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**COBRE EM SOLOS ARENOSOS SOB VINHEDOS E
RESPOSTA BIOQUÍMICA-FISIOLÓGICA DE
VIDEIRAS EM PRODUÇÃO**

TESE DE DOUTORADO

Alcione Miotto

Santa Maria, RS, Brasil.

2012

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RESPOSTA BIOQUÍMICA-FISIOLÓGICA DE
VIDEIRAS EM PRODUÇÃO**

Alcione Miotto

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**COBRE EM SOLOS ARENOSOS SOB VINHEDOS E RESPOSTA
BIOQUÍMICA-FISIOLÓGICA DE VIDEIRAS EM PRODUÇÃO**

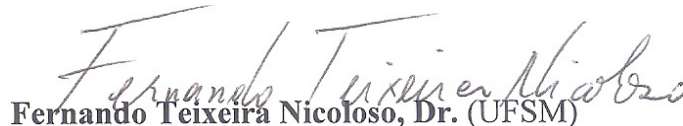
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RESUMO

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

COBRE EM SOLOS ARENOSOS SOB VINHEDOS E RESPOSTA BIOQUÍMICA-FISIOLÓGICA DE VIDEIRAS EM PRODUÇÃO

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No cultivo de videiras, o uso de fungicidas cúpricos para o controle das doenças fúngicas causam o acúmulo excessivo de Cu em solos, o que pode provocar alterações nutricionais às plantas. O trabalho objetivou estudar o acúmulo e formas de Cu em solos arenosos e de caráter ácidos cultivados com vinhedos na região da Campanha Gaúcha do Rio Grande do Sul e as respostas fisiológico-bioquímicas de videiras em produção. O estudo foi realizado nos anos de 2009 a 2011 em vinhedos da cultivar *Cabernet Sauvignon* cultivados na região da Campanha Gaúcha, município de Sant'Ana do Livramento, RS. Primeiramente foram investigados os teores de Cu total (Cu_T) e disponível (Cu_{EDTA}) em 19 vinhedos com diferentes idades e históricos de aplicações de fungicidas a base de Cu. Além disso, também investigou-se a distribuição vertical de Cu_T e Cu_{EDTA} , a quantidade de Cu_T acumulado por hectare e as formas de Cu no solo (fracionamento químico) em três vinhedos com 5, 11 e 31 anos de cultivo. Um solo coletado em uma área sob campo natural, próximo aos vinhedos, foi utilizado para fins de comparação. Na segunda parte do estudo foram avaliados os teores e acúmulo de Cu nos órgãos das videiras e teores de macronutrientes e parâmetros bioquímicos em limbos foliares em vinhedos cultivados em solos com teores crescentes de Cu. As amostras de tecidos das videiras foram coletadas nos ciclos vegetativos 2009/2010 e 2010/2011, em vários estádios fenológicos, antes e também após a aplicação de fungicidas a base de Cu. Os resultados mostram que os vinhedos com mais de 25 anos de cultivo apresentaram teores médios de 90 mg kg^{-1} de Cu_T na camada superficial do solo. Do total de Cu acumulado nos solos, aproximadamente, 77% é Cu_{EDTA} , sendo portanto, potencialmente disponível para as plantas. A principal forma de acúmulo de Cu nos solos dos vinhedos foi ligada à fração mineral (ligados aos óxidos de ferro e manganês), que representou 75% do Cu_T , seguido pela matéria orgânica que complexa apenas 20% do total. Não foram observadas alterações importantes na fração residual de Cu, de maior força de ligação, o que indica a prevalência de formas potencialmente disponíveis. Nas videiras foram observados incrementos nos teores de Cu nas folhas e evidência de estresse oxidativo que foram correlacionados com o aumento dos teores de Cu no solo. Contudo, o aumento da disponibilidade de Cu nos solos pouco afeta os teores e o acúmulo de Cu em videiras em produção e não altera seu estado nutricional. O uso de fungicidas a base de Cu é o principal fator determinante do aumento dos teores de Cu nos órgãos anuais das videiras, especialmente, nas folhas e râquis, mas o resíduo de Cu nas bagas não está relacionado à quantidade aplicada do elemento.

Palavras-chave: Fungicida cúprico, fracionamento químico de cobre, contaminação ambiental, metal pesado, nutrição de plantas, estresse oxidativo.

ABSTRACT

Tese de Doutorado
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COPPER IN VINEYARDS SANDY SOILS AND RESPONSE BIOCHEMICAL- PHYSIOLOGICAL OF THE GRAPEVINE IN PRODUCTIVE SEASON

AUTHOR: ALCIONE MIOTTO

ADVISOR: CARLOS ALBERTO CERETTA

Place and date of defense: Santa Maria, December 20, 2012.

