hinders cell elongation and differentiation processes, as well as reduces the assimilation of carbon (Adrees et al., 2015; Baldi et al., 2018b). SO4 showed the highest plant height and shoot dry mass values in control solution and the lowest reduction in comparison to the other rootstocks (approximately 50%) when high Cu concentration was added. On the other hand, Isabel presented the highest RGR values in control solution, but also the highest reduction in comparison to the other rootstocks (by 62%) due to the addition of a high Cu concentration. The effect observed in rootstocks can be explained by differences in each genetic material, since they are selected from *Vitis* species and hybrids with different plant vigor, rooting ability, water and nutrient absorption efficiency, nutrition status, photosynthetic efficiency and carbon assimilation, and tolerance to environmental stress (Pongrácz 1983, Christensen 2003; Holland et al., 2018). Despite the ability to trigger defense mechanisms against excessive Cu availability, such as accumulation of the excessive metal in the root system linked to cell wall or storage in the vacuole, regulation of transporters for Cu absorption and translocation in plants, exudation of organic compounds (such as phenolic compounds, organic acids and phytosiderophores) that can complex Cu in the rhizosphere region and the activation of antioxidant enzymes (Yruela, 2005; 2009; Marschner et al., 2012; Adrees et al., 2015; Brunetto et al., 2016).

The addition of Ca and P to Cu-excess solution as amendments did not have significant effect on growth parameters, mainly on plants height, shoots dry matter and RGR, in the different rootstocks of grapevines in nutritive solution. Different from what was observed by Baldi et al. (2018a, b; c) in grapevines cultivated in soil presenting high Cu

concentration with P application as amendment, which found a beneficial effect of P addition on the efficiency of the photosynthetic apparatus, on nutrient acquisition and, consequently, on plant growth. Lack of response from P application in nutrition solution on phytotoxicity caused by Cu excess can be attributed to the high concentration of Cu in solution and the added P concentration was not enough to reduce Cu availability in the solution absorbed by plants, which, consequently, did not favor plant growth. This behavior was also observed by Soares et al. (2006) in solution with high Zn (225 µM) and P (2 mM) concentration - P did not influence Zn availability in the solution; thus, responses from the mitigation of phytotoxicity caused by Zn were of nutrition and biochemical order in *Trema micrantha* (L.) BLUM. plants.

On the other hand, Ca application had positive effect on the root system of rootstocks SO4 and IAC 572 in comparison to the treatment with high Cu concentration in nutrition solution. This outcome can be explained by the beneficial effect of Ca on root system structure, such as increased cell wall thickness, and by the mitigation of root thickening and root shortening due to toxicity caused by Cu (Chen et al., 2013). Reduced toxicity effect caused by Cu excess in grapevines due to the application of limestone (composed of a CaCO₃ and MgCO₃ mixture, at Ca:Mg ratio of 2:1) was observed by Ambrosini et al. (2015) with the reduction of Cu available in solution and the increase in absorbed Ca and Mg concentrations, so this process has favored the root system structure in plants. Trentin et al. (2019) also observed the beneficial effect of limestone application on soil presenting high Cu concentration, since it reduces the toxicity effect on young grapevines.

The highest Cu concentration in all rootstocks was observed in the root system, and the highest values of it were shown by plants grown in high Cu concentration solution. This outcome can be explained by the greatest Cu availability in solution for plant absorption, as well as by the fact that there was Cu accumulation in roots linked to cell wall and maybe to storage in vacuole in order to reduce Cu translation to the shoot, which is an organ more sensitive to metal excess (Kabata-Pendias, 2011; Ambrosini et al., 2015; Guimarães et al., 2016; Baldi et al., 2018a). Plants presented clear changes in the root system, such as darkening, apex thickening and larger number of secondary roots due to Cu excess (Ambrosini et al., 2015; Guimarães et al., 2016). Copper (Cu) accumulation in the root system also explained the lower Cu concentration in the shoot, despite the Cu addition to the nutrition solution (Baldi et al., 2018a; 2018b). Despite the lowest Cu concentration in the shoot, losses in plant growth can be related to the indirect effects of high Cu concentration in the solution and in root system on the absorption and translocation of other nutrients. High Cu

concentration in the solution can influence ions' homeostasis in plants through antagonism and/or synergism for nutrient absorption and translocation, for example, Mn, Fe, Zn, Ca and P (Kopittke and Menzies, 2006; Kopittke et al., 2011; De Conti et al., 2018; Marastoni et al., 2019).

Rootstocks grown in solution with high Cu concentration + P did not present increased P content in the shoot and root system, except for Isabel. This behavior can be related to the high concentration of Cu and the P dose used in the experiment (62 mg L^{-1}), since Baldi et al., (2018a) observed that P concentrations in the tissue of grapevines decreased due to high Cu concentrations in solution; however, P addition (100 mg kg^{-1}) to the soil increased P concentration in grapevine tissue. The concentrations of Ca in the root system of all rootstocks were higher than that in solution added with Cu + Ca; this outcome may be explained by the greater Ca addition to the solution and by the retention of Ca in membrane structure and in cell wall (Chen et al., 2013; Ambrosini et al., 2015).

Copper (Cu) excess can change the structure and functioning of the photosynthetic apparatus in plants, hindering photosynthetic activity (Baldi et al., 2018b; Cambrollé et al., 2013; 2015). Parameters related to photosynthesis, especially the net photosynthetic rate (A), stomatal conductance (Gs), transpiration rate (E) and instantaneous efficiency of carboxylation (A/Ci), increased 21 days after Cu treatment when compared to 7 days after it (Figures 3 and 4). The lowest A and A/Ci values were observed for rootstocks grown in solution added with Cu in comparison to plants in control conditions. This outcome can be explained by the toxicity effect caused by Cu excess on the tissue (Cambrollé et al., 2013; 2015; Tiecher et al., 2018). Paulsen 1103 presented the higher A and A/Ci values in control solution than the other cultivated rootstocks, as well as presented lower Gs values, which have favored WUE increased in these plants. Similar results were described by Cambrollé et al. (2015) for grapevines grown under high Cu levels (23mM), which have presented a considerable decline in A and Gs due to effects caused by metal excess in the photosynthetic apparatus. After 21 days of Cu treatment, it was possible to verify the effect of the amendments on Cu toxicity, especially Ca for the SO₄, IAC 572 and Isabel rootstocks and P for the Paulsen 1103 and IAC 572 rootstocks. Although plants have presented higher A and A/Ci values when they were grown in solution added with Cu + P and Cu + Ca than in the treatment with only Cu addition to the solution, there was no increase in P concentration in the shoot and root, as well as in plant growth.

Phosphorus (P) addition can help to mitigate the inhibition of the photosynthetic activity caused by metal excess due to binding and formation of stable molecules with Cu in

solution, decreasing the absorption and translocation of Cu to shoots (Cao et al., 2002; Miretzky and Fernandez-Cirelli, 2008; Baldi et al., 2018a; 2018b; 2018c). Moreover, the addition of P favors the photosynthetic process and mineral nutrition of plants (Marschner et al., 2012; Baldi et al., 2018b). On the other hand, plants grown in solution added with Cu + Ca may have had their photosynthetic efficiency favored due to the greater accumulation of Cu in the root system and less translocation to the shoots provided by the increase of Ca in the plants, which favors the increase in cell wall thickness, reducing the thickening effects and shortening the root system due to the toxicity caused by Cu (Chen et al., 2013). In addition, Ca is an important element for the photosynthesis process, structural component of photosystem II and the regulation of stomata opening and closing process (Hochmal et al., 2016), contribute on plants' nutrition status and play an important role in the maintenance of membrane and cell wall integrity (Ambrosini et al., 2015). These results corroborate the ones reported by Hippler et al. (2018), who observed increased effects of toxicity caused by Cu excess on the photosynthetic activity of Swingle citrumelo seedlings grown under low Ca availability in nutrition solution.

Calcium (Ca) addition did not favor plant growth under high Cu concentration conditions, but it stimulated the synthesis of photosynthetic pigment, chlorophyll *a*, total chlorophyll and carotenoids in rootstocks Paulsen 1103, IAC 572 and Isabel. This outcome can be related to Ca effect on pigment degradation reduction (Min et al., 2013). Isabel also presented increased pigment synthesis in all solution added with high Cu concentration, and it can be related to the increased pigment concentration for shoot unit, due to the smaller leaf expansion caused by P shortage induced by Cu excess in plants (Marschner et al., 2012; Valentimuzzi et al., 2015b).

From the principal component analysis, we can observe the formation of two distinct groups (Figure 7). These groups were separated based on the multivariate correlation between rootstocks, treatments, and response variables. Group 1, to the right of the PC1 axis, was mainly formed by all rootstocks cultivated under the control condition (dashed green ellipse). Thus, group 1 was associated with positive response variables, such as those related to photosynthetic parameters, which directly regulate the grapevine's growth, such as plant height, shoot, and roots dry matter. But also, for SO4 grapevines grown high Cu content by using Ca as amendment (Cu + Ca) (part of dashed orange ellipse). These grapevines showed higher concentrations of Ca, Zn, and P in roots. This may indicate a possible interaction between these elements, from the formation of calcium and zinc phosphates, which may have favored a lower Cu absorption by the vines. In this way, the SO4 grapevines, even grown in

an environment with excess copper, managed to present good values of chlorophyll a, which indicates, good functioning of the photosynthetic apparatus. Group 2 formed to the left of PC1 (red, blue, and orange dashed ellipses), was composed of all grapevines rootstocks grown in copper excess, excess of copper with P and Ca, except SO4 (Cu + Ca). In this case, the beneficial effect of the amendments was very small to copper excess. Being that, phosphorus addition (Cu + P) as an amendment was very similar to the effect of copper excess without any amendment (Cu) for all rootstocks. Even belonging to this group, the addition of calcium as an amendment, provided slight improvements to the grapevines environment in copper excess. One of these reasons may be linked to the Ca increase in the shoot, conferring greater rigidity to the cell wall. The PCA was not as efficient in separating the rootstocks by their efficiency. Even so, from this analysis, we can see that Paulsen 1103 rootstocks tended to support the copper excess better than the other rootstocks tested. On the other hand, the Isabel genetic material proved to be highly sensitive to high copper concentrations, and its cultivation is not indicated in regions with a long history of cupric fungicides application.

5. Conclusions

High Cu concentrations in the solution impaired growth, nutrient uptake and photosynthetic activity of all grapevine rootstocks. Calcium (Ca) and phosphorus (P) addition had positive effect on the photosynthetic activity of all rootstocks in comparison to the solution with high Cu concentration. However, it was not enough to revert growth to control levels. Calcium (Ca) application as amendment also favored root system growth in rootstocks SO4 and IAC 572, as well as the synthesis of photosynthesizing pigments in rootstocks Paulsen 1103, IAC 572 and Isabel. However, based on an overall evaluation, the present results have suggested that rootstocks Paulsen 1103 and SO4 can be more tolerant to phytotoxicity caused by Cu, whereas Isabel is more sensitive. Moreover, P and Ca application was not efficient in mitigating phytotoxicity caused by high Cu concentration in grapevine plants growth in nutrition solution.

6. Contributions

Edicarla Trentin: study design, accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Letícia Morsch and Jacson Hindersmann: accomplishment of the experiment, laboratory analysis, interpretation of results and writing of the article. Camila Peligrinotti Tarouco and Fernando T. Nicoloso: study design, biochemical analysis in plants, photosynthetic analysis, accomplishment of the experiment and writing of

the article. Lincon Oliveira Stefanello, Lessandro De Conti, Isley Cristiellem Bicalho da Silva and Carina Marchezan: statistical analysis, interpretation of results and writing of the article. Paulo Ademar Avelar Ferreira, Felipe Klein Ricachenevsky, Carlos Alberto Cerretta and Gustavo Brunetto: study design, interpretation of results and writing of the article.

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Figure 1. Grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control), with high Cu content (Cu), higher Cu and P (Cu + P) and with higher Cu and Ca (Cu + Ca).

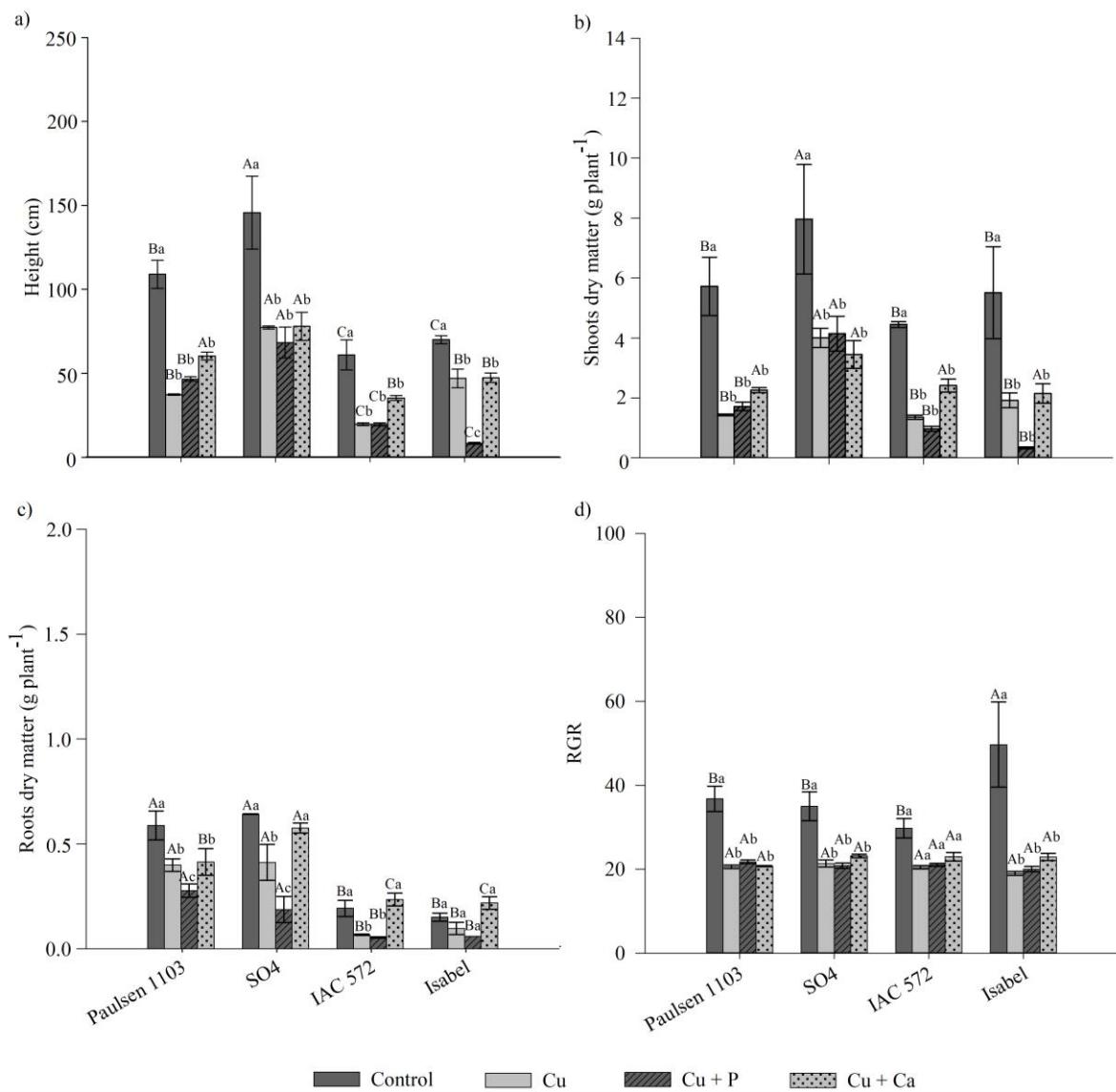


Figure 2. Height (a), shoot (b) and root (c) dry mass yield, and relative growth rate (RGR) (d) of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) and with higher Cu and Ca (Cu + Ca) content. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P<0.05$).

Table 1. Increment values of height and number of leaves of grapevines rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutritive solution (control), with high Cu content (Cu), higher Cu and P (Cu + P), and with higher Cu and Ca (Cu + Ca) during 21 days.

Rootstocks	Treatments	Height (cm)	Number of leaves
Paulsen 1103	Control	41.68 ± 9.43	28.25 ± 3.88
	Cu	0.00 ± 0.00 ⁽¹⁾	4.25 ± 0.63
	Cu + P	5.00 ± 1.78	5.00 ± 0.82
	Cu + Ca	8.00 ± 1.47	0.00 ± 0.00
SO4	Control	52.13 ± 14.55	19.50 ± 4.87
	Cu	14.25 ± 0.31	2.65 ± 0.85
	Cu + P	0.00 ± 0.00	0.00 ± 0.00
	Cu + Ca	18.00 ± 1.87	4.50 ± 0.20
IAC 572	Control	29.50 ± 6.09	16.00 ± 2.68
	Cu	0.00 ± 0.00	0.00 ± 0.00
	Cu + P	0.00 ± 0.00	0.00 ± 0.00
	Cu + Ca	21.00 ± 6.74	0.00 ± 0.00
Isabel	Control	42.50 ± 2.25	9.00 ± 0.82
	Cu	0.00 ± 0.00	4.65 ± 0.85
	Cu + P	2.50 ± 0.35	1.50 ± 0.20
	Cu + Ca	11.25 ± 0.31	3.00 ± 0.41

⁽¹⁾Means followed by zero represents no increment in these values during the time of experiment conduction.

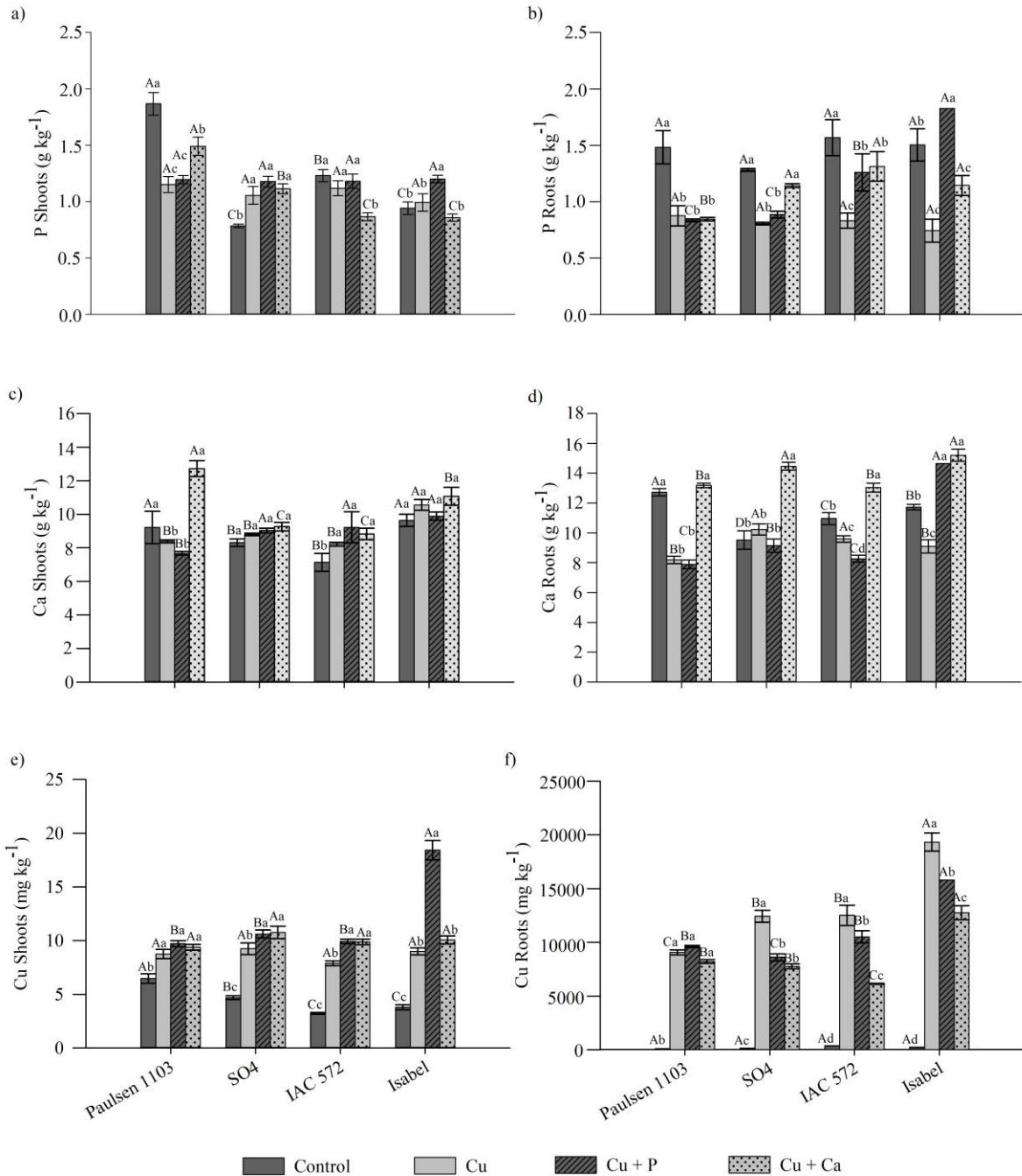


Figure 3. Phosphorus, Calcium and Copper concentration in the shoot (a, c, e) and roots (b, d, f), respectively, of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P<0.05$).

Table 2. Percentage of P, Ca and Cu in the shoot and roots of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control), with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content for 21 days.

Rootstocks	Treatments	P (%)		Ca (%)		Cu (%)	
		Shoots	Roots	Shoots	Roots	Shoots	Roots
Paulsen 1103	Control	55.72	44.28	42.00	58.00	8.25	91.75
	Cu	56.80	43.20	50.61	49.39	0.10	99.90
	Cu + P	58.88	41.12	49.32	50.68	0.10	99.90
	Cu + Ca	63.83	36.17	49.10	50.90	0.11	99.89
SO4	Control	37.98	62.02	46.61	53.39	3.74	96.26
	Cu	56.69	43.31	46.25	53.75	0.07	99.93
	Cu + P	57.06	42.94	49.67	50.33	0.12	99.88
	Cu + Ca	49.39	50.61	39.04	60.96	0.14	99.86
IAC 572	Control	43.98	56.02	39.41	60.59	0.92	99.08
	Cu	57.34	42.66	46.12	53.88	0.06	99.94
	Cu + P	48.39	51.61	52.72	47.28	0.09	99.91
	Cu + Ca	39.76	60.24	40.38	59.62	0.16	99.84
Isabel	Control	38.49	61.51	45.12	54.88	1.82	98.18
	Cu	57.19	42.81	53.70	46.30	0.05	99.95
	Cu + P	39.62	60.38	40.29	59.71	0.12	99.88
	Cu + Ca	42.85	57.15	42.13	57.87	0.08	99.92

Table 3. Translocation index (TI) and bioaccumulation factor (BAF) of Cu in grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control), with high Cu content (Cu), higher Cu and P (Cu + P) content and higher Cu and Ca (Cu + Ca) content for 21 days.

Rootstocks	Treatments	TI	BAF
Paulsen 1103	Control	0.101	342.12
	Cu	0.001	2.31
	Cu + P	0.001	2.57
	Cu + Ca	0.001	2.48
SO4	Control	0.039	247.85
	Cu	0.001	2.44
	Cu + P	0.001	2.81
	Cu + Ca	0.001	2.85
IAC 572	Control	0.009	171.14
	Cu	0.001	2.09
	Cu + P	0.001	2.62
	Cu + Ca	0.002	2.61
Isabel	Control	0.019	201.21
	Cu	0.000	2.38
	Cu + P	0.001	4.87
	Cu + Ca	0.001	2.66

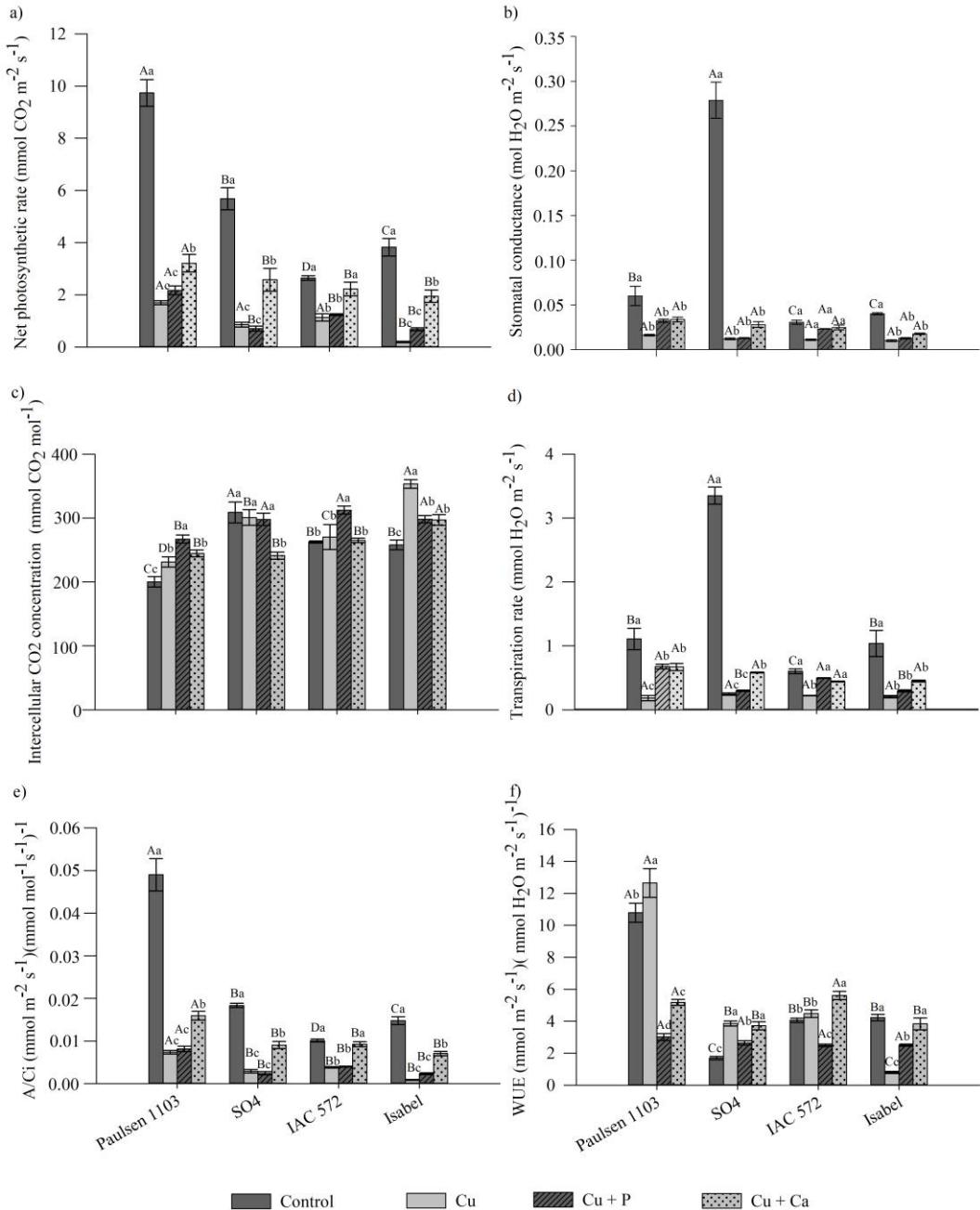


Figure 4. Net photosynthetic rate (A) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (a), stomatal conductance (Gs) ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (b), intercellular CO₂ concentration (Ci) ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) (c), transpiration rate (E) ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (d), water use efficiency (WUE) [$(\mu\text{mol m}^{-2} \text{ s}^{-1}) / (\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1})^{-1}$] (e) and instantaneous efficiency of carboxylation (A/Ci) [$(\mu\text{mol m}^{-2} \text{ s}^{-1}) / (\mu\text{mol mol}^{-1} \text{ s}^{-1})^{-1}$] (f) in leaves of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content, 7 days after treatment addition. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P < 0.05$).

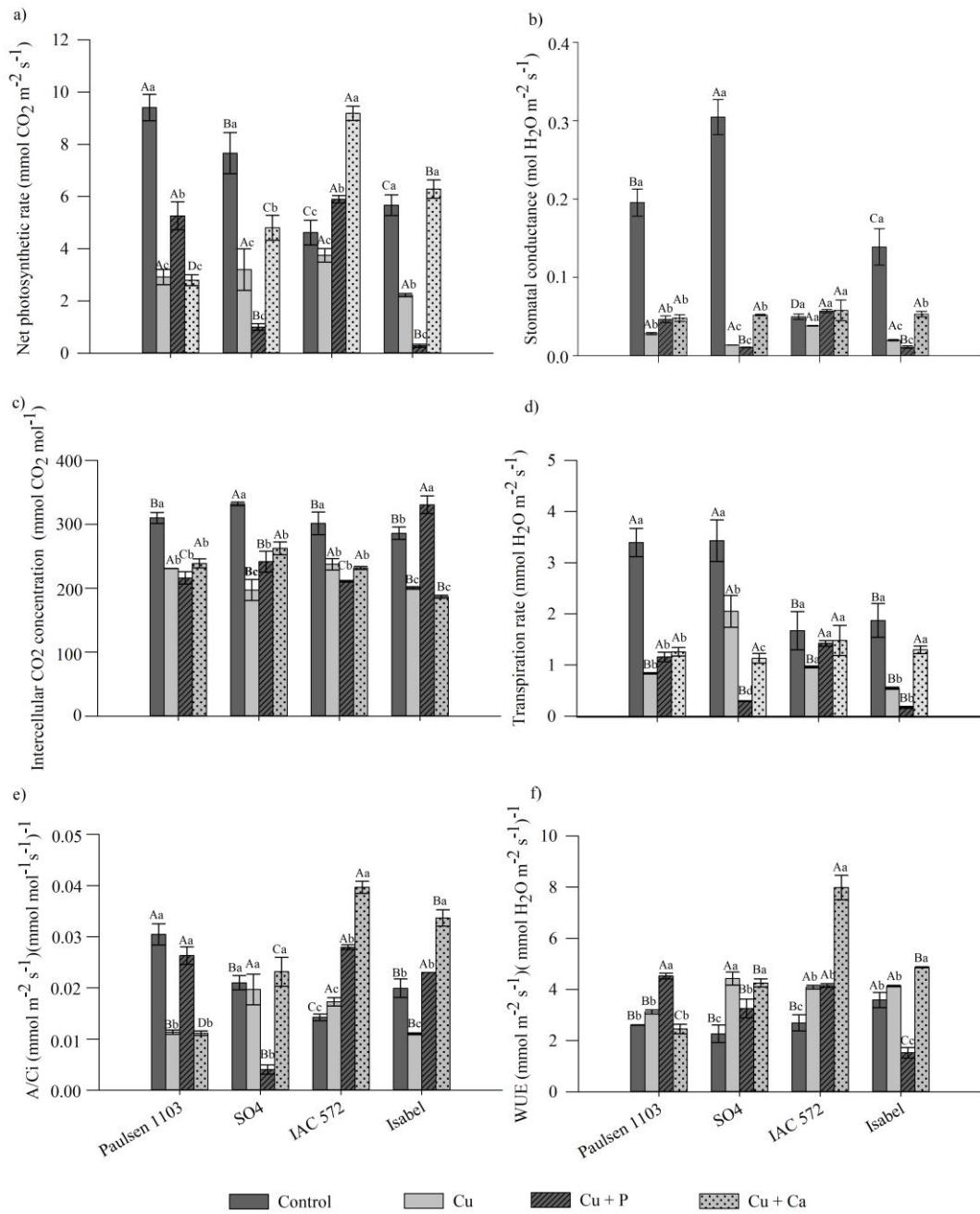


Figure 5. Net photosynthetic rate (A) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (a), stomatal conductance (G_s) ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (b), intercellular CO_2 concentration (C_i) ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) (c), transpiration rate (E) ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (d), water use efficiency (WUE) [$(\mu\text{mol m}^{-2} \text{ s}^{-1})(\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1})^{-1}$] (e) and instantaneous efficiency of carboxylation (A/Ci) [$(\mu\text{mol m}^{-2} \text{ s}^{-1})(\text{mmol mol}^{-1} \text{ s}^{-1})^{-1}$] (f) in leaves of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content, 21 days after treatment addition. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P<0.05$).

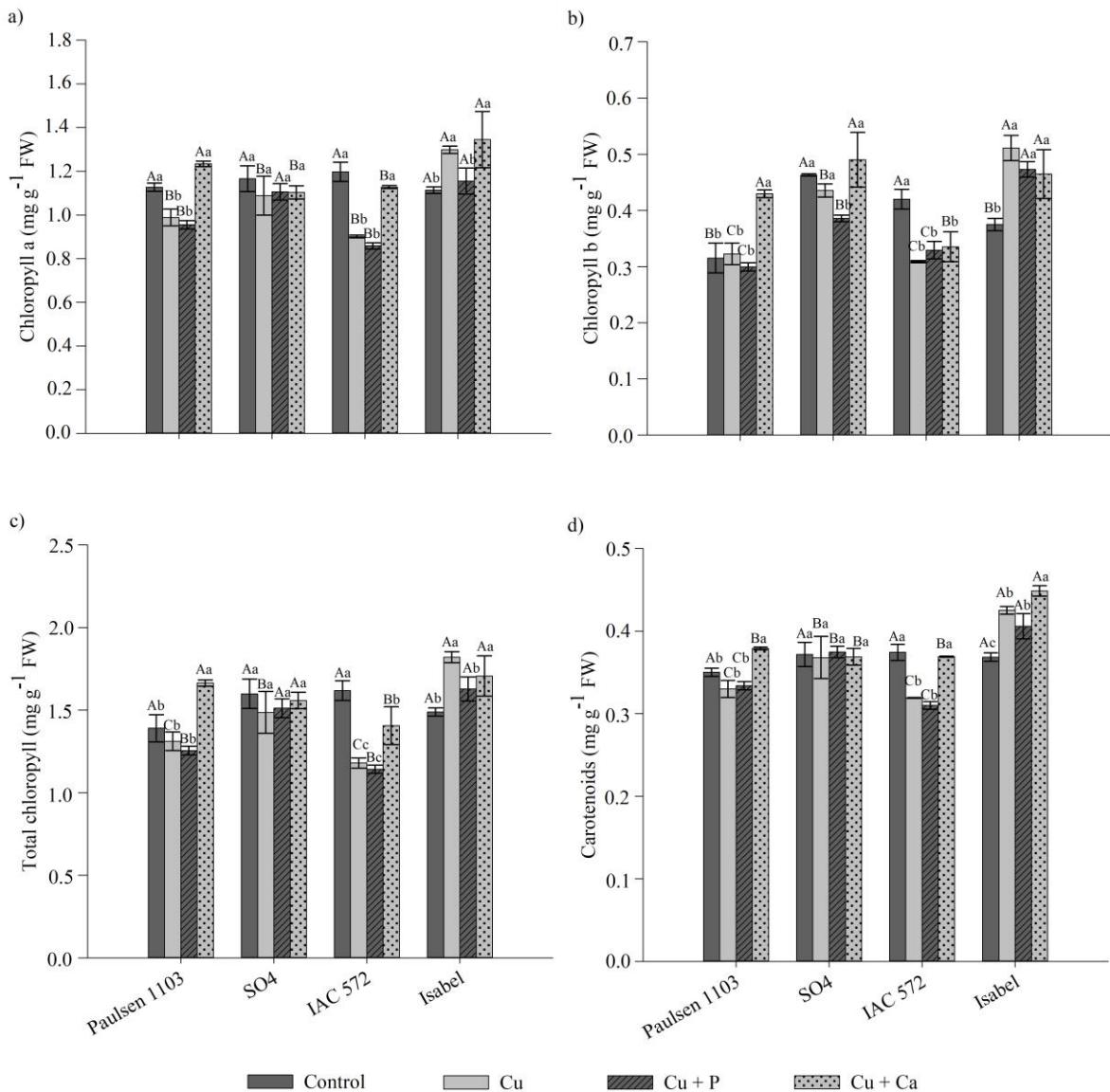


Figure 6. Chlorophyll *a* (Chl *a*) (a), chlorophyll *b* (Chl *b*) (b), total chlorophyll (Total Chl) (c) and carotenoid contents (d) in leaves of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P<0.05$).