The use of Cu-based fungicides in the cultivation of grapevines for controlling fungal diseases results in excessive accumulation of Cu in soils, which may cause nutritional problems to plants. The present work aimed to investigate the accumulation and forms of Cu in acid sandy soils cultivated with vineyards in the Campanha Gaúcha region and the biochemical and physiological responses of grapevines in productive season. The study was conducted from 2009 to 2011 in the vineyards of the cultivar *Cabernet Sauvignon*, grown in the Campanha Gaúcha region, municipality of Sant'Ana do Livramento, RS. First, the total (Cu_T) and available (Cu_{EDTA}) concentration of Cu were assessed in 19 vineyards at different ages and historic of applications of Cu-based fungicides. In addition, assessments were carried out for the vertical distribution of Cu_T and Cu_{EDTA} , the amount of accumulated Cu_T per hectare and the forms of Cu in the soil (chemical fractionation) in three vineyards aged 5, 11 and 31 years of cultivation. A soil collected from an area under natural field, near the vineyards, was used for comparison purposes. In the second part of the study, concentration and accumulation of Cu in the organs of the grapevines and the concentrations of macronutrients and biochemical parameters in leaf blades were investigated in vineyards grown in soils with increasing Cu concentrations. Tissue samples were collected from vines in the 2009/2010 and 2010/2011 vegetative cycles, at various phenological stages, before and after the application of Cu-based fungicides. The results reveal that the vineyards aged over 25 years of cultivation presented average concentrations of 90 mg kg^{-1} of Cu_T in the surface layer of the soil. Approximately 77% of the total Cu accumulated in the soil is Cu_{EDTA} , and thus potentially available to plants. The main form of accumulation of Cu in the soils of the vineyards was related to the mineral fraction (bound to iron and manganese oxides), which represented 75% of Cu_T , followed by the organic matter that complexes only 20% of the total. No significant changes were observed in the residual fraction of Cu, with higher bond strength, which indicates the prevalence of potentially available forms. In the grapevines, increased Cu concentrations were observed in leaves, as well as evidence of oxidative stress, which were correlated with increased concentrations of Cu in soil. However, the increased availability of Cu in soils slightly affects the concentrations and content of Cu in grapevines in productive season and does not change their nutritional status. The use of Cu-based fungicides is the main determinant of increased concentration of Cu in annual organs of grapevines, especially leaves and rachis, but the residue of Cu in the berries is not related to the amount applied to the element.

Keywords: Copper-based fungicide, chemical fractionation of copper, environmental contamination, heavy metals, plant nutrition, Oxidative stress.


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1 INTRODUÇÃO GERAL

O cultivo de videiras a campo em regiões chuvosas só é possível com o uso de fungicidas para o controle das doenças fúngicas. Alguns dos principais fungicidas utilizados na viticultura usam cobre (Cu) na sua composição, sendo a calda bordalesa o produto mais conhecido. Estes fungicidas são aplicados várias vezes durante um único ciclo de produção e ao longo dos anos causam o aumento dos teores de Cu em solos de vinhedos. Importantes estudos realizados em diversos países mostram que o acúmulo de Cu em solos sob vinhedos é um importante problema ambiental, pois ocorre em extensas áreas pelo mundo. Contudo, mesmo nos países mais desenvolvidos não há uma recomendação consolidada para a utilização de fungicidas cúpricos. Em adição, os órgãos governamentais de controle estabelecem, de forma genérica, os teores máximos permitidos de Cu em solo e não regulamentam a quantidade e frequência das aplicações.

Assim, embora o acúmulo de Cu em solos cultivados com vinhedos tenha sido estudado intensamente nos tradicionais países produtores de uvas, a interpretação dos teores observados em um determinado solo é uma tarefa difícil. Os teores totais de Cu nos solos são determinados por diversos fatores, como a quantidade aplicada, perdas por erosão ou lixiviação e capacidade de sorção, atributo que pode ser bastante diferente entre os solos. Como resultado da interação destes fatores são encontrados na literatura relatos de teores de Cu em solos cultivados com vinhedos variando de ~20 a mais de 1.500 mg kg⁻¹.

Contudo, dependendo das características dos solos, o aumento do teor total não é seguido de um aumento proporcional dos teores disponíveis de Cu. Isto ocorre porque a disponibilidade de Cu em solos é regulada por um grande número de reações químicas. Este metal apresenta alta afinidade com os grupos funcionais da matéria orgânica (MO), óxidos de ferro (Fe), alumínio (Al) e manganês (Mn), e por este motivo é pouco móvel no solo, acumulando-se principalmente na camada superficial. Outro fator preponderante da disponibilidade de Cu é o pH do solo. Em condições de pH neutro ou alcalinos, como ocorre nos solos calcários, o Cu pode ser sorvido pelos carbonatos ou precipitar na forma de Cu(OH)₂ ou CuCO₃. Por outro lado, em solos com pH ácido a sorção de Cu nos óxidos de Fe e Al é dificultada devido à sua maior carga positiva. Assim, a capacidade de um solo de sorver Cu depende principalmente do pH, concentração e constituintes da fração argila.

A mais de três décadas, a região da Campanha Gaúcha do RS salienta-se como uma importante produtora de uvas viníferas do Sul do Brasil. Esta região oferece boas condições

edafoclimáticas para o cultivo da videira e, por isso, nos últimos anos tem recebido investimentos para aumentar a área plantada. Contudo, mesmo com verões mais secos e precipitação pluvial anual de 1.300 mm, os frequentes eventos pluviométricos da primavera e início de verão aumentam a incidência de doenças fúngicas nas videiras e a necessidade de fungicidas. Dentre os principais fungicidas utilizados, destacam-se os cúpricos, cujo uso resulta no acúmulo de Cu nos solos. Esta situação merece maior atenção, pois os solos da Campanha Gaúcha apresentam carácter ácido, baixos teores de argila (em geral < 10%), de matéria orgânica (<1%) e, portanto, baixa capacidade de sorção de Cu. Estudos prévios realizados por Giroto (2010) e Farias (2012) mostraram que em solos arenosos da Campanha Gaúcha teores totais de Cu entre 50 a 90 mg kg⁻¹ causam toxidez em plantas de aveia e batata, ressaltando a necessidade de mais estudos.

Na literatura são encontrados diversos estudos sobre o acúmulo e disponibilidade de Cu nos solos cultivados com vinhedos. Porém, os efeitos do excesso de Cu nos solos sobre a nutrição das videiras são pouco conhecidos, sendo os estudos escassos e discordantes. Testes realizados em vasos com mudas de videiras mostram que o excesso de Cu no solo causa redução do crescimento e acúmulo do metal em raízes, com pouca translocação para a parte aérea. Por outro lado, estudos realizados em condições de campo com videiras em produção cultivadas em solos com excesso de Cu mostram acúmulo do metal no caule, ramos e também elevados teores nas folhas. Em geral, não são observados sintomas de toxidez de Cu em videiras em produção, mas o excesso de Cu no solo pode interferir negativamente na nutrição das plantas e também causar problemas ao seu metabolismo.

2 HIPÓTESES

O uso frequente de fungicidas cúpricos no cultivo da videira na região da Campanha Gaúcha do RS aumenta os teores de Cu em solos sob os vinhedos. Por causa das características dos solos, o Cu acumulado aumenta as formas solúveis e trocáveis, aumentando a sua percolação nos perfis dos solos.

O aumento da disponibilidade de Cu em solos de vinhedos aumenta a sua absorção pelas videiras em produção. O Cu absorvido é acumulado principalmente em raízes, mas parte é translocado para os órgãos aéreos das videiras porém não afeta os parâmetros nutricionais ou causa estresse oxidativo nas folhas.

2.1 Objetivo geral

Avaliar o acúmulo e a disponibilidade de cobre nos solos arenosos sob vinhedos da região da Campanha Gaúcha e seus efeitos sobre a fisiologia de videiras em produção.

ARTIGO I - ACÚMULO, DISPONIBILIDADE E FORMAS DE COBRE EM SOLOS ARENOSOS E DE CARÁTER ÁCIDO CULTIVADOS COM VIDEIRAS

Copper accumulation, availability and fractions in sandy acidic vineyard soils¹

Abstract

The use of copper-based fungicide in control of fungal leaf diseases in vineyards can cause excessive accumulation of Cu in soils. In soils of an acidic nature, with a sandy texture and low organic matter (OM) content, greater Cu availability is expected, which may increase the risk of toxicity to plants and environmental contamination. The study aimed to evaluate the evolution of total content, available content and fractions of Cu in profiles of sandy soils of an acidic nature and planted to grapevines from 2 to 32 years. The study was carried out in vineyards located in the South of Brazil. Nineteen vineyards with different histories of application of copper-based fungicides were selected. Soil samples were collected and submitted to analysis of total content, available content and chemical fractionation of Cu. All the soils are Ultisols and had a sandy texture, low OM content and an acidic nature. The vineyards with more than 25 years under cultivation had mean contents of 90 mg kg⁻¹ of total Cu in the layer from 0.00-0.10 m. Around 77% of the Cu accumulated in the soils proved to be potentially available to the plants. The greatest total and available Cu contents were verified in the soil surface layers, where up to 75% of the total Cu is in the clay minerals and only 20% is complexed in the OM. Important alterations were not observed in the residual fraction of Cu, which indicates the lack of existence of many high binding energy sites.

Keywords: Copper-based fungicide, chemical fractionation of copper, environmental contamination, *Vitis vinifera*.

Introduction

Copper-based fungicides are frequently applied in cultivation of grapevines for control of fungal leaf diseases. This practice leads to accumulation of copper (Cu) in the soil and raises the potential for toxicity to plants and environmental contamination.

¹ Artigo elaborado de acordo com as normas da revista Soil Science Society of America Journal (Anexo A).

In Brazil, the main and oldest winegrowing region is the Serra Gaúcha (mountainous region of Rio Grande do Sul) where some soils may contain up to 665 mg kg⁻¹ total Cu (Casali et al., 2008). Nevertheless, Cu content in the soil may have wide variation in vineyard soils, for example: 20 to 500 mg kg⁻¹ in France (Brun et al., 1998; Flores-Veles et al. 1996); 35 to 600 mg kg⁻¹ in Spain (Arias et al, 2004; Nóvoa-Muñoz et al., 2007); 50 to 300 mg kg⁻¹ in Italy (Viti et al., 2008; Toselli et al., 2009); 100 to 210 mg kg⁻¹ in Greece (Vavoulidou et al., 2005) and 40 to 250 mg kg⁻¹ in Australia (Pietrzak and McPhail, 2004; Wightwick et al., 2008). Such amplitude may mainly be caused by physicochemical differences among the soils and the climatic conditions, among them, average rainfall, which determines the incidence of fungal leaf diseases and, consequently, the quantity of Cu applied (Vavoulidou et al., 2005; Komárek et al., 2010).