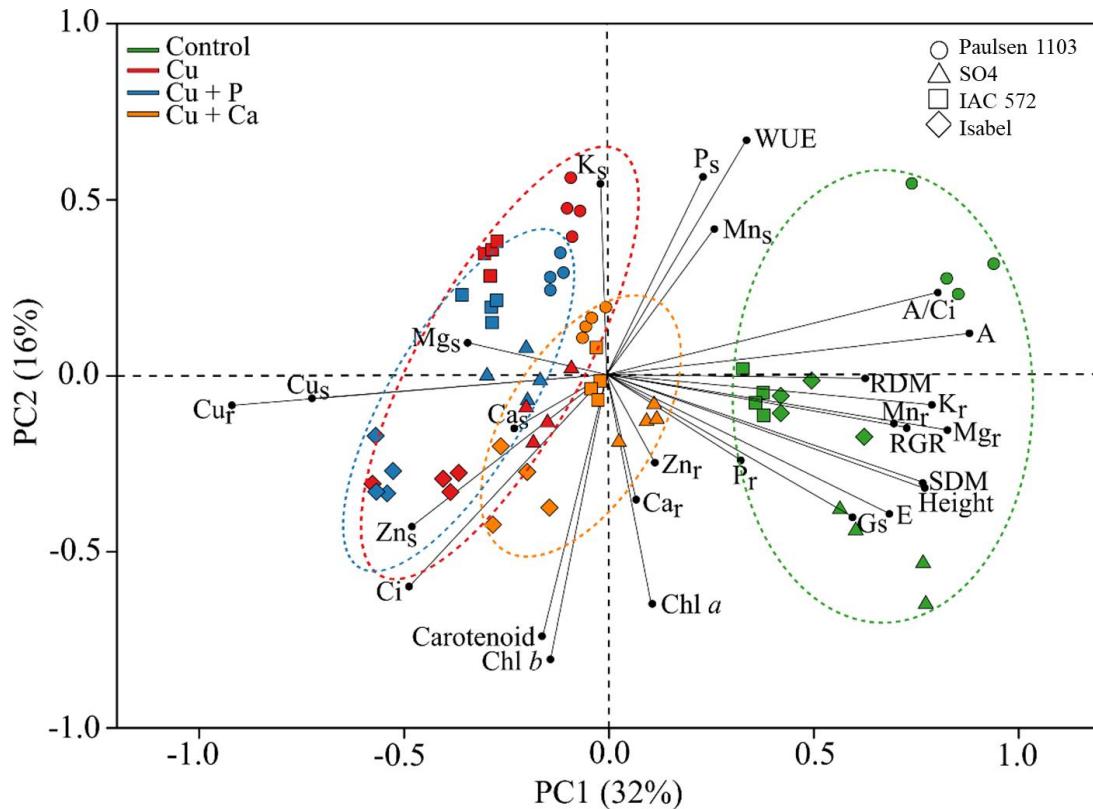


Figure 7. Multivariate principal component analysis (PCA) based on photosynthetic [net photosynthetic rate (A), stomatal conductance (Gs), intercellular CO₂ concentration (Ci), transpiration rate (E), water use efficiency (WUE) and instantaneous efficiency of carboxylation (A/Ci)] parameters, on photosynthesizing pigments [chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids], on P, K, Ca, Mg, Cu, Zn and Mn concentration in tissues [shoot (s) and root (r)] and on plant growth parameters [plant height (h), root dry mass (RDM) and shoot dry mass (SDM), and relative growth rate (RGR)] in four grapevine rootstocks [Paulsen 1103 (circle), SO4 (triangle), IAC 572 (square) and Isabel (rhombus)] grown in standard nutrition solution (control) (green) with high Cu content (red), higher Cu and P (Cu + P) content (blue) and with higher Cu and Ca (Cu + Ca) content (orange).

Supplementary Material

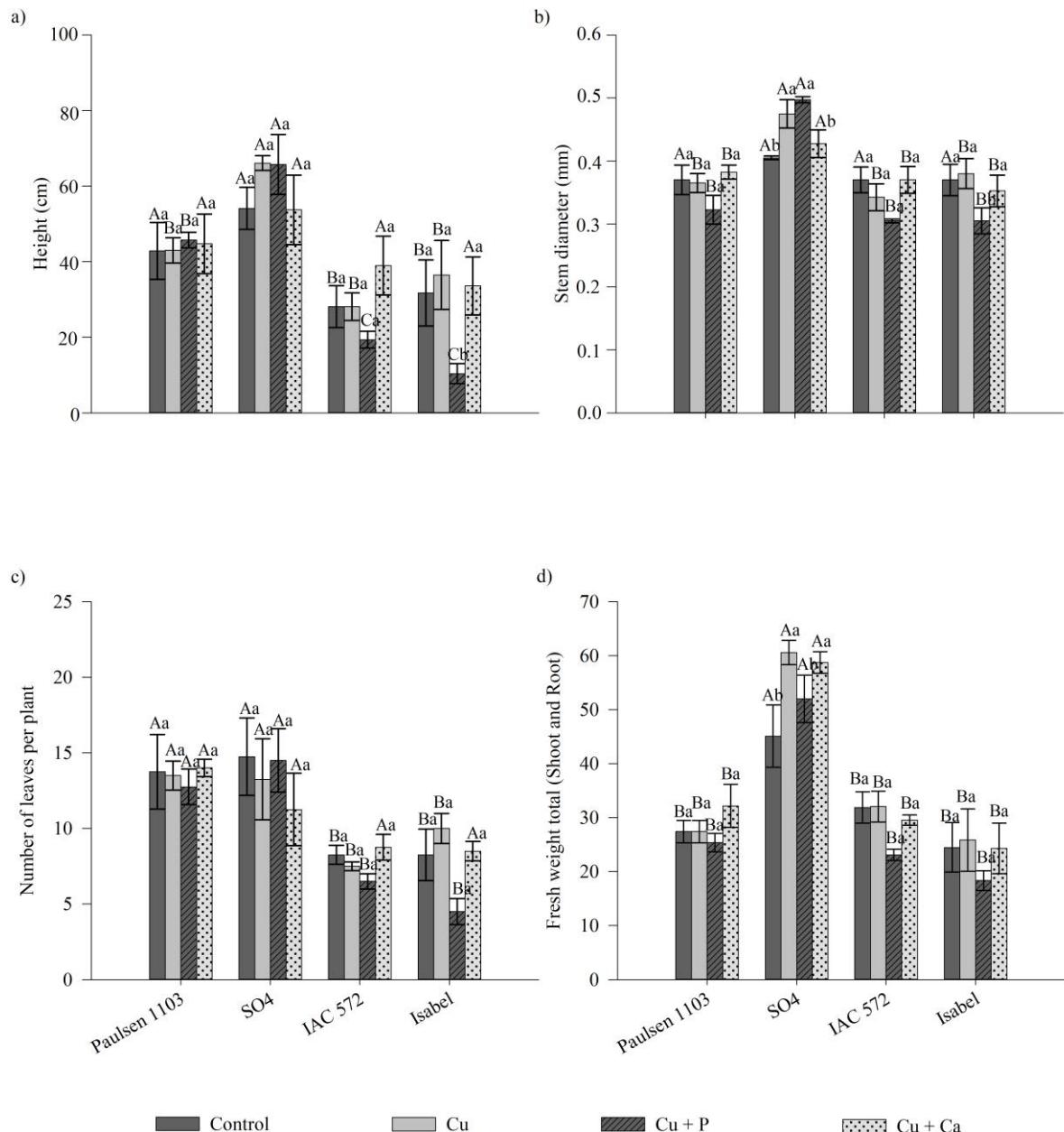


Figure 1. Height (a), steam diameter (b), number of leaves (c) and total fresh weight (shoot and root) (d) of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content in the beginning of the experiment. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P<0.05$).

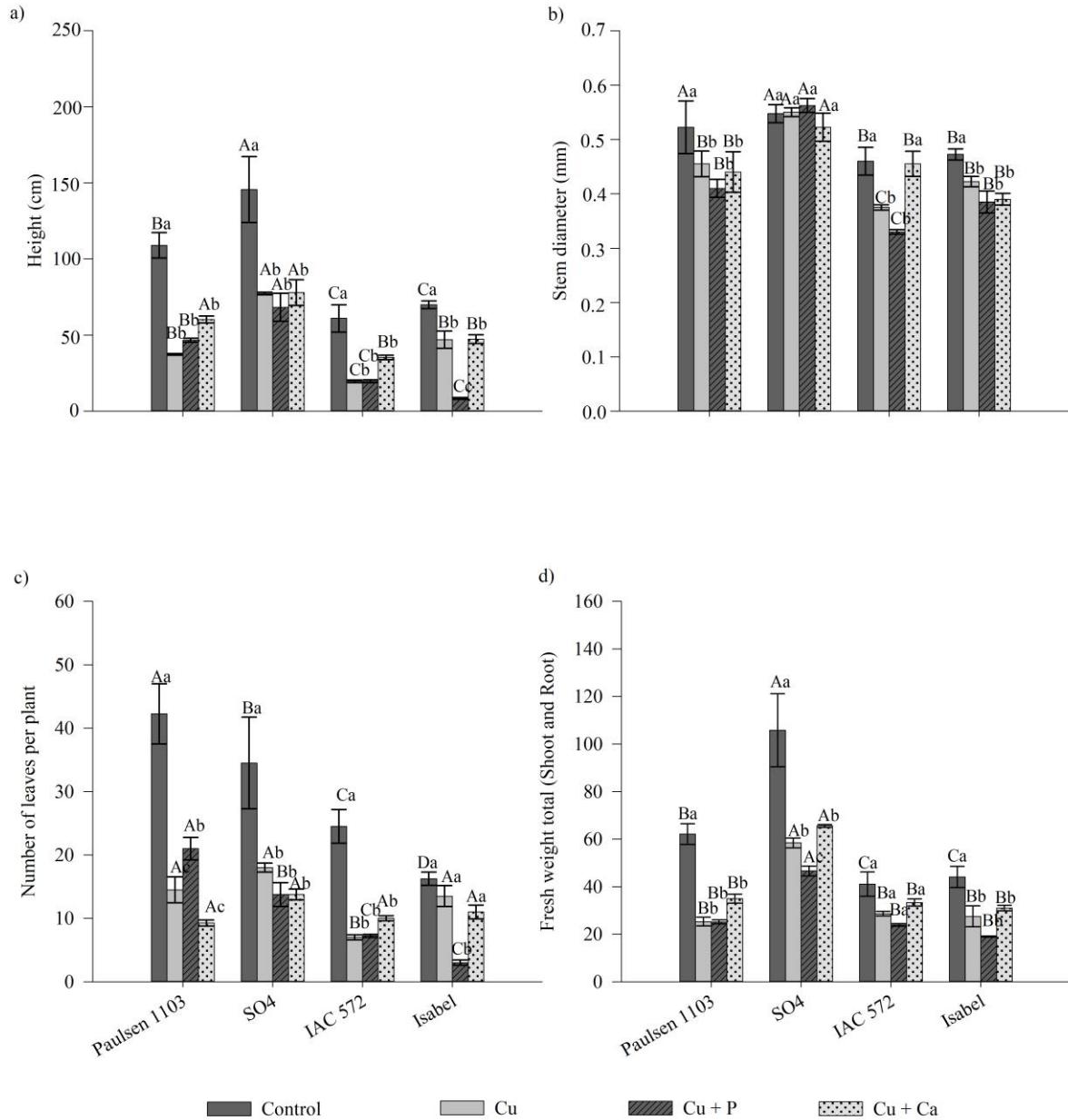


Figure 2. Height (a), steam diameter (b), number of leaves (c) and total fresh weight (shoot and root) (d) of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content at the end of the experiment. Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% ($P<0.05$).

Table 1. Macronutrient concentration in the shoot and roots of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content.

Rootstocks	Treatments	P	K	Ca	Mg
		(g kg ⁻¹)	(g kg ⁻¹)	(g kg ⁻¹)	(g kg ⁻¹)
Shoots					
Paulsen 1103	Control	1.86 Aa ⁽¹⁾	19.43 Aa	9.22 Ab	4.62 Aa
	Cu	1.15 Ac	17.14 Ab	8.39 Bb	4.56 Aa
	Cu + P	1.19 Ac	17.64 Ab	7.68 Bb	3.98 Ba
	Cu + Ca	1.49 Ab	19.97 Aa	12.72 Aa	4.75 Aa
SO4	Control	0.78 Cb	13.95 Bb	8.31 Ba	3.96 Aa
	Cu	1.05 Aa	14.89 Bb	8.80 Ba	4.09 Aa
	Cu + P	1.18 Aa	17.61 Aa	9.03 Aa	4.58 Ba
	Cu + Ca	1.11 Ba	16.54 Ba	9.26 Ca	4.03 Ba
IAC 572	Control	1.23 Ba	18.23 Aa	7.13 Bb	3.91 Aa
	Cu	1.12 Aa	18.87 Aa	8.22 Ba	4.67 Aa
	Cu + P	1.18 Aa	16.63 Ab	9.22 Aa	4.15 Ba
	Cu + Ca	0.87 Cb	15.28 Bb	8.823 Ca	4.00 Ba
Isabel	Control	0.94 Cb	15.28 Ba	9.64 Aa	4.01 Ab
	Cu	0.99 Ab	17.85 Aa	10.56 Aa	4.92 Aa
	Cu + P	1.2 Aa	16.00 Aa	9.89 Aa	5.43 Aa
	Cu + Ca	0.86 Cb	15.89 Ba	11.07 Ba	3.84 Bb
Roots					
Paulsen 1103	Control	1.48 Aa	21.29 Aa	12.73 Aa	4.41 Ba
	Cu	0.88 Ab	7.92 Ac	8.18 Bb	1.92 Ab

	Cu + P	0.83 Cb	6.59 Bc	7.89 Cb	1.97 Ab
	Cu + Ca	0.84 Bb	11.28 Cb	13.18 Ba	1.44 Cc
SO4	Control	1.28 Aa	12.07 Bb	9.52 Db	3.63 Ca
	Cu	0.80 Ab	8.48 Ac	10.23 Ab	2.29 Ac
	Cu + P	0.89 Cb	8.28 Ac	9.15 Bb	2.04 Ac
	Cu + Ca	1.14 Aa	18.42 Aa	14.47 Aa	2.92 Ab
IAC 572	Control	1.56 Aa	20.87 Aa	10.96 Cb	3.29 Ca
	Cu	0.83 Ac	3.73 Bc	9.59 Ac	1.63 Ac
	Cu + P	1.26 Bb	4.08 Cc	8.27 Cd	1.54 Bc
	Cu + Ca	1.31 Ab	13.38 Bb	13.04 Ba	2.38 Bb
Isabel	Control	1.50 Ab	19.86 Aa	11.72 Bb	5.11 Aa
	Cu	0.74 Ad	5.00 Bc	9.10 Bc	1.99 Ab
	Cu + P	1.83 Aa	8.07 Ab	14.65 Aa	2.04 Ab
	Cu + Ca	1.14 Ac	9.09 Db	15.20 Aa	1.57 Cc

⁽¹⁾Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% (P<0.05).

Table 2. Micronutrient concentration in the shoot and roots of grapevine rootstocks (Paulsen 1103, SO4, IAC 572 and Isabel) grown in standard nutrition solution (control) with high Cu content (Cu), higher Cu and P (Cu + P) content and with higher Cu and Ca (Cu + Ca) content.

Rootstocks	Treatments	Cu	Mn	Zn
		(mg kg ⁻¹)	(mg kg ⁻¹)	(mg kg ⁻¹)
Shoots				
Paulsen 1103	Control	6.46 Ab ⁽¹⁾	145.19 Ab	31.69 Aa
	Cu	8.74 Aa	169.39 Aa	34.12 Ba
	Cu + P	9.71 Ba	109.64 Ac	23.96 Ca
	Cu + Ca	9.38 Aa	141.93 Ab	28.07 Ba
SO4	Control	4.68 Bc	64.97 Ba	23.78 Bb
	Cu	9.24 Ab	80.16 Ca	39.72 Ba
	Cu + P	10.61 Ba	91.32 Aa	42.42 Ba
	Cu + Ca	10.76 Aa	74.63 Ba	35.91 Ba
IAC 572	Control	3.23 Cc	141.87 Aa	34.21 Aa
	Cu	7.89 Ab	101.91 Bb	25.31 Ca
	Cu + P	9.92 Ba	72.11 Bc	26.96 Ca
	Cu + Ca	9.88 Aa	78.76 Bc	31.50 Ba
Isabel	Control	3.80 Cc	152.78 Aa	38.58 Ad
	Cu	9.00 Ab	115.57 Bb	61.43 Ab
	Cu + P	18.42 Aa	71.77 Bc	100.46 Aa
	Cu + Ca	10.06 Ab	123.61 Ab	51.93 Ac
Roots				
Paulsen 1103	Control	71.93 Ab	253.93 Ba	51.97 Ba
	Cu	9057.04 Ca	41.42 Bb	31.62 Bb

	Cu + P	9609.02 Ba	40.58 Ab	28.61 Db
	Cu + Ca	8211.087 Ba	29.30 Ab	25.52 Cb
SO4	Control	120.45 Ac	203.04 Ba	46.19 Bb
	Cu	12428.54 Ba	73.93 Bb	52.92 Aa
	Cu + P	8600.60 Cb	50.74 Ab	45.20 Cb
	Cu + Ca	7752.70 Bb	68.22 Ab	58.21 Aa
IAC 572	Control	349.18 Ad	347.64 Aa	87.43 Aa
	Cu	12503.89 Ba	75.31 Bb	55.40 Ac
	Cu + P	10503.96 Bb	66.23 Ab	64.97 Ab
	Cu + Ca	6127.47 Cc	65.35 Ab	49.30 Bc
Isabel	Control	204.69 Ad	200.30 Ba	57.33 Ba
	Cu	19321.61 Aa	154.55 Aa	61.00 Aa
	Cu + P	15794.53 Ab	ND	54.64 Ba
	Cu + Ca	12748.42 Ac	56.06 Ab	42.48 Bb

⁽¹⁾Means followed by uppercase letters represent the comparison to rootstocks in each treatment; lowercase letters represent the comparison to treatments in each rootstock, both through Scott-Knott test at 5% (P<0.05).

5.5 ESTUDO V

Potential of vermicompost and limestone in reducing copper toxicity in young grapevines grown in Cu-contaminated vineyard soil⁵

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Potential of vermicompost and limestone in reducing copper toxicity in young grapevines grown in Cu-contaminated vineyard soil

Abstract

Foliar fungicide application in grapevines increases the content of heavy metals such as copper (Cu) in vineyard soils, which may reach phytotoxic levels. The application of soil amendments such as limestone and vermicompost may reduce Cu availability and phytotoxicity. The study aimed to assess growth, physiological status and changes in root morphology in young grapevines grown for 12 months in Cu-contaminated soil with and without the application of soil amendments. Samples of a Typic Hapludalf soil were collected in a vineyard with more than 30 years of cultivation. The following treatments were used: 1) control (without amendment), 2) application of organic vermicompost (86.7 g kg⁻¹) and 3) application of limestone (3 Mg ha⁻¹). Grapevines (Paulsen 1103 rootstock) were transplanted and grown for 12 months in PVC soil columns. We assessed parameters of growth, photosynthesis and root morphology. Grapevines grown in soil treated with limestone showed increased growth, dry matter yield and photosynthetic efficiency. The highest Cu concentrations in root tissue were found in grapevines grown in control soil. The application of vermicompost in this study did not alleviate Cu toxicity. Grapevines grown in soil treated with vermicompost showed high manganese (Mn) concentration in shoots before the winter pruning, reflecting the high Mn concentrations in soil solution that caused Mn phytotoxicity, resulting in plant death after the winter pruning. The vermicompost used in this study is not suitable for agronomic use. The use of limestone was an effective strategy to reduce Cu availability and phytotoxicity.

Keywords: Heavy metal, Rootstock, Amendments, Phytotoxicity.

1. Introduction

The prevention and control of foliar fungal diseases such as grape downy mildew (*Plasmopara viticola*) in vineyards around the world is performed by continuous applications of copper fungicides (especially Bordeaux mixture), which can lead to increased Cu concentration in vineyard soils (Brunetto et al., 2014; Tiecher et al., 2016; 2017). Because of decreasing grape yields, old vineyards are eradicated and soils are prepared for the plantation of young grapevines. However, this practice may stimulate the mineralization of soil organic matter that forms complexes with Cu. This further increases Cu availability to plants and

possibly causes phytotoxicity to young grapevines if Cu soil concentration is in excess (Weng et al., 2002; De Conti et al., 2016).

Cu is a micronutrient that is a cofactor of many proteins involved in electron transport in photosynthesis, mitochondrial respiration, as well as carbon and nitrogen metabolism (Yruela, 2005; 2009; Kabata-Pendias, 2011). However, high Cu concentration in tissue can be toxic to plants (Michaud, 2008; Lequeux et al., 2010; Cambrollé et al., 2015). Copper can bind to sulfhydryl groups of proteins and inhibit their function, as well as restricts the uptake of other essential ions (Yruela, 2009; Marastoni et al., 2019). High contents of Cu may cause changes in root architecture and morphology, reduction of growth of the main root, thickening of the root apex and increase of lateral root density (Lequeux et al., 2010).

Cooper (II) and Cu⁺ redox cycles in cells catalyze the production of hydroxyl radicals through Fenton chemistry, which are toxic and cause damage to DNA, lipids and proteins (Halliwell and Gutteridge, 1984). Excess Cu in shoot tissues may interfere in the biosynthesis of photosynthetic components, resulting in a decrease of photosynthetic pigment content, changes in the structure of the chloroplast and the composition of the thylakoid membrane (Cambrollé et al., 2013; 2015). Cooper may form complexes with chlorophyll, impairing the process of converting light into chemical energy in the form of NADPH and ATP, and the release of O₂ (Yruela, 2005; 2009; Kabata-Pendias, 2011). Also, the increase in thylakoid membrane fluidity occurs through lipid peroxidation. This affects the activity of the photosystems, especially PSII, which is more sensitive to Cu toxicity, inhibiting the oxygen-evolving complex and causing losses by fluorescence (Yruela, 2005; 2009; Kabata-Pendias, 2011).

Thus, the use of amendments (e.g., limestone and vermicompost) capable of reducing Cu availability and phytotoxicity to plants is necessary in Cu-contaminated vineyards prepared for future cultivation of young grapevines (Chen et al., 2013; Ambrosini et al., 2015; Santana et al., 2015). Limestone promotes the increase in soil pH, causing deprotonation of acidic functional groups of reactive soil particles. This increases cation exchange capacity (CEC) and Cu adsorption, which decreases bioavailability and uptake potential for plants (Ambrosini et al., 2015; Brunetto et al., 2016). The addition of organic waste such as vermicompost may promote increased nutrient availability in soil. However, it may also increase soil organic carbon content and soil organic matter, which can complex Cu, thus decreasing its availability and toxicity potential (Santana et al., 2015; Brunetto et al., 2016).

Studies using the application of limestone and vermicompost are reported in literature as viable strategies to minimize Cu phytotoxicity to grapevines or to cover crop species in

vineyards (Chen et al., 2013; Juang et al., 2014; Ambrosini et al., 2015; 2017; Santana et al., 2015). However, most studies were performed using contaminated soils in short incubation experiments in laboratories or greenhouses, which increases the availability of Cu in soil and its phytotoxic potential (Chen et al. 2013; Cambrollé et al., 2015). This makes it difficult to understand which amendment is most efficient in decreasing Cu availability and toxicity potential for longer exposures. Thus, this study aims at assessing growth, physiological status and changes in root morphology in young grapevines grown for 12 months in Cu-contaminated soil with and without soil amendment application.

2. Material and methods

2.1 Description of the experiment

Samples of a Typic Hapludalf soil were collected at 0-0.20 m in a vineyard with more than 30 years of cultivation and a history of annual Cu-based fungicide applications. The vineyard was located near the city of Santana do Livramento, in the Campanha Gaúcha of the state of Rio Grande do Sul, Brazil ($30^{\circ}47'34.5''S$ and $55^{\circ}22'5.5''W$). The soil was air-dried, grounded, and sieved through a 2 mm mesh. Part of the soil was subjected to chemical analysis (Tab. 1) as described (Tedesco et al. 1995).

The equivalent to 3 Mg ha⁻¹ limestone (PRNT 100%) and 30 g C kg⁻¹ as organic vermicompost (equivalent to 86.7 g kg⁻¹ vermicompost) were added to separate portions of 144 kg of soil. Limestone and vermicompost were applied to soil and homogenized by hand. Afterwards, 24 kg of soil were incubated for 30 days in plastic bags. Every two days, the incubated soil was stirred and the plastic bags were opened to eliminate CO₂. Distilled water was added to maintain the maximum water holding capacity (MWHC) at 70%. Incubation was carried out in a greenhouse with average temperature of 26 ° C and relative humidity of 50%. The amounts of limestone and vermicompost used in this study were based on an incubation experiment by Ferreira et al. (2018), who used different doses of the two amendments to reduce Cu availability. Vermicompost was produced from grape residue subjected to aerobic composting, followed by vermicomposting with *Eisenia andrei* Bouché (1972). The chemical composition of the vermicompost is described in Santana et al. (2015).

Twenty-four kg of soil of each amendment were placed in PVC soil columns (0.55 m in height and 0.25 m in diameter) arranged on tables in a greenhouse. In January 2016, one grapevine (*Vitis vinifera*) plant (Paulsen 1103, commonly used as rootstock) was transplanted onto each column. The seedlings were produced through in vitro micropagation. The grapevines were grown until January 2017, totaling 12 months. Average temperature of 26 °

C and the relative humidity of 50% were maintained throughout cultivation period. In the winter pruning, six viable buds were kept in each plant. After pruning, only one main shoot was kept per plant with a tutor.

2.2 Height, diameter, dry matter yield and tissue nutrient concentration

Shoot height and stem diameter were measured every 15 days after the transplanting (DAT) of grapevines. Winter pruning was carried out eight months after transplanting and the residual plant material was reserved for subsequent determination of tissue nutrient concentration. At 12 months after transplanting, grapevines were harvested. Shoots were cut close to the soil surface and stored, and roots were separated from the soil by hand, washed in running water, and then in distilled water. Shoots collected at winter pruning and at harvest and roots collected at harvest were dried in an oven with forced air circulation at 65 °C until constant weight. Afterwards, root and shoot dry matter were measured, and tissue samples were ground in a Wiley mill. Part of the tissue was prepared and subjected to nitric-perchloric digestion (Embrapa, 2009). Calcium (Ca), magnesium (Mg), Mn, Cu, zinc (Zn) and iron (Fe) concentrations in the extract were determined in an atomic absorption spectrophotometer (AAS, Varian SpectrAA-600, Australia). Potassium (K) concentration was determined by flame photometer (DM62, Digimed, Brazil). Phosphorus (P) concentration was determined using the methodology of Murphy & Riley (1962) by colorimetry in a spectrophotometer (SF325NM, Bel Engineering, Italy). The remaining tissue was subjected to sulfur digestion (Tedesco et al., 1995) to determine nitrogen (N) concentration, which was carried out according to the Kjeldahl method in a steam distiller (TE-0364, Tecnal, Brazil) (Bremner and Mulvaney, 1982).

2.3 Root morphology

Twelve months after the transplanting of grapevines, the soil of the columns was stratified in layers of 0.0-0.05, 0.05-0.10, 0.10-0.20, 0.20-0.30, 0.30-0.40 and 0.40-0.45 m. Soil and root samples were collected at each layer. Roots were washed in running water and then in distilled water. Afterwards, roots were scanned in an Epson Expression 11000 scanner equipped with additional light (TPU), with a resolution of 600 dpi. Finally, the images were analyzed in WinRhizo Pro 2013 software, and root length, surface area, average diameter and volume were determined.

2.4 Photosynthetic pigment content

At 12 months after the transplanting of grapevines, four leaves were collected in the upper third of the shoot and immediately placed in liquid N₂ and stored at -80 °C. Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid contents were determined as described (Hiscox and Israelstam, 1979). Absorbance was determined at 470, 645 and 663 nm for Chl a, Chl b and carotenoids, respectively, using a spectrophotometer (SF325NM, Bel Engineering, Italy). Photosynthetic pigment contents were estimated using the equation of Lichtenthaler (Lichtenthaler, 1987).

2.5 Chlorophyll a fluorescence

Chlorophyll a fluorescence analysis was carried out 8 and 12 months after the transplanting of the grapevines using a JUNIOR-PAM fluorometer (Walz, Germany). Fluorescence readings were performed in leaves of the upper third of the shoot between 8:00 am and 10:00 am (Ferreira et al., 2015). Prior to readings, leaves went through a 30-min dark adaptation period to determine the minimal level of initial fluorescence (F₀). Leaves were subjected to a pulse of saturating light (10,000 µmol m⁻² s⁻¹) for 0.6 s to determine maximum fluorescence (F_m). According to parameters determined for fluorescence, we calculated maximum quantum yield of PSII (F_v/F_m), effective quantum efficiency of PSII (Y(II)) and non-photochemical quenching (NPQ) (Maxwell and Johnson, 2000). Maximum electron transport rate (ETRmax) was determined during the dark period, before dawn (5:00-6:00 am) (Ferreira et al., 2015). ETR was evaluated and calculated through the emission of light curves (photosynthetically active radiation, PAR) (Rascher et al., 2000).

2.6 Photosynthetic activity

Photosynthetic activity was assessed 8 and 12 months after the transplanting of grapevines. Measurements were performed using an infra-red gas analyzer (Li-6400, Li-COR Inc., Neb., USA) in leaves of the upper middle third of the shoot. Net photosynthetic rate (A), internal CO₂ concentration (Ci), transpiration rate (E), stomatal conductance to CO₂ (Gs), water use efficiency (WUE) and instantaneous carboxylation efficiency (A/Ci) (by Ribulose-1,5-bisphosphate carboxylase/oxygenase) were determined in a leaf chamber, with CO₂ concentration of 400 µmol mol⁻¹, temperature of 20/25 °C, relative humidity of 50 ± 5% and photon flux density of 1000 µmol m² s⁻¹. A, Ci, E and Gs were calculated using equations of Von Caemmerer and Farquhar (1981).

2.7 Statistical analysis

The results were submitted to analysis of variance in Sisvar software, version 4.0 (Ferreira, 2008). When the effect of treatments was significant, the means were separated by the Tukey test at 5%.

3. Results

3.1 Soil and amendments

Soil used in this study showed a pH of 5.5, a total organic C of approximately 6 g kg⁻¹ and available Cu of 122 mg kg⁻¹ (Table 1). The addition of lime increased pH to 7.7, exchangeable Ca to 694 mg kg⁻¹, and available Mn to 177 mg kg⁻¹. The addition of vermicompost increased total organic C to 17 g kg⁻¹, soil available P (270 mg kg⁻¹), K (288 mg kg⁻¹), Ca (911 mg kg⁻¹), and Mg (737 mg kg⁻¹) (Table 1). Addition of vermicompost increased the concentration of most of the nutrients in the soil solution (Tab. 1).

3.2 Height, diameter and dry matter yield

The addition of limestone showed a positive effect on grapevine growth: i.e. shoot height and dry matter before the winter pruning (Figure 1a, c), stem diameter (Figure 1b), shoot and root dry matter yield at plant harvest after 12 months of cultivation (Figure 1a, b, c, d). The use of vermicompost resulted in increased plant growth comparable to limestone treatment up until winter pruning. However, plants treated with vermicompost did not sprout after the winter pruning (Figure 1a). For this reason, data of plants cultivated in soil treated with vermicompost are restricted to the first nine months.

3.3 Tissue nutrient concentration

Before winter pruning, the highest P, K, Ca, Mg and Mn concentrations were found in shoots of grapevines grown in soil treated with vermicompost (Table 2). In particular Mn was 54 and 76% higher than in the control and limestone-treated plants, respectively. Shoot N, Cu, Zn and Fe concentrations were lower in grapevines grown in soils treated with limestone and vermicompost in comparison to those of the control soil (Table 2).

After the winter pruning, shoot P concentration was higher in grapevines grown in soil treated with limestone than those of the control soil (Table 2), whereas the reverse was found for shoot K, Cu, Zn and Mn concentrations (Table 2). Root P concentration was higher in grapevines treated with limestone than those of the control soil (Table 2). On the other hand,

root Mg, Cu, Zn, Fe and Mn concentrations of grapevines grown in soil treated with limestone were 35, 178, 274, 54 and 55% lower than those of the control soil, respectively (Table 2).

3.4 Root morphology

Since grapevines grown in soil treated with vermicompost did not survive after the winter pruning, we assessed root morphology of plants grown in the control and limestone-added soils. The highest values of root length, surface area and volume were found at 0.0-0.1 m of depth in the soil treated with limestone (Figure 2a, b, d). On the other hand, root length, surface area and volume at 0.1-0.2 m were lower in grapevines grown in soil treated with limestone compared to the control treatment (Figure 2a, b, d). At 0-0.05 m-depth, roots of grapevines grown in soil treated with limestone had a lower diameter in comparison to the untreated control (Figure 2c). Root volume was not affected by treatments (Figure 2d).

3.5 Photosynthetic pigment content and chlorophyll a fluorescence

After 12 months of cultivation, Chl a, Chl b, total Chl and carotenoid leaf contents were higher in grapevines grown in soil treated with limestone compared to untreated control (Figure 3).

Although with different intensity, in all the treatments, ETR increased with increasing intensity of photosynthetically active radiation (PAR) emitted on the leaves of grapevines (Figure 4a, b). PAR promoted a significant increase in ETR starting from 285 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The highest values of ETR were found in grapevines grown in soil amended with limestone, followed by the vermicompost (at least until winter pruning) and control (Figure 4a). After the winter pruning, limestone kept inducing a higher ETR than untreated control leaves (Figure 4b).

Before and after the winter pruning, the highest values of initial fluorescence (F_0) were found in leaves of grapevines grown in soil treated with limestone and vermicompost (Figure 5a). Before the winter pruning, the highest values of maximum quantum yield of PSII (F_v/F_m) and effective quantum efficiency of PSII ($Y(\text{II})$) were found in leaves of grapevines amended with limestone, followed by vermicompost (at least before winter pruning) and control (Figure 5b, c). Before the winter pruning, non-photochemical quenching (NPQ) was lowest in grapevines amended with limestone, followed by those treated with the vermicompost and untreated control (Figure 5d).

After the winter pruning, the values of maximum quantum yield of PSII (F_v/F_m) and effective quantum efficiency of PSII ($Y(\text{II})$) were higher in leaves of grapevines grown in soil

with limestone than the control (Figure 5b, c). Non-photochemical quenching (NPQ) was lower in leaves of grapevines grown in soil treated with limestone than those of the untreated control soil (Figure 5d).