The total Cu content in soils also depends on its sorption capacity with functional groups of soil reactive particles, which is a result of diverse components. Sorption capacity increases in accordance with the content of clay minerals, oxides and hydroxides of Fe, Al and Mn, carbonates and organic matter (OM) (McBride, 1994; Bradl, 2004). Soil pH (Chaignon et al., 2003; Bradl, 2004), Cation Exchange Capacity (CEC) and the quality of the OM are important factors that regulate sorption of metals and, therefore, also Cu availability (Brun et al., 2001; Arias et al., 2005). Soils with neutral to alkaline pH values may, in general, bear higher Cu content when compared to soils of an acidic nature, this being explained by the presence of carbonates that favor precipitation and adsorption of Cu (Bradl, 2004). On the other hand, there are also studies that indicate that in soils of an acidic nature, Cu availability is greater (Brun et al., 2001; Chaignon et al., 2003; Pietrzak and McPhail, 2004), which increases its potential for causing toxicity to plants and water contamination.

Studies of Cu availability there is widespread use of total content as a reference for comparing different situations. Nevertheless, although it is a useful indicator, total Cu content is weakly correlated with its availability (Brun et al., 1998; Brun et al., 2001). Available Cu content is controlled by the complex physicochemical reactions of Cu with the diverse soil components (Bradl, 2004), it difficult obtaining more exact diagnoses. Perhaps the best manner of evaluating Cu availability in soils is through cultivation of plants (Brun et al., 1998; Chaignon et al., 2009). However, due to the time necessary and the difficulty of establishing a routine, single or sequential chemical extraction methods are used. As a result, normally single extraction methods are used for determining Cu availability, such as diluted acids, saline solutions (CaCl₂, MgCl₂, NH₄OAc, etc...), chelating agents (DTPA, EDTA) or a

mixture of them (Schramel et al., 2000). One of the most used extraction methods for evaluating availability to plants is EDTA.

Sequential extraction methods are usual when one desires to obtain Cu forms in the soil. Nevertheless, although it is a target of critical analysis due to possible problems in interpretations of the results generated by analytical limitations (Schramel et al., 2000), chemical fractionation is highly important for research on metals in soils (Kennedy et al., 1997). Applying extraction methods with different extraction forces up to complete soil dissolution, the total of accumulated metals is divided into five or more fractions, depending on the method adopted (Tessier et al., 1979; Kennedy et al., 1997). Upon selecting physicochemical fractions that affect metal availability, the method offers indications of the chemical species and of the bond strength of the metal with the soil, which allow inferences in relation the availability in a general way and potential danger of toxicity to plants or transfer to the environment.

The purpose of the present study was to evaluate the evolution of the total content and available content of Cu and of the Cu fractions in profiles of sandy soil of an acidic nature with different periods of time under cultivation of grapevines.

Materials and Methods

The study was conducted with soils from commercial vineyards in the Campanha Gaúcha region, in Sant'Ana do Livramento, located in the state of Rio Grande do Sul, in the extreme south of Brazil. The Ultisol is predominant in the region and is derived from arenite with high silicon content, with sandy texture and lightly sloped to rolling topography (Streck et al., 2002). The climate in the region is subtropical, classified as Cfa (Köppen classification) with average rainfall of 1388 mm year⁻¹ (IPA, 1989). Although summers are drier because of high evapotranspiration, frequent rainfalls occur during the plant and productive cycle of the grapevines, requiring frequent applications of fungicides, especially copper-based fungicides, for prevention of fungal leaf diseases. The quantities of Cu applied via fungicides prior to 1993 ranged from 10 to 15 kg Cu ha⁻¹ year⁻¹. The quantities of Cu applied since 1993 are presented in Table 2.

In all the vineyards studied, the training system was espalier trellis. Up to the end of the 1980s, the soil between the grapevine rows was frequently submitted to harrowing for weed control, and plants located in the plant row were desiccated using herbicide. After 1990, between-row weeds were cut periodically and the residues remained on the soil surface and

the weeds in the plant row continued to be desiccated. In June 2009, 19 commercial vineyards were selected, with increasing ages and, consequently, different histories of copper-based fungicide application (Table 1). The vineyards were placed in five classes according to time under cultivation and management: less than or equal to five years, from six to 10 years, from 11 to 15 years, longer than 25 years, longer than 25 years under cultivation after plowing (Table 1). The last group was composed of three soils that after 30 years under cultivation were plowed for new planting of grapevines. In addition, a natural field (NF) area with no agricultural use, adjacent to the vineyards, was selected, with the Cu soil contents being used as a reference. In the vineyards and in the NF area, soil was collected at the layers of 0.00-0.10 and 0.10-0.20 m. The samples were collected with a spade and each sample was composed of 12 subsamples collected at random in the rows and between rows of each vineyard. The soil was dried, passed in a sieve with a 2 mm screen and submitted to analysis of total Cu (CuEPA) and available Cu (CuEDTA), as described below.