3.6 Photosynthetic activity

Before and after the winter pruning, the highest values of photosynthetic rate (A), stomatal conductance (Gs) and instantaneous carboxylation efficiency (A/Ci) were found in leaves of grapevines treated with limestone, followed by the vermicompost and control (Figure 6a, b, f). The highest transpiration rate (E) and water use efficiency (WUE) were found in leaves of grapevines grown in soils with limestone and vermicompost (Figure 6d, e). Before the winter pruning, the highest values of the intracellular CO₂ concentration (Ci) were found in leaves of grapevines grown in soils with limestone and vermicompost (Figure 6c).

After the winter pruning, the highest values of A, Gs, E and A/Ci were found in leaves of grapevines grown in soil with limestone (Figure 6a, b, d, f). On the other hand, the Ci and WUE were not affected by soil application of amendments (Figure 6c, e).

4. Discussion

The highest growth and dry matter yield of grapevines grown in Cu-contaminated soil treated with limestone may have occurred because of the increase in soil pH. This promotes the deprotonation of the surface acidic functional groups of minerals and soil organic matter, which increases cation exchange capacity. Consequently, it increases Cu adsorption, decreasing its content in the soil solution (Brunetto et al., 2016). Also, with increasing pH, there may have been an increase in the concentration of anions such as carbonate (CO₃-2), hydroxyls (OH-) and phosphate (PO₄-3) in soil solution, which may bind Cu and form complexes (Wang et al., 2012; Brunetto et al., 2016). In addition, liming may increase the availability of macronutrients such as Ca and Mg, facilitating uptake by plants and reducing the effect of Cu toxicity (Juang et al., 2014; Ambrosini et al., 2015). Higher Ca concentration in shoots may facilitate the formation of calcium oxalate crystals, which incorporate heavy metals such as Cu in their structure, thus decreasing its phytotoxic effect (Nakata and Franceschi, 2005). Higher Mg concentration in tissue may compete with Cu ions and prevent the replacement of the central Mg ion of the chlorophyll molecule (Yruela, 2009). We found higher P concentration in tissue (both in shoots and roots) in grapevines treated with limestone after winter pruning. The rise of soil pH can reduce adsorption of P forms, consequently, P availability increases. The high soil availability of P and less Cu absorption

support the plants growth and nutrient accumulation in the treatment with limestone. Available P, beside the nutritional importance for plant growth and development, may form a metal-phosphate complex in roots and reduce Cu transport to shoots (Juang et al., 2014; Rosa et al., 2014; Ambrosini et al., 2015).

The low Cu, Zn and Mn concentrations in shoots and roots of grapevines treated with limestone may be attributed to the increase of soil pH (7.7), which decreases micronutrient availability compared to the lower pH of the control soil (5.5). Higher Cu, Zn and Mn concentrations in roots versus shoots may be a defense mechanism used by plants (Ambrosini et al., 2015; Cambrollé et al., 2013). Excess of Cu in root tissue may remain in the apoplast (possibly attached to the cell wall) or compartmentalized in the vacuole of the root cells, decreasing transport to shoots and the phytotoxic effect (Cambrollé et al., 2013; Ambrosini et al., 2015; Tiecher et al., 2017).

Grapevines of the limestone treatment showed greater root length and surface area compared to those of the control soil. This was most likely because the symptoms of toxicity caused by excess Cu in soil were minimized by raising the pH, which decreases the availability of Cu and other heavy metals in soil (Ambrosini et al., 2015). It may also be a result of the increasing Ca and Mg concentrations in soil (Chen et al., 2013; Juang et al., 2014). However, plants grown in Cu-contaminated solution without limestone application had shorter and thicker roots. This was likely caused by excess Cu in tissue, which inhibits the process of cell division and elongation at the root apex (Ambrosini et al., 2015).

Strikingly, grapevines grown in soil treated with vermicompost showed no sprouting after the winter pruning, and died after nine months of exposure. This response indicates that the vermicompost used in this study is not suitable for agronomic use. The phytotoxic effect was probably caused by the high concentration of nutrients that increased soil salinity, and promoted high nutrient concentration in plant tissues. Vermicompost treatment increased macronutrient (P, K, Ca and Mg) and micronutrient (Mn) concentrations in shoots. This was most likely due to the decomposition of the vermicompost and the increase of these nutrients in soil and in solution, thus increasing uptake by the grapevines (Santana et al., 2015). Thus, the formation of soluble organic complexes that are mobile in soil solution may occur, facilitating the approximation of cations to the roots of the grapevines (Bolan et al., 2003; De Conti et al., 2016). In this study, the soil of the vermicompost treatment had a higher Mn concentration in solution (5.55 mg L⁻¹) compared to the control treatment (0.55 mg L⁻¹). Soils with pH lower than 5.5 favor Mn availability, which in turn can results in higher accumulation in shoot tissues, as its uptake is poorly regulated by plants (Shao et al., 2017).

High Mn concentrations may interfere with the uptake and use of other mineral elements, as well as affect energy metabolism, decrease photosynthetic rates and cause oxidative stress in plants (Kabata-Pendias, 2011).

Excess Cu in shoot tissues affects physiological and biochemical processes such as photosynthesis (Kabata-Pendias, 2011). Previous studies have shown that Cu concentrations greater than 20 mg kg⁻¹ in shoot tissues inhibit photosynthesis (Bibi and Hussain, 2005; Vassilev et al., 2002). In this study, we found that leaves of grapevines grown in the control soil with high Cu concentration (17.55 mg kg⁻¹) showed reduced net photosynthetic rate. This behavior may be related to low chlorophyll content in leaves. High Cu concentration in leaf tissue may cause degradation of the internal structure of chloroplasts by replacing the central Mg ion with Cu in the chlorophyll molecules. This contributes to reduced synthesis and pigment content in leaves (Küpper et al., 2002; Yruela, 2005; 2009).

Also the integrity and/or function of the photochemical apparatus and photosynthetic pigment content (Cambrollé et al., 2013; Cambrollé et al., 2015) may be involved in Cu toxicity. However, there was no direct relationship between net photosynthetic rate and stomatal conductance, as we did not find a decrease of intercellular CO₂ concentration associated to Cu excess (Cambrollé et al., 2013; 2015). The application of amendment increased net photosynthetic rate, transpiration rate, stomatal conductance to CO₂, water use efficiency and instantaneous carboxylation efficiency (by Ribulose-1,5-bisphosphate carboxylase/oxygenase). This was because there was less Cu uptake and transport in soil with the presence of amendments (especially limestone), which minimizes the effects of Cu phytotoxicity (Juang et al., 2014; Ambrosini et al., 2015).

The lowest values of initial fluorescence (F_0), maximum quantum yield of PSII (F_v/F_m) and effective quantum efficiency of PSII ($Y(II)$) found in leaves of control treatment indicate a state of photoinhibition caused by excess Cu in tissue (Cambrollé et al., 2013; Tiecher et al., 2017). The lowest values found for F_v/F_m may be attributed to the lower values of F_m , indicating a decrease in the fraction of open reaction centers (Maxwell and Johnson, 2000) and/or lower leaf chlorophyll content (Cambrollé et al., 2013; 2015; Tiecher et al., 2016; 2017).

Non-photochemical energy quenching (NPQ) in the form of heat is a protection mechanism of the leaves to excess light, preventing the formation of reactive oxygen species (ROS), which may damage proteins, lipids and membrane structure (Maxwell and Johnson, 2000; Cambrollé et al., 2012). The highest values of NPQ were found in leaves of control grapevines. This means the plants are dissipating a higher amount of energy in the form of

heat, due to the toxic effect caused by excess Cu in shoot tissue (Cambrollé et al., 2013; 2015; Tiecher et al., 2016; 2017).

The higher accumulation of photosynthetic pigments in leaves of grapevines grown in soil treated with limestone may be explained by the improved nutritional condition of these plants and lower Cu uptake and translocation, which contributed to minimize the phytotoxic effect of Cu (Ambrosini et al., 2015; Tiecher et al., 2017).

5. Conclusions

Young grapevines of the Paulsen 1103 rootstock grown in Cu-contaminated soil treated with limestone to reduce Cu phytotoxicity showed higher growth, adequate functioning of the photosynthetic apparatus and few changes in root morphology. On the other hand, vermicompost was not an effective alternative as a soil amendment, resulting in plant death, which is likely due to a phytotoxic effect caused by excess Mn in solution. Our results indicate that careful evaluation of vermicomposts is necessary for their agronomic uses in excessive Cu conditions. Limestone, on the other hand, is a good amendment to decrease the effects of Cu toxicity in grapevine plants.

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Table 1. Chemical properties and chemical characterization of the soil solution of a Typic Hapludalf soil collected at 0-0.20 m in a vineyard with more than 30 years of cultivation and incubated for 30 days without amendment (control) and with the application of limestone and vermicompost.

Properties	Control	Limestone	Vermicompost
pH in H ₂ O (1:1 ratio)	5.50	7.70	5.50
Total organic C (sulfochromic digestion) (g kg ⁻¹)	6.06	6.08	17.06
Available P (extracted by Mehlich-1) (mg kg ⁻¹)	59.04	59.40	269.88
Available K (extracted by Mehlich-1) (mg kg ⁻¹)	40.10	37.42	287.57
Exchangeable Al (extracted by KCl 1 mol L ⁻¹) (mg kg ⁻¹)	0.00	0.00	0.00
Exchangeable Ca (extracted by KCl 1 mol L ⁻¹) (mg kg ⁻¹)	400.14	693.75	911.50
Exchangeable Mg (extracted by KCl 1 mol L ⁻¹) (mg kg ⁻¹)	184.35	372.20	736.65
Available Cu (extracted by Mehlich-1) (mg kg ⁻¹)	122.39	134.51	124.06
Available Zn (extracted by Mehlich-1) (mg kg ⁻¹)	35.62	36.30	41.92
Available Mn (extracted by Mehlich-1) (mg kg ⁻¹)	141.99	177.09	175.04
Soil solution chemical characterization	<hr/>		
Al (mg L ⁻¹)	0.150	0.274	0.144
Cu (mg L ⁻¹)	0.144	0.230	0.632
Zn (mg L ⁻¹)	0.482	0.003	0.485
Mn (mg L ⁻¹)	0.555	0.220	5.556
Fe (mg L ⁻¹)	0.027	0.072	0.010
Ca (mg L ⁻¹)	100.478	128.235	937.843
Mg (mg L ⁻¹)	105.986	120.622	767.036
K (mg L ⁻¹)	40.614	24.381	703.481
P (mg L ⁻¹)	0.155	0.152	0.649
NO ₃ ⁻ (mg L ⁻¹)	130.787	72.726	844.103
NH ₄ ⁺ (mg L ⁻¹)	3.337	3.502	10.282
SO ₄ ²⁻ (mg L ⁻¹)	46.346	60.847	138.243
Dissolved organic carbon (DOC) (mg L ⁻¹)	20.491	47.023	162.355
pH	5.981	7.392	5.233

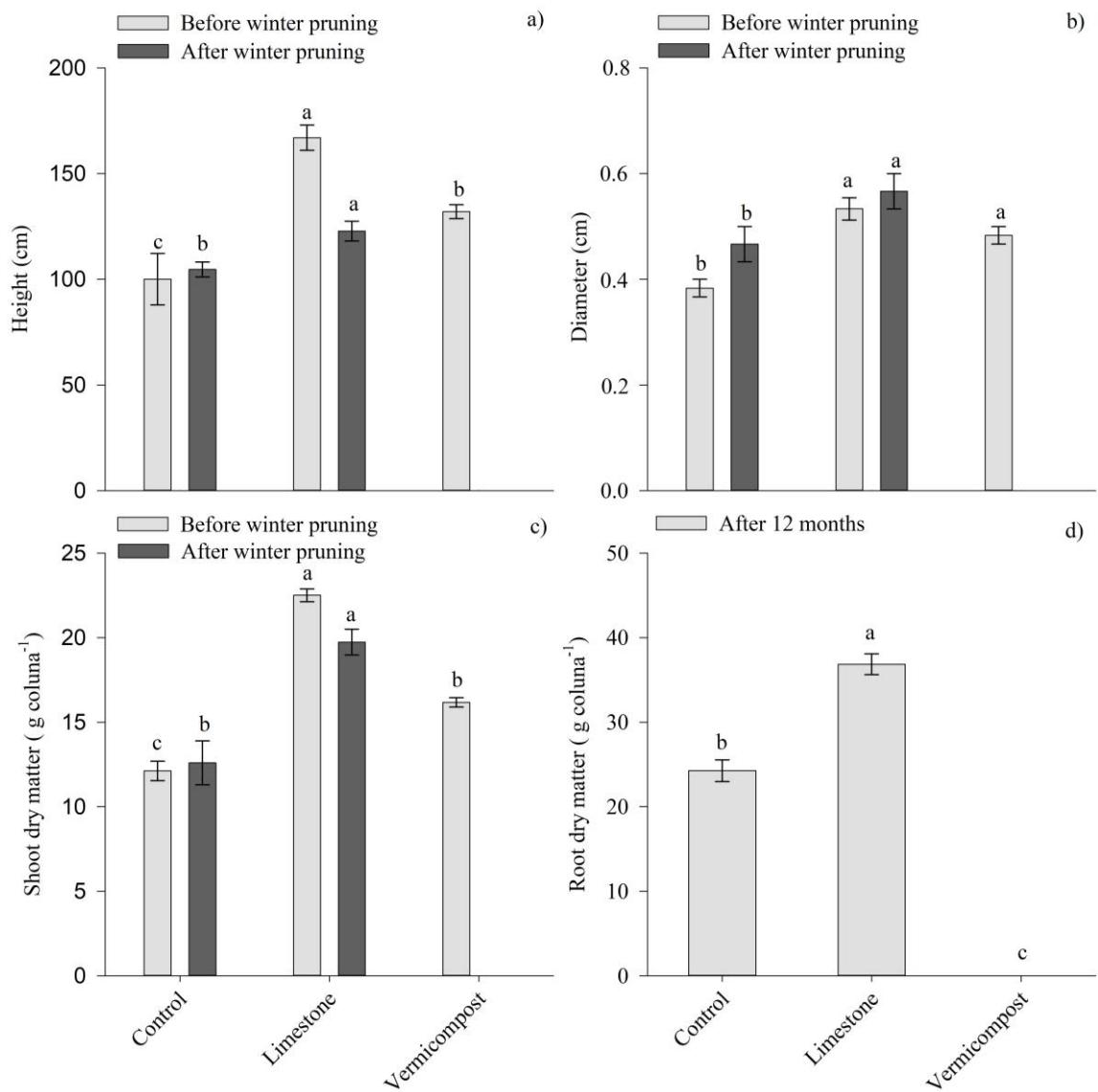


Figure 1. Height (a), stem diameter (b), shoot (c) and root (d) dry matter yield of grapevines (Paulsen 1103 rootstock) grown in soil with high Cu content without amendment (control) and with the application of limestone and vermicompost. Histograms with the same lowercase letter are not statistically different by the Tukey test at 5%. Bars represent standard error ($n = 4$).

Table 2. Nutrient concentration in shoots and roots of grapevines (Paulsen 1103 rootstock) grown in soil with high Cu content without amendment (control) and with the application of limestone and vermicompost before and after winter pruning.

Nutrient	Control	Limestone	Vermicompost	CV%
Shoots before winter pruning				
N (g kg ⁻¹)	39.31 a ⁽¹⁾	32.29 b	32.05 b	5.61
P (g kg ⁻¹)	3.06 b	1.73 c	11.36 a	11.16
K (g kg ⁻¹)	132.30 b	69.30 c	189.00 a	9.29
Ca (g kg ⁻¹)	13.60 c	18.88 b	28.25 a	9.21
Mg (g kg ⁻¹)	9.71 c	13.43 b	20.68 a	6.11
S (g kg ⁻¹)	4.41 a	5.53 a	4.24 a	25.19
Cu (mg kg ⁻¹)	17.55 a	5.01 b	5.21 b	11.85
Zn (mg kg ⁻¹)	76.53 a	34.59 b	44.44 b	18.55
Fe (mg kg ⁻¹)	100.40 a	80.40 b	81.12 b	9.67
Mn (mg kg ⁻¹)	187.25 b	98.78 c	407.30 a	24.49
Shoots after winter pruning				
N (g kg ⁻¹)	17.59 a	16.39 a	-	12.05
P (g kg ⁻¹)	1.17 b	1.48 a	-	16.42
K (g kg ⁻¹)	11.17 a	8.57 b	-	16.74
Ca (g kg ⁻¹)	19.88 a	15.55 a	-	27.39
Mg (g kg ⁻¹)	6.44 a	5.68 a	-	20.75
S (g kg ⁻¹)	1.72 a	1.40 a	-	28.10
Cu (mg kg ⁻¹)	36.86 a	26.73 b	-	12.65
Zn (mg kg ⁻¹)	150.76 a	74.69 b	-	18.01
Fe (mg kg ⁻¹)	39.21 a	44.71 a	-	25.20
Mn (mg kg ⁻¹)	126.98 a	80.26 b	-	25.13
Roots after winter pruning				
N (g kg ⁻¹)	11.95 a	11.71 a	-	17.26
P (g kg ⁻¹)	0.98 b	1.31 a	-	13.60
K (g kg ⁻¹)	2.83 a	2.62 a	-	15.19
Ca (g kg ⁻¹)	18.68 a	18.69 a	-	27.76
Mg (g kg ⁻¹)	4.25 a	3.14 b	-	30.16
S (g kg ⁻¹)	0.85 a	0.56 b	-	25.99

Cu (mg kg ⁻¹)	428.86 a	154.23 b	-	19.32
Zn (mg kg ⁻¹)	524.46 a	140.03 b	-	8.93
Fe (mg kg ⁻¹)	610.53 a	396.38 b	-	36.45
Mn (mg kg ⁻¹)	83.14 a	53.68 b	-	15.98

⁽¹⁾Means followed by the same lowercase letter in rows (treatments) do not differ from each other by the Tukey test at 5% (P<0.05).

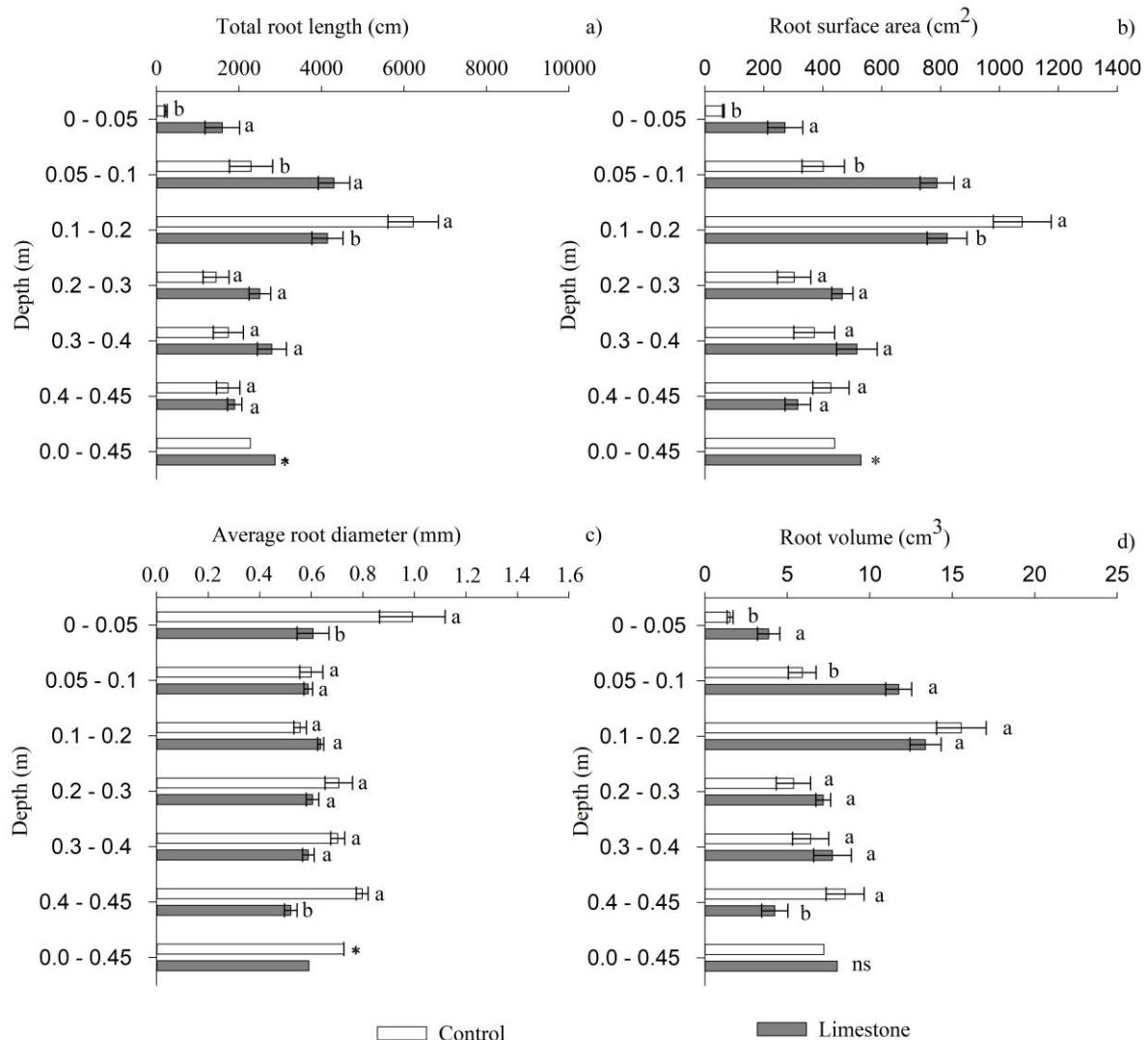


Figure 2. Total root length (a), root surface area (b), average root diameter (c) and root volume (d) of grapevines (Paulsen 1103 rootstock) grown in soil with high Cu content without amendment (control) and with the application of limestone. The treatments were compared at each depth, and throughout the profile of the soil column. Means followed by the same lowercase letter do not differ from each other by the Tukey test at 5%. nsNon-significant and *Significant by the Tukey test at 5%.

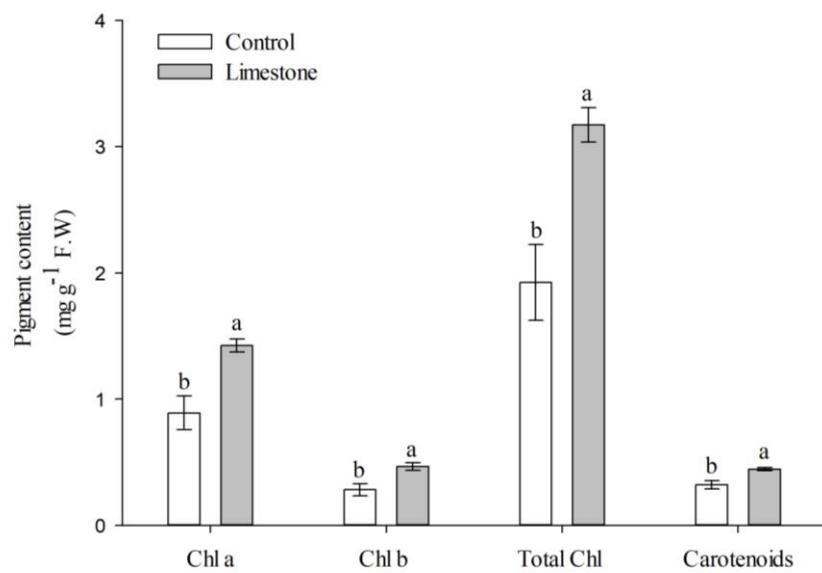


Figure 3. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (Total Chl) and carotenoid contents in leaves of grapevines (Paulsen 1103 rootstock) 12 months after the transplanting of grapevines in soil with high Cu content without amendment (control) and with the application of limestone and vermicompost. Means of the treatments followed by the same lowercase letter are not statistically different by the Student t-test at 5%. Bars represent standard error ($n = 4$).

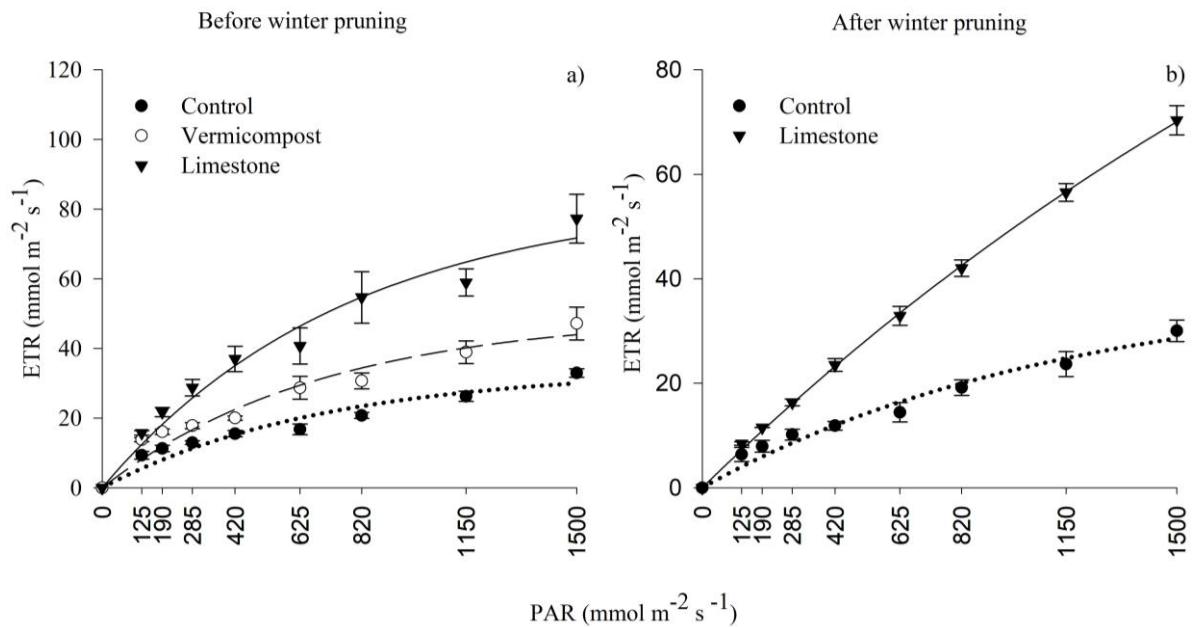


Figure 4. Electron transport rate (ETR) and photosynthetically active radiation (PAR) in leaves of grapevines (Paulsen 1103 rootstock) grown in soil with high Cu content without amendment (control) and with the application of limestone and vermicompost before (a) and after winter pruning (b).

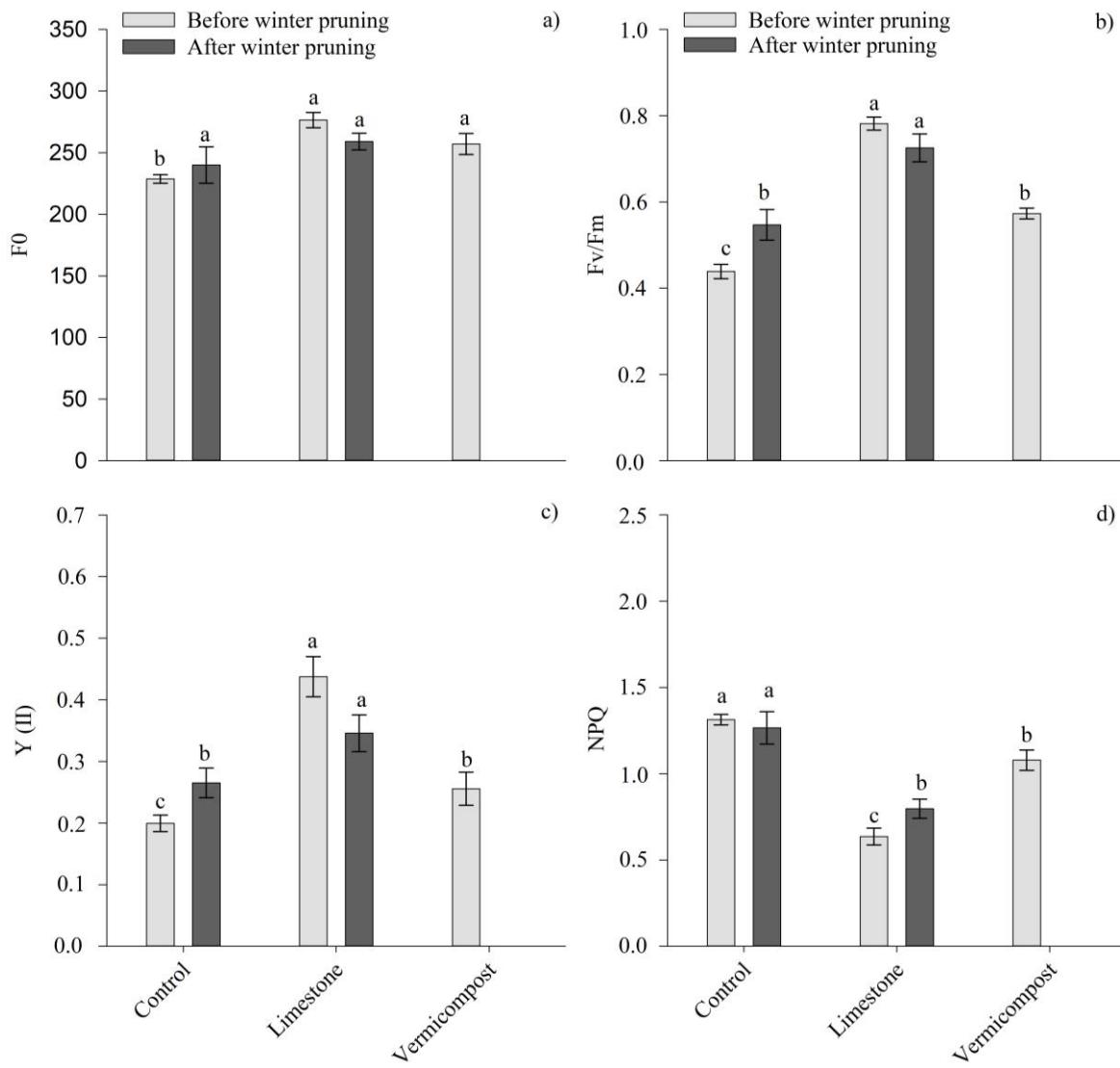


Figure 5. Initial fluorescence (F_0), maximum PSII quantum yield (F_v/F_m) (b), effective quantum efficiency of PSII ($Y(II)$) (c) and non-photochemical quenching (NPQ) (d), before and after winter pruning, in leaves of grapevines (Paulsen 1103 rootstock) grown in soil with high Cu content without amendment (control) and with the application of limestone and vermicompost. Histograms with the same lowercase letter are not statistically different by the Tukey test at 5%. Bars represent standard error ($n = 4$).

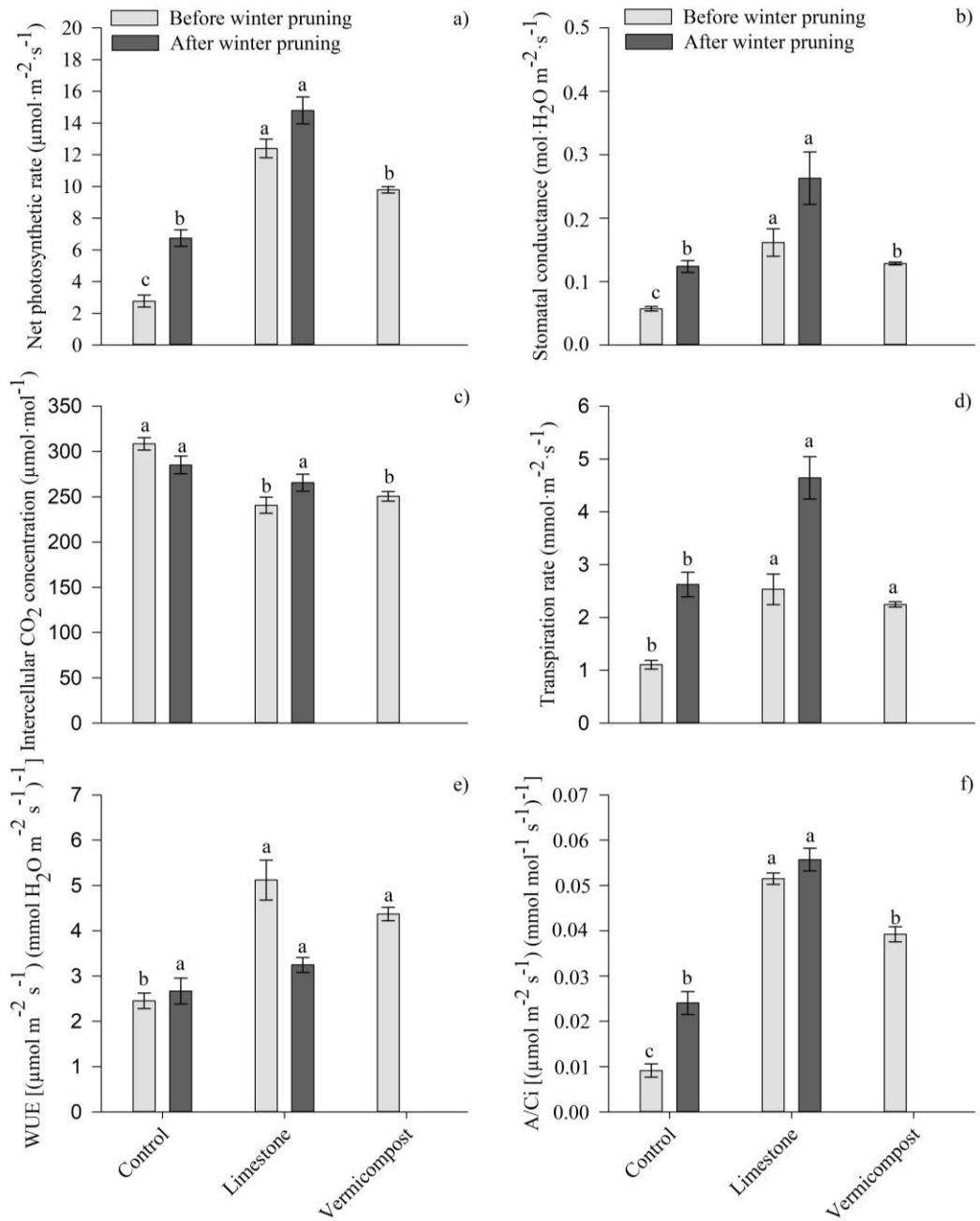


Figure 6. Net photosynthetic rate (A) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (a), stomatal conductance (Gs) ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (b), intercellular CO_2 concentration (Ci) ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) (c), transpiration rate (E) ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (d), water use efficiency (WUE) [$(\mu\text{mol m}^{-2} \text{ s}^{-1})(\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1})^{-1}$] (e) and instantaneous efficiency of carboxylation (A/Ci) (Ribulose-1,5-bisphosphate carboxylase/oxygenase) [$(\mu\text{mol m}^{-2} \text{ s}^{-1})(\text{mmol mol}^{-1} \text{ s}^{-1})^{-1}$] (f), before and after winter pruning, in leaves of grapevines (Paulsen 1103 roostock) grown in soil with high Cu content without amendment (control) and with the application of limestone and vermicompost. Histograms with the same the same lowercase letter are not statistically different by the Tukey test at 5%.