In July 2009, three Cabernet Sauvignon vineyards were selected, designated as vineyard 1 (VN1), vineyard 2 (VN2) and vineyard 3 (VN3), among the 19 vineyards. VN1 was five years old, VN2, 11 years old and VN3, 31 years old. These three vineyards exhibited average conditions among those 19 previously sampled in terms of values of clay, OM, CEC and soil depth, as well as soil topography. The history of application of Cu in VN1, VN2 and VN3 from 1993 to 2009 is described in Table 2. The history of application of Cu in the vineyards before 1993 cannot be recovered. In October 2009, three trenches were open in the three vineyards with dimensions of 1.0 x 1.5 x 0.6 m in a transversal direction to the grapevine row, sufficient for sampling the plant row, the projection of the canopy of the plants and the middle of the between-row area. In the NF area adjacent to the vineyards, three trenches of 1.0 x 1.0 x 0.6 m were also opened, but at random in the area. Soil samples were then collected in the layers of 0.00-0.05, 0.05-0.10, 0.10-0.15, 0.15-0.20, 0.20-0.25, 0.25-0.30, 0.30-0.40 and 0.40-0.60 m. From equal masses of soils collected in the layers cited above, mixtures were made to compose samples from the 0.00-0.05, 0.05-0.15 and 0.15-0.30 m layers for the vineyards and NF. The soil was air dried at 45°C until reaching constant mass, passed through a sieve with a 2 mm screen and set aside for analyses. In the same layers, undeformed soil samples were collected for determination of apparent density (mass/volume), using metallic cylinders of 5.72 cm diameter and 4.0 cm height. The soil content contained in the metallic rings was dried in a laboratory oven at 105°C for 24 h and, soon after, its mass was determined using an analytical balance.

Part of the soil samples from the 19 vineyards and NF were submitted to granulometric analysis in which the pipette method was used (Embrapa, 1997). Soil from the 0.00-0.20 m layer of the NF area was submitted to X-ray diffractometry analysis for identification of the main clay minerals. For that purpose, a sample of the clay fraction was placed on glass slides and analyzed in a Phillips X-ray diffractometer (PW 3710), equipped with a Cu tube. Angular variation from 4 to 35° 2 θ was used with step recording (step = 0.02° 2 θ /s) and time of 0.5 seconds per step. The diffractogram showed high intensity peaks corresponding to the 1:1 kaolinite clay minerals. Peaks related to 2:1 clay minerals showed very low intensity.

Another part of the soil samples were submitted to pH in water analysis, using the soil:water suspension at the proportion of 1:1 v/v, after 30 minutes of equilibrium (Tedesco et al., 1995). The OM content was determined by the Walkley-Black method (Embrapa, 1997). The CEC at pH 7.0 (CEC_{pH7.0}) was calculated by the sum of the exchangeable cations Ca²⁺ + Mg²⁺ + K⁺ plus estimated H+Al (Tedesco et al., 1995). The exchangeable Ca²⁺ and Mg²⁺ contents were extracted using the KCl 1 mol L⁻¹ solution and determined in an atomic absorption spectrophotometer (AAS) GBC (932 AA). The exchangeable K⁺ content was extracted using the Mehlich 1 solution and determined in flame photometer (Tedesco et al., 1995). The value of H+Al was estimated by the equilibrium pH among the soil, water and SMP buffer solution (Shoemaker et al., 1961) at the proportion of 2:2:1, m/v/v (Tedesco et al., 1995). The estimate was made with the use of the equation $H+Al = (e^{10.665 - 1.1488pH_{SMP}})/10$ (CQFS-RS/SC, 2004).

The last part of the soil samples was submitted to analysis of the plant-available Cu contents (CuEDTA) using the EDTA solution (Chaignon et al., 2009). For that purpose, 0.25 g dry soil samples were added to 15 mL capacity centrifuge tubes and agitated for 2 h with 10 mL of the solution containing disodium EDTA 0.01 mol L⁻¹ + NH₄CH₃COO 1.0 mol L⁻¹, with pH corrected to 7.0. After agitation, the suspension was centrifuged, filtered and the Cu content was determined in AAS. The “total” Cu content in the soils (CuEPA) was determined by the 3050B method of the USEPA (United States Environmental Protection Agency) (USEPA, 1996). Samples of 1.0 g of soil were transferred to a 250 mL capacity beaker covered with watch glass for reflux and in heating plate oxidated with HNO₃ and H₂O₂ and afterwards attacked with concentrated HCl. At the end of extraction, the remaining particles were separated by filtering, and the Cu content was determined in AAS. With the CuEPA contents, the quantities of Cu accumulated per hectare in the 0.00-0.60 m layer of VN1, VN2, VN3 and NF were calculated. For this calculation, the mean contents of CuEPA from the

eight layers evaluated were multiplied by the total mass of the soil layer (volume x apparent density). The values were expressed in kg ha^{-1} .