6. DISCUSSÃO GERAL

Aplicações frequentes de fungicidas cúpricos para prevenção e controle de doenças foliares em vinhedos resultam no incremento da concentração de Cu no solo. São aplicadas anualmente em torno de 30 kg de Cu em áreas de vinhedos, especialmente na forma de calda bordalesa ($\text{Ca}(\text{OH})_2 + \text{CuSO}_4$) (BRUNETTO et al., 2014; COUTO et al., 2014). Solos de vinhedos antigos na região da Campanha Gaúcha, por exemplo, aqueles em Santana do Livramento (RS), podem apresentar até 63 mg kg^{-1} de Cu total em vinhedos com 30 anos de cultivo (MIOTTO et al., 2010) e concentrações de Cu total de até 665 mg kg^{-1} em vinhedos com 40 anos de cultivo na região da Serra Gaúcha, em Bento Gonçalves (RS) (CASALI et al., 2008). A disponibilidade de Cu em solução para absorção pelas plantas é maior na região da Campanha Gaúcha devido as características do solo, textura mais arenosa e com menor teor de matéria orgânica, que apresentam menor reatividade ao Cu em relação aos solos mais argilosos e com maior conteúdo de matéria orgânica da região da Serra Gaúcha.

A disponibilidade do Cu no solo é dependente da concentração de carbono orgânico dissolvido, pH e textura do solo (CHAIGNON et al., 2009; KIM et al., 2010; DE CONTI et al., 2016). Solos com pH mais ácido apresentam maior disponibilidade de Cu na forma de Cu^{2+} e Cu^+ , o qual está disponível e ativo na solução do solo para absorção pelas plantas e, consequentemente, intensificando os efeitos de toxidez (Estudo I). O pH ideal para o cultivo de videiras é de 6,0 (CQFS-RS/SC, 2016), sendo que condições de pH mais elevado favorecem a redução da disponibilidade de Cu através da formação de espécies solúveis e insolúveis na forma de hidroxilas, diminuindo problemas de toxidez e melhorando a disponibilidade de outros macronutrientes para absorção pelas plantas (KIM et al., 2010; PÉREZ-ESTEBAN et al., 2014; DE CONTI et al., 2016).

Em resposta a condições adversas de fatores bióticos e abióticos, as plantas podem promover alterações químicas, físicas e biológicas na região da rizosfera (HINSINGER et al., 2005). As plantas podem aumentar ou diminuir o pH rizosférico de acordo com a espécie vegetal, concentração de Cu e pH do solo (BRAVIN et al., 2009a). Em condições de excesso de Cu, as plantas podem aumentar os valores de pH do solo através da exsudação de OH^- , reduzindo a biodisponibilidade de Cu e, consequentemente, sintomas de toxidez (CHAIGNON et al., 2009; HINSINGER et al., 2009; BRAVIN et al., 2009a; 2012). A exsudação de compostos orgânicos também pode contribuir para reduzir efeitos de toxidez nas plantas através da formação de quelatos com o Cu na região da rizosfera e no interior das

raízes, e também promove alterações nos valores de pH (CHAIGNON et al., 2009; KIM et al., 2010; KABATA-PENDIAS, 2011; DE CONTI et al., 2018a).

Com base neste contexto, no estudo I foram observadas maiores concentrações de composto fenólicos e flavonóides nos exsudatos radiculares de plantas de pepino e aveia cultivadas em solução com alta concentração de Cu (50 µM), o que pode atuar como estratégia das plantas em tentar reduzir a disponibilidade de Cu e sua absorção pelas plantas. Alterações no pH rizosférico e exsudação de compostos orgânicos são respostas específicas de cada espécie vegetal a altas concentrações de metais no solo (MEIER et al., 2012; MONTIEL-ROZAS et al., 2016). Neste mesmo estudo I, as plantas de aveia e pepino quando cultivadas em alta concentração de Cu (50 µM) e baixo pH (4,5) apresentaram sintomas de toxidez mais severos em relação as plantas cultivadas em condições de pH mais básico. As plantas apresentaram alterações nos valores de pH da solução, no intuito de aumentar o valor de pH em solução com pH 4,5 e baixar o pH em solução com pH 7,5. Sendo que em solução com alta concentração de Cu, 100% do Cu²⁺ apareceu disponível em solução com pH 4,5, 98% em solução com pH 6,0 e apenas 30% de Cu²⁺ em solução com pH 7,5, ou seja, 70% do Cu²⁺ apareceu ligado com OH⁻, formando moléculas como CuOH⁺, Cu₂(OH)₂²⁺, Cu(OH)₂ e Cu₃(OH)₄²⁺ em solução nutritiva.

Altas concentrações de Cu ocasionam problemas de toxidez às plantas, especialmente videiras jovens transplantadas em áreas de renovação de vinhedos antigos. O excesso de Cu disponível prejudicou o crescimento e desenvolvimento das plantas, conforme observado na em todos os estudos da presente Tese e observado na figura 1, uma redução no conteúdo de matéria fresca e seca de parte aérea e raízes (Estudos I, II, III, IV, e V). Sintomas visuais de toxidez por excesso de Cu são o murchamento e amarelecimento das folhas, evoluindo para folhas secas e senescentes, consequentemente, em casos mais severos, levando a morte das plantas (Figura 1, porta-enxerto Isabel). A redução do crescimento das plantas é reflexo direto dos efeitos tóxicos ocasionados pelas altas concentrações de Cu no tecido, especialmente no sistema radicular das plantas prejudicando a absorção de água e nutrientes pelas plantas (AMBROSINI et al., 2015; 2018). As análises morfológicas de raízes realizadas no estudo I da Tese demonstraram de forma clara a redução da biomassa radicular no tratamento com alta concentração de Cu e pH 4,5 de pepino e aveia em relação aos demais tratamentos. Altas concentrações afetam a capacidade de diferenciação celular no sistema radicular das plantas, ocasionando o engrossamento e encurtamento do ápice radicular, bem como o escurecimento das raízes, e aparecimento de raízes secundárias (AMBROSINI et al., 2015; 2018; GUIMARÃES et al., 2016). Estas alterações morfológicas no sistema radicular foram

observadas de forma clara nos experimentos com porta-enxertos em solução nutritiva (Estudos II, III, IV), conforme observado na figura 2. As maiores concentrações de Cu foram observadas no sistema radicular de todas as plantas cultivadas em alta concentração de Cu (Estudos I, II, III, IV, e V), o qual está localizado principalmente no apoplasto, ligado a parede celular ou também armazenado no vacúolo (AMBROSINI et al., 2015; GUIMARÃES et al., 2016).

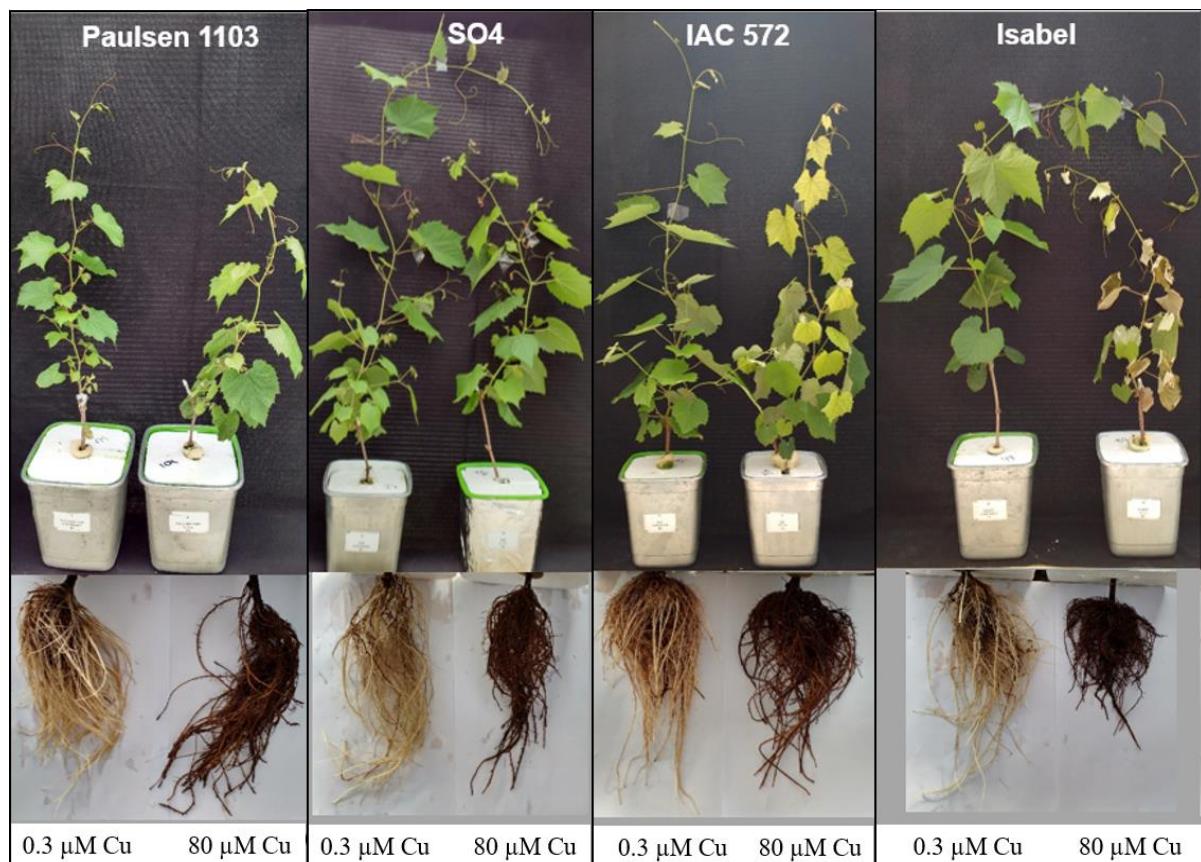


Figura 1- Imagem dos porta-enxertos (PE) de videira (Paulsen 1103, SO4, IAC 572 e Isabel), após 15 dias de cultivo em solução nutritiva controle (0,3 μM Cu) e com alta concentração de Cu (80 μM Cu), proveniente do estudo III.



Figura 2 - Imagem do sistema radicular do porta-enxerto (PE) Paulsen 1103 após 15 dias de cultivo em solução nutritiva controle ($0,3 \mu\text{M Cu}$) e com alta concentração de Cu ($80 \mu\text{M Cu}$), proveniente do estudo III.

Além de prejudicar a absorção de nutrientes devido a alterações anatômicas e morfológicas no sistema radicular das plantas, o excesso de Cu disponível pode influenciar na homeostase mineral nas plantas (DE CONTI et al., 2018a), competindo por absorção com outros nutrientes devido ao efeito de maior concentração do metal, o que pode apresentar variação de acordo com as espécies vegetais e/ou cultivares (MARASTONI et al., 2019a). Altas concentrações de Cu podem influenciar na absorção e translocação de nutrientes, como Mn, Fe, Zn, Ca e P (KOPITTKE e MENZIES, 2006; KOPITTKE et al., 2011; MARASTONI et al., 2019a). Consequentemente, alterando o equilíbrio nutricional das plantas, prejudicando o crescimento e aumentando a sensibilidade ao excesso de metal (Estudos II, III, IV).

Na parte aérea das plantas, altas concentrações de Cu prejudicam a síntese de pigmentos fotossintetizantes, provocam alterações na estrutura e funcionamento do aparato fotossintético, consequentemente, prejudicando a eficiência fotossintética, assimilação de carbono e crescimento das plantas (Estudos III, IV, V). O excesso de Cu no tecido também favoreceu a formação de espécies reativas de oxigênio (ROS), conforme observado nos estudos II e III, onde os porta-enxertos de videira apresentaram um incremento nas concentrações de H_2O_2 e peroxidação lipídica mensurada através da concentração de malondialdeído (MDA), indicando estresse oxidativo em função das altas concentrações de Cu no tecido (MARSCHNER, 2012; ADREES et al., 2015). Em resposta ao aumento na concentração de ROS, as plantas apresentaram maiores níveis de atividade das enzimas POD

e SOD no tecido, como mecanismos de controle bioquímico para evitar estresse oxidativo (MARSCHNER, 2012; ADREES et al., 2015).

Diante da problemática de contaminação dos solos de vinhedos por altas concentrações de Cu e toxicidade as plantas, comprometendo a produtividade destas áreas, é necessário o estudo de estratégias de cultivo para minimizar os problemas de toxidez. A utilização de porta-enxertos (PE) que apresentem tolerância a as altas concentrações de Cu no solo, bem como, a aplicação de substâncias químicas com efeito amenizante ao excesso de Cu, como fósforo, cálcio, calcário e vermicomposto podem ser estratégias de cultivo empregadas em áreas de vinhedos contaminados (Estudos III, IV e V).

As plantas podem responder de diferentes maneiras quando submetidas a condição de estresse abiótico por excesso de metal, de acordo com o genótipo, concentração de Cu e do tempo de exposição das plantas ao metal (YRUELA, 2003; 2009; FIDALGO et al., 2013; MARASTONI et al., 2019a). Existem poucas informações em relação a capacidade de tolerância de PE de videira ao excesso de metal pesado no solo, especialmente de Cu. Desta forma, os estudos III e IV da Tese foram realizados no intuito de verificar a capacidade de tolerância de diferentes PE ao excesso de Cu. Os PE de videira apresentaram diferente comportamento quando submetidos a alta concentração de Cu em solução nutritiva (Estudos III, IV), o que está diretamente ligado a características intrínsecas de cada material genético. Os materiais SO4 e IAC 572 são altamente vigorosos, apresentando rápido crescimento e aporte de biomassa, no entanto, quando cultivados em altas concentrações de Cu apresentaram grandes reduções de crescimento. Por outro lado, o PE Paulsen 1103 apresentou menores variações nos parâmetros de crescimento, alta eficiência fotossintética e assimilação de carbono. Paulsen e SO4 apresentaram os maiores valores de dissipação não fotoquímica de energia (NPQ) em solução com alta concentração de Cu, entretanto apresentaram os maiores valores de taxa de transporte de elétrons (ETR) em solução com alta concentração de Cu. E Isabel, como pé-franco, apresentou os menores valores de ETR em solução com alta concentração de Cu e maior sensibilidade ao excesso de Cu em solução, através de sintomas visuais de murchamento e amarelecimento das folhas (Estudo III). O PE Paulsen apresentou menores alterações nos parâmetros de crescimento e fisiológicos quando cultivado em solução com alta concentração de Cu em relação a solução controle, indicando que este genótipo pode ser mais tolerante ao excesso de Cu.

Além da utilização de PE com maior tolerância ao excesso de Cu, a utilização de amenizantes visando a redução da biodisponibilidade de Cu no solo pode ser utilizado como estratégia de cultivo em áreas contaminadas. Amenizantes tem como princípio a redução da

biodisponibilidade do Cu no solo através da indução de processos químicos como adsorção a superfícies minerais, formação de complexos estáveis com ligantes orgânicos, precipitação superficial e troca iônica (BRUNETTO et al., 2016). Assim, substâncias amenizantes podem desempenhar um papel importante na diminuição da absorção de Cu pelas plantas e consequentemente translocação para a parte aérea e problemas de toxidez, melhorando o crescimento e a produtividade das culturas. Diante disso, os estudos IV e V da Tese foram conduzidos no intuito de verificar a eficiência da utilização de amenizantes ao excesso de Cu sobre porta-enxertos de videira. No estudo IV foram utilizados o P e Ca como tratamento amenizante ao excesso de Cu em solução nutritiva, enquanto que no estudo V foram utilizados o calcário e vermicomposto como tratamento amenizante no solo.

No estudo IV, a aplicação de 62 mg L⁻¹ de P e 400 mg L⁻¹ de Ca não apresentaram efeito amenizante a altas concentrações de Cu (60 µM) em solução nutritiva sobre parâmetros de crescimento dos PE de videira. A adição de Ca apresentou efeito benéfico sobre o crescimento do sistema radicular dos PE SO4 e IAC 572 em relação as plantas cultivadas em solução com alta concentração de Cu, o que pode ser explicado pelo efeito do Ca sobre o fortalecimento da estrutura da parede celular nas raízes através do aumento da espessura da parede celular, minimizando os efeitos de toxidez por excesso de Cu nas raízes, como encurtamento e engrossamento do ápice radicular, evitando sintomas de toxidez o que pode favorecer a eficiência fotossintética das plantas (CHEN et al., 2013; AMBROSINI et al., 2015). Por outro lado, houve uma maior eficiência fotossintética das plantas cultivadas em solução com alta concentração de Cu e adição de P e Ca como amenizantes, o que pode ser explicado pelo efeito benéfico do P e Ca sobre a nutrição mineral das plantas, bem como, manutenção da estrutura e funcionamento do aparato fotossintético das plantas (BALDI et al., 2018a; 2018 b; 2018c; CHEN et al., 2013) e, especialmente, para o Ca o fortalecimento do sistema radicular das plantas (CHEN et al., 2013).

No estudo V, utilizando calcário e vermicomposto como tratamentos amenizantes ao excesso de Cu no solo foi observado um efeito positivo da aplicação de calcário na redução da biodisponibilidade de Cu para absorção pelas plantas, bem como dos sintomas de toxidez. O calcário promove o aumento dos valores de pH do solo e, com isso, ocorre a desprotonação de grupos funcionais ácidos das partículas reativas do solo, aumentando a capacidade de troca de cátions (CTC) e adsorção de Cu, consequentemente, diminuindo a biodisponibilidade de Cu no solo e potencial de absorção pelas plantas (JORIS et al., 2012; AMBROSINI et al., 2015; BRUNETTO et al., 2016). Enquanto que a aplicação de vermicomposto não apresentou efeitos positivos depois de 12 meses de condução do experimento, resultando na não brotação

e morte das videiras após a realização da poda hibernal. No tratamento com aplicação de vermicomposto também foi observado uma alta concentração de Mn disponível em solução para absorção pelas plantas, que juntamente com o Cu prejudicou o crescimento e desenvolvimento das plantas.

A utilização de estratégias de manejo de forma integrada, PE mais tolerantes e aplicação de amenizantes, como calcário para manutenção do pH do solo próximo a 6,0, adubação adequada e manutenção de cobertura verde no solo afim de incrementar os valores de matéria orgânica do solo podem contribuir para a manutenção dos cultivos e redução da biodisponibilidade de Cu em áreas contaminadas. Além destas medidas corretivas e preventivas ao problema de toxidez por excesso de metal pesado no solo, também é importante tentar reduzir as aplicações de fungicidas nas áreas de cultivo, rotacionar o princípio ativo dos produtos utilizados, bem como utilizar produtos com ação sistêmica visando aumentar o efeito residual e reduzir o número de aplicações.

7. CONCLUSÕES GERAIS

As plantas de pepino e aveia apresentaram capacidade de alterar os valores de pH na solução tentando alcançar uma condição ideal de crescimento, visando aumentar ou diminuir a disponibilidade de nutrientes. Essa capacidade foi menos expressiva no tratamento com 50 μM de Cu e pH 4,5, onde as plantas sofreram maior efeito de toxicidade por excesso de Cu.

A exsudação de compostos orgânicos, flavonóides e compostos fenólicos, aumentaram nas plantas (pepino e aveia) cultivadas em solução com alta concentração de Cu (50 μM Cu), que pode atuar como uma estratégia para diminuir a biodisponibilidade e absorção de Cu pelas plantas.

Altas concentrações de Cu prejudicaram o crescimento, aporte de biomassa na parte aérea e sistema radicular, estado nutricional, provocaram alterações na morfologia de raízes nas plantas (pepino e aveia), bem como nos porta-enxertos (PE) de videiras.

Todos os PE de videira apresentaram efeitos negativos sobre atividade fotossintética quando cultivados em solução com alta concentração de Cu em função de alterações na estrutura e funcionamento do aparato fotossintético. Além disso, houve aumento da concentração de H_2O_2 e peroxidação lipídica de membranas em função do excesso de Cu, bem como ativação e síntese de enzimas antioxidantes para controlar o estresse oxidativo.

As maiores concentrações de Cu foram observadas no sistema radicular das plantas, possivelmente no simplasto, ligado a parede celular ou armazenado no vacúolo, minimizando

a quantidade de Cu translocada para a parte e evitando sintomas de toxidez em órgãos mais sensíveis das plantas.

Os PE avaliados apresentaram diferentes respostas ao excesso de Cu em solução de acordo com características intrínsecas de cada material genético. Isabel, como pé franco, apresentou maior sensibilidade detectada visualmente ao excesso de Cu, através do murchamento e amarelecimento das folhas. Os PE SO4 e IAC 572 por serem bastante vigorosos apresentaram os melhores resultados de crescimento, enquanto que Paulsen 1103 apresentou melhor eficiência fotossintética. As plantas de Paulsen 1103 e SO4 tenderam a apresentar melhor comportamento em relação ao excesso de Cu em solução, apresentando potencial de tolerância ao excesso de Cu. O uso de PE tolerantes ao excesso de Cu disponível pode ser uma alternativa de cultivo em locais de vinhedos contaminados com excesso de metais pesados.

A adição de Ca e P como tratamentos amenizante em solução nutritiva apresentaram efeito positivo sobre a atividade fotossintética de todos os PE, em relação a solução com alta concentração de Cu. No entanto, não foi suficiente para promover alterações nos parâmetros de crescimento das plantas, consequentemente, não foram amenizantes eficientes ao excesso de Cu em solução.

A aplicação de calcário como amenizante da fitotoxidez do Cu no solo favoreceu o crescimento do PE de videira Paulsen 1103, funcionamento do aparato fotossintético e reduziu as alterações morfológicas no sistema radicular. Por outro lado, a aplicação de vermicomposto como tratamento amenizante não foi uma alternativa efetiva, ocasionando a morte das videiras, o que pode estar relacionado ao efeito de fitotoxidez pelo excesso de Mn disponível em solução.

8. PERSPECTIVAS DE ESTUDOS FUTUROS

I – Realização de maiores estudos visando a seleção de porta-enxertos de videira tolerantes a altas concentrações de Cu em solução nutritiva;

II – Verificar o efeito de altas concentrações de Cu em porta-enxertos de videira em cultivo com solo contaminado de vinhedos por um período de tempo superior como, por exemplo, um ano de cultivo;

III – Avaliar os efeitos das altas concentrações de Cu sobre plantas de videira enxertadas (porta-enxerto e variedade de produção);

IV – Avaliar a interação do Cu com outros elementos e exsudação de ácidos orgânicos em porta-enxertos de videira cultivados em solo contaminado por altas concentrações de Cu;

V – Observar o efeito da utilização de estratégias de cultivo de forma integrada, porta-enxertos de videira mais tolerantes e aplicação de amenizantes, em solo de vinhedos contaminado por altas concentrações de Cu;

VI – Observar o efeito da utilização de estratégias de cultivo, porta-enxertos de videira mais tolerantes e aplicação de amenizantes, em condições de cultivo à campo.

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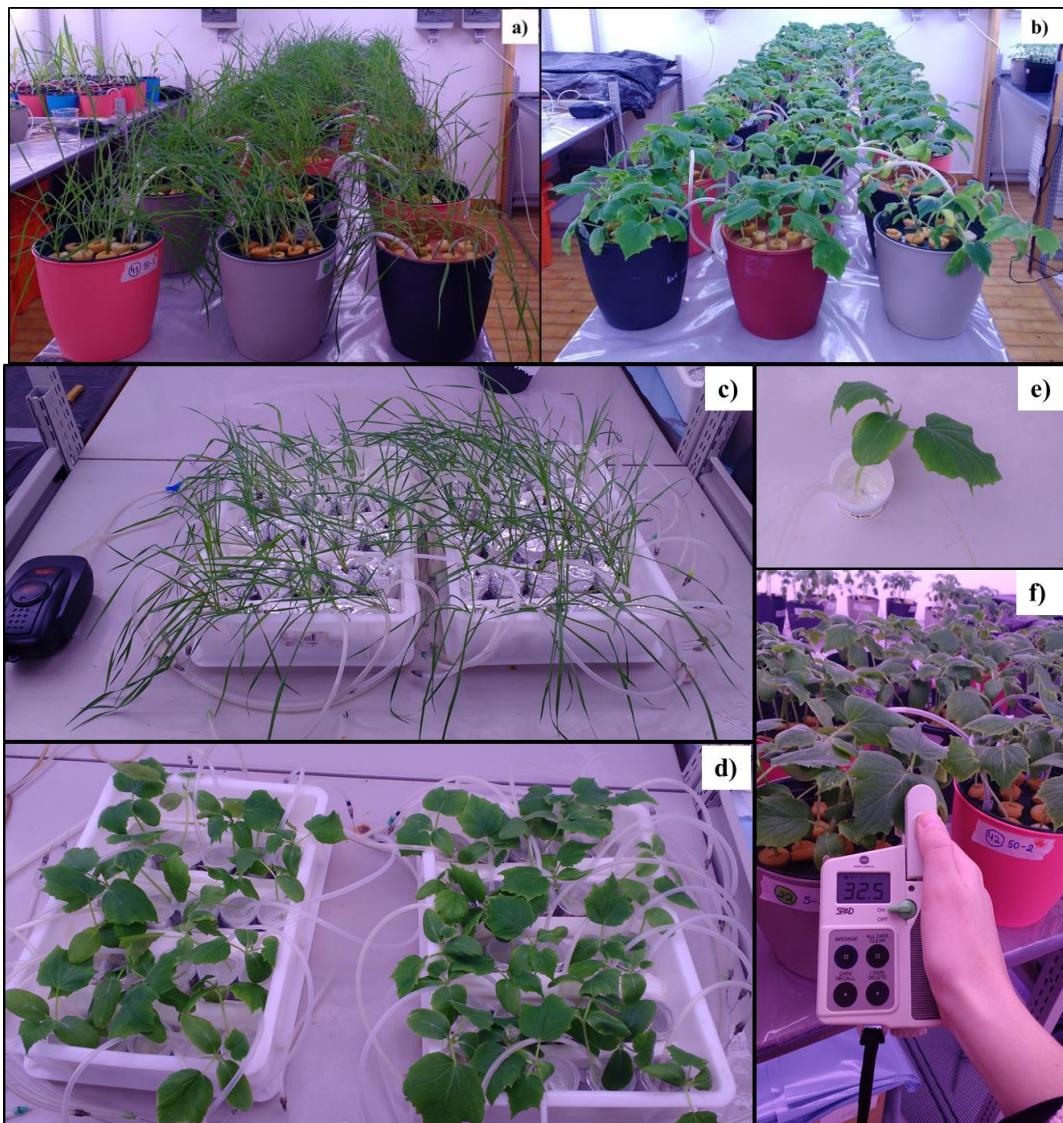
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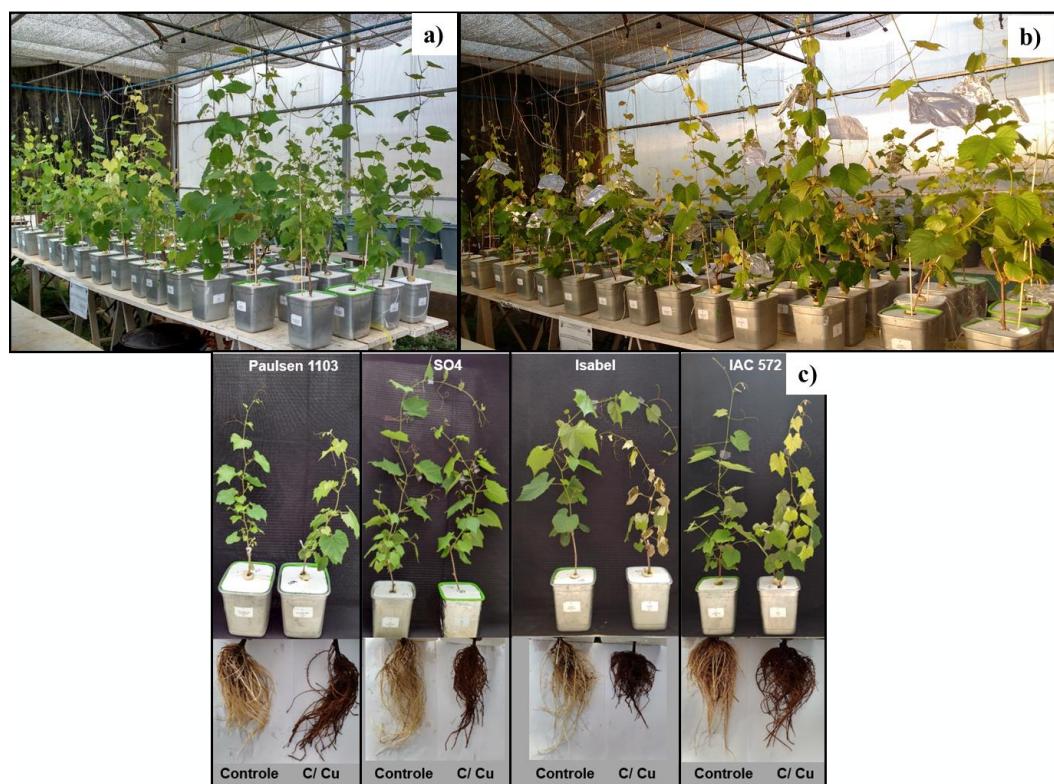
APÊNDICES



Apêndice 1: Imagem do experimento, correspondente ao Artigo I, conduzido em sala de crescimento com plantas de aveia (a) e pepino (b), cultivados em solução nutritiva com diferentes valores de pH (4,5, 6,0 e 7,5) e concentrações de Cu (0,2, 5 e 50 μ M). Coleta de exsudatos radiculares (c, d, e) e avaliação do índice SPAD (f).



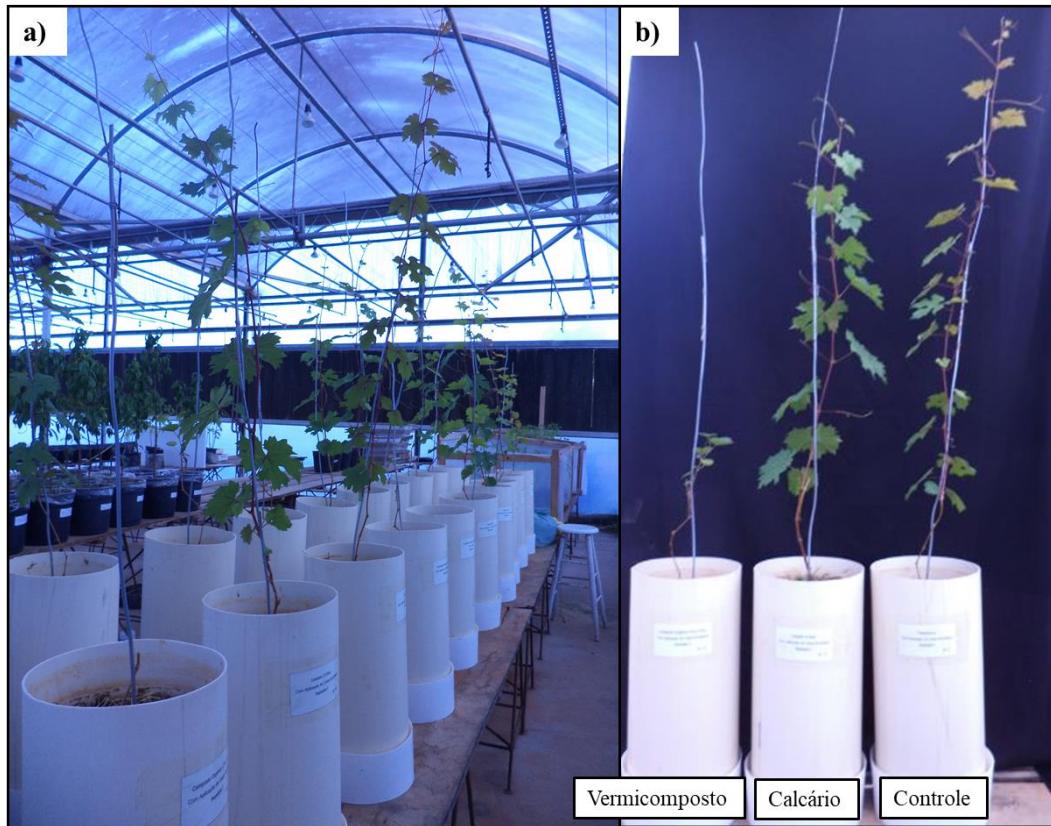
Apêndice 2: Imagem do experimento, correspondente ao Artigo II, conduzido em sala de crescimento com os porta-enxertos de videira Magnolia (a, b) e Paulsen 1103 (c, d), cultivados com diferentes concentrações Cu em solução nutritiva (controle, 20, 40 e 80 µM).



Apêndice 3: Imagem do experimento, correspondente ao Artigo III, conduzido com diferentes porta-enxertos de videira (a) cultivados em solução nutritiva controle e com excesso de Cu (80 µM). Folhas cobertas com papel alumínio para análise de fluorescência da clorofila a (b) e o efeito do excesso de Cu na parte aérea e sistema radicular das plantas (c).



Apêndice 4: Imagem do experimento, correspondente ao Artigo IV, conduzido com diferentes porta-enxertos de videira (a) cultivados em solução nutritiva controle, com excesso de Cu (60 μM) e com aplicação de P e Ca como amenizantes. Efeito do excesso de Cu na parte aérea e sistema radicular das plantas (b).



Apêndice 5: Imagem do experimento conduzido em colunas de pvc, correspondente ao Artigo V (a), e o efeito do excesso de cobre observado nas videiras jovens (Paulsen 1103) de acordo com os tratamentos aplicados, controle, vermicomposto e calcário (b).

Apêndice 6: Receita de grostoli

Caracterização:

O grostoli, também chamado de “crostoli”, “cueca virada” ou “orelhas de gato” é uma receita de origem européia. Chegou ao Brasil juntamente com os imigrantes. Na Europa, o grostoli é um doce típico de Natal e Ano Novo, na Itália é comum no carnaval. Enquanto que no Brasil não apresenta uma data em especial, é consumido durante o ano inteiro.

É uma receita tradicional e deliciosa para o café da manhã e lanches da manhã ou da tarde, que traz no gostinho a lembrança do aconchego da casa dos pais e dos nonos. O grostoli pode ser preparado de várias formas, ficando mais fofinho ou sequinho, podendo ser polvilhado com açúcar e canela, de acordo com o gosto de cada um.

Materiais e soluções:

- 3 ovos;
- 5 colheres de sopa de açúcar;
- 3 colheres de sopa de cachaça;
- 1 colher de sopa de manteiga;
- 1 colher de sopa de fermento químico;
- 1 pitada de sal;
- 2 xícaras de farinha de trigo.

Procedimento de análise:

Misturar bem todos os ingredientes em uma bacia, até a massa ficar homogênea. Deixar a massa descansar por 2 a 3 horas. Espichar a massa com o auxílio de um rolo de macarrão. Cortar a massa em retângulos, fazer um corte central em cada retângulo e virar um dos lados por dentro deste corte para dar o formato do grostoli. Fritar na banha ou óleo quente. Polvilhar com açúcar e canela, opcional.

Determinação:

Grostoli é um ótimo acompanhamento para um café ou chimarrão, especialmente na companhia de pessoas queridas, família e amigos.

Observação: Não importa para onde vamos, o importante é não esquecer das nossas origens e de tudo que nos foi ensinado.