Soil from the 0.00-0.05, 0.05-0.15 and 0.15-0.30 m layers of VN1, VN2, VN3 and NF were submitted to chemical fractionation of Cu (Tessier et al., 1979). In triplicate, 1.0 g of soil was transferred to 50 mL capacity Falcon type polypropylene centrifuge tubes, to which the following extractors were applied in sequence: 1) *Soluble fraction* (CuS) - 8 mL of Mill-Q water; 2) *Exchangeable fraction* (CuE) – 8 mL of MgCl_2 solution 1.0 mol L^{-1} at pH 7.0; 3) *Fraction bound to clay minerals* (CuMin) – 20 mL of the NH_2OHHCl solution 0.04 mol L^{-1} in CH_3COOH 25 % (v/v) at pH 2.0; 4) *Fraction bound to organic matter* (CuOM) – 3 mL of the HNO_3 solution 0.02 mol L^{-1} + 8 mL of H_2O_2 at 30% corrected to pH 2.0 with HNO_3 ; 5) *Residual fraction* (CuR) – total digestion with HF and HClO_4 . Total Cu (CuT) was analyzed in 1.0 g samples of non-fractionated soil, according to CuR. After each extraction, the samples were centrifuged at 3500 RPM for 30 min and an aliquot of supernatant was filtered and set aside for determination of Cu content. Determination of the CuS contents was performed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Perkin Elmer, Optima 2100 DV) and all the others fraction in AAS.

The results were presented with mean values and standard deviations. The differences among soil layers and vineyards were submitted to analysis of variance (ANOVA) and when significant differences ($P < 0.05$) were observed, the Tukey test was applied for comparison of means.

Results

The 19 soils of the vineyards collected in the 0.00-0.10 and 0.10-0.20 m layers showed similar clay, silt, sand and OM contents (Table 3). The mean clay content of the soils was 61 g kg^{-1} , and most showed contents lower than 100 g kg^{-1} and sand values from 800 to 900 g kg^{-1} . Mean OM contents of the soils of the vineyards were 10.5 g kg^{-1} in the 0.00-0.10 m layer and 7.1 g kg^{-1} in the 0.10-0.20 m layer, a little greater than the original contents of the soils, which are 9.0 and 4.8 g kg^{-1} (Table 3). There was no correlation between time under cultivation and the OM content in the soil (data not shown). The values of pH in water of the vineyard soils in the 0.00-0.10 m layer were similar to 6.0; in the 0.10-0.20 m layer near 5.5, greater than the pH in water of the soil of the NF area (Table 3). The $\text{CEC}_{\text{pH}7.0}$ values of the vineyard soils ranged from 3.0 to more than $6.0 \text{ cmol}_c \text{ kg}^{-1}$ (Table 3). There was a positive correlation between values of $\text{CEC}_{\text{pH}7.0}$ and the OM contents ($r=0.93$, $P < 0.01$, in the 0.00-

0.10 layer and $r=0.92$, $P < 0.01$, in the 0.10-0.20 m layer), which shows a strict relationship between these attributes. The values of pH in water and the clay, silt and sand contents of the soils do not correlate with the values of $CEC_{pH7.0}$ (data not presented).

Applications of Cu-based fungicides led to accumulation of the metal in the soil. The natural content of CuEPA of the soil was 3.0 mg kg^{-1} , as observed in the NF (Fig. 1a). In the vineyard soils, the 0.00-0.10 m layer showed, on average, 13.8; 21.7 and 31.2 mg kg^{-1} in vineyards with less than 5, from 6-10 and 11-15 years under cultivation, respectively. In the oldest vineyards of more than 25 years under cultivation, the mean CuEPA content was 90.3 mg kg^{-1} , approximately 30 times greater than the content observed in the NF area. In the 0.10-0.20 m layer, the content was observed to range from 6.0 to 10.0 mg kg^{-1} for the vineyards ≥ 15 years under cultivation, increasing to 32.0 mg kg^{-1} in the soils of the vineyards with more than 25 years under cultivation (Fig. 1a).

The CuEDTA contents showed behavior similar to that observed in the CuEPA contents. The natural content of CuEDTA in the soil of the NF area was 0.65 mg kg^{-1} for both the layers evaluated (0.00-0.10 and 0.10-0.20 m) (Fig. 1b). In vineyards ≥ 15 years under cultivation, the mean contents of CuEPA of the 0.00-0.10 m layer ranged from 8.9 to 22.0 mg kg^{-1} , reaching 73.8 mg kg^{-1} in vineyards with more than 25 years under cultivation (Fig. 1b). In the 0.10-0.20 m layer, the mean CuEPA content was 4.7 mg kg^{-1} in vineyards with up to 15 years under cultivation, increasing to 22.0 mg kg^{-1} in vineyard with more than 25 years under cultivation. Wide variation was observed in the CuEDTA contents and also CuEPA contents in soils of vineyards with similar ages, especially those with more than 15 years under cultivation.

Tilling the soil reduced the Cu contents in the uppermost soil layer. In the 0.00-0.10 and 0.00-0.20 m layers, the mean CuEPA and CuEDTA contents were 90.4 and 73.3 for the vineyard soils >25 years of age, and 40.0 and 28.9 mg kg^{-1} in soils with the same time under cultivation but turned over by tillage, respectively (Fig. 1a and 1b).

The CuEDTA contents of the 0.00-0.10 and 0.10-0.20 m layers showed positive linear correlation with the CuEPA contents (Fig. 2). The equation ($\text{CuEDTA} = -1.93 + 0.79\text{CuEPA}$) indicates that nearly 77% of the CuEPA present in the soil is extractable by EDTA.

Analysis of the soil layers up to 0.60 m depth showed that accumulation of Cu in the vineyard soils occurred mainly in the surface layers (Fig. 3). The natural Cu contents are constant in the soil profile, being less than 3.0 mg kg^{-1} for CuEPA and similar to 1.0 mg kg^{-1} for CuEDTA. In VN1, VN2 and VN3, the Cu accumulated mainly to the depth of the 0.05, 0.20 and 0.30 m layer, respectively. The greatest contents were verified in VN3 where, in the

0.00-0.05 m layer, contents of 113 and 98 mg kg⁻¹ for CuEPA and CuEDTA, respectively, were observed, decreasing with increased depth (Fig. 3). A large gradient was observed in the Cu content among the layers evaluated since in the first three layers (0.00-0.05, 0.05-0.10, 0.10-0.15 m) of VN2 and four layers (0.00-0.05, 0.05-0.10, 0.10-0.15, 0.15-0.20 m) of VN3, the CuEPA contents decreased around 50% for each 0.05 m of depth.

The total sum of CuEPA accumulated in the 0.00-0.60 m layer of the soils of vineyards under cultivation for 5, 11 and 31 years resulted in a net increase of 15, 66 and 175 kg of Cu ha⁻¹, respectively (Table 4). VN3 showed the greatest accumulated quantity of CuEPA, which is greater in all the layers above 0.20 m. As for VN2, the accumulated quantity of CuEPA is greater than in VN1 and NF only in the 0.00-0.10 m layer. No difference was observed in the accumulated quantity of Cu in the soil of VN1 in relation to the soil of the NF in all the layers evaluated, although the soil of VN1 exhibited greater quantities in the 0.00-0.05 m layer (Table 4 and Fig. 3a). The quantity of Cu applied in VN1, VN2 and VN3 was less than the quantity present in the 0.00-0.60 m layer for the three vineyards (Table 4). The difference is a little more than 8 kg for VN1 and near 20 kg for VN2 and VN3. It is fitting to highlight that the quantity of Cu applied from the year 1978 to 1993 is not taken account of in the soil of VN3, due to the absence of data. Nevertheless, it is estimated that the quantity applied in these 15 years is from 150 to 225 kg ha⁻¹, resulting from an application rate of 10 to 15 kg of Cu year⁻¹.

The accumulation of Cu in the soils with grapevines led to large alterations in their fractions in the soil. The natural CuT content (digestion with HF) of the studied soils was a little higher than 4 mg kg⁻¹ in the layers up to 0.30 m, as observed in the soil of the NF area (Table 5). As for VN1, VN2 and VN3, the CuT contents were 10.5, 51.1 and 92.1 in the 0.00-0.05 m layer respectively, and decreased with depth (Table 5). Except in the 0.15-0.30 m layer of VN1, the CuT contents were always greater in the vineyard soils in comparison to the soil of the NF area. The CuEPA and CuT contents were in general very similar among the soils and the layers evaluated, indicating that the mineral matrix of the soil particles contains little Cu in the composition (Table 5).

The increase in Cu content in the soils planted to grapevines was also detected by the different extractors used in fractionation. In comparison to the soil of the NF area, the soils planted to grapevines exhibited, in general, greater content in the CuSol, CuE, Cu-Min and Cu-OM fractions (Table 5). The contents obtained in all the Cu fractions were greater in VN3 in all the layers evaluated (0.00-0.05, 0.05-0.15 and 0.15-0.30 m). In the soil of VN2, alterations in the Cu contents were proportional to the time under cultivation (11 years) and

the quantity of accumulated Cu, being greater in the layers above 0.15 m. As for the soil of VN1, alterations in the fractions were less and restricted to the 0.00-0.05 m layer.

In the soil of the NF area, the CuR and Cu-OM fractions exhibited the majority of CuT of the soil (Table 5 and Fig. 4). However, in vineyard soils, it may be observed that the Cu-Min fraction became most important in Cu sorption, while the CuR content remained constant (Table 4 and Fig. 4). In the VN3 soil, the clay minerals adsorbed 74% of the total Cu of the 0.00-0.15 m layer and 52% of the 0.15-0.30 m layer (Fig. 4). Similar behavior was observed in relation to the quantity adsorbed by the clay minerals in the 0.00-0.05 m layer of VN1 and 0.00-0.15 m layer of VN2.

The greatest content of the Cu-OM fraction was 22.1 mg kg^{-1} , observed in the 0.00-0.05 m layer of the VN3 soil (Table 5), which already exhibited 92.1 mg kg^{-1} of CuT in this same layer and percolation of Cu in the profile (Fig. 3). The percentage of Cu complexed by the OM of the soils under vineyards is little changed in relation to the soil of the NF area, where it represents from 17 to 33% of the total. In the vineyard soil, the Cu-OM contents represent from 14 to 24% of the sum of the fractions of the 0.00-0.30 m layer, except in the surface layer of VN1 (Fig. 4).

The accumulation of Cu in the vineyard soils also increased the CuE and CuSol contents. The greatest alterations in the CuE contents were observed in VN3 in the 0.00-0.05 and 0.05-0.15 m layers and the VN2 0.00-0.05 m layer (Table 4). Increases from 4.5 to 8.8 times in the CuE contents coincide with CuT contents greater than 50 mg kg^{-1} . On the other hand, CuT contents less than 15 mg kg^{-1} had little change on the CuE fraction (Table 4). Data also show that the percentage of CuE in relation to the total extracted decreased as the CuT content increased (Fig. 4).

The CuSol fraction proved to be quite sensitive to the variations of CuT contents. In the 0.00-0.05 m layer, where there was the greatest accumulation, the CuSol increased from undetectable contents ($<40 \text{ } \mu\text{g kg}^{-1}$) of the NF to 42.5, 292.4 and $616.9 \text{ } \mu\text{g kg}^{-1}$ for the VN1, VN2 and VN3 soils respectively. The CuSol contents in the vineyards decreased with depth, but in VN3, the 0.15-0.30 m layer still exhibited $188.6 \text{ } \mu\text{g kg}^{-1}$, which indicates greater availability. In fact, in vineyard soils, in the layers where there was Cu accumulation, the CuSol fraction represented from 0.5 to 1.4% of the sum of the fractions (Fig. 4).

Discussion

The Ultisols used in this study have low OM and clay contents (Table 3) and the predominance of 1:1 clay minerals (kaolinite), which gives them low sorption capacity of

metals like Cu. Kaolinite shows low isomorphous substitution, which results in low density of negative charges (CEC), which is abundant in 2:1 clay minerals and important for sorption of metals (McBride, 1994). In addition, the soils used in this study originated from acidic rocks and do not have carbonates in their composition; therefore, they have an acidic nature. In soils of a limestone origin, in the presence of carbonates and neutral to alkaline pH, Cu may be adsorbed to the carbonates or precipitate in the form of hydroxides or carbonates (Bradl, 2004). These chemical reactions allow greater Cu contents in these soils without presenting harm to plants (Brun et al., 1998; Chaignon and Hinsinger, 2003).

In installation of vineyards, soil liming is recommended to raise the pH in water up to 6.0 (CQFS-RS/SC, 2004), which explains the greater pH of the vineyard soils in comparison to the soil of the NF area (Table 3). Nevertheless, the acidic nature of the soils persists and acid pH values favor maintenance of more available forms of Cu (McBride, 1994; Bradl, 2004). Studies show that in soils of an acidic nature, Cu is more available, which could lead to its toxicity to plants (Brun et al., 2001; Chaignon et al., 2003). Nevertheless, the main objective of liming is not to decrease Cu availability through the increase in pH values of the soil, but rather neutralization of Al^{+3} , which is considered sufficient for good plant growth when the pH in water values reach 5.5 (CQFS-RS/SC, 2004). Liming sufficient for raising the pH up to 6.5 to 7.0 could decrease Cu availability, but the magnitude of this effect and its results on grapevine nutrition still require further studies.

On the other hand, the maintenance of OM contents in the soils of the vineyards examined in this study is the result of soil management techniques adopted as of the 1990s. In this decade, there was the introduction of cover crops like oats (*Avena strigosa*) and clovers (*Trifolium spp.*) in the vineyards, which together with the senescent leaves and residues from trimming the grapevines increased the input of organic matter in the soil (Brunetto et al., 2011). In this same decade, the between-row soils of the grapevines was no longer turned over, which resulted in maintenance of the cover crop, in slower decomposition of the organic residues and, consequently, in reduction of erosion. Maintenance of OM contents is of great importance for fertility of soils of variable charge, especially those with a sandy texture. It is known that in these soils, a large part of the CEC is derived from negative charges of the OM (Syers et al., 1970), like those observed in this study. The increase of CEC results in greater capacity of retaining nutrients like Ca^{2+} , Mg^{2+} and K^+ , which improves plant nutrition and may help to decrease the phytotoxic effects of Cu (Marschner, 1995). In addition, OM controls Cu availability because it has sites with high affinity and binding energy capable of strongly complexing this metal (Besnard et al., 2001; Croué et al., 2002; Bradl, 2004).

In the vineyards studied, it was observed that the use of Cu-based fungicides for around 30 years resulted in relatively high contents of the metal in the soils, if the physicochemical limitations of the soils are considered. Nevertheless, the total contents observed in our study (Fig. 1a and 3a) are similar and in many cases less than that reported in the literature. Diverse studies show that in soils of an acidic nature of different textures planted to vineyards, the total Cu contents ranged from 40 to 665 mg kg⁻¹ (Flores-Veles et al., 1996; Brun et al., 1998; Casali et al., 2008; Fernández-Calviño et al., 2009). In Australia, in soils of an acidic and sandy nature planted to grapevines, total mean contents from 30 to 113 mg kg⁻¹ were reported for the 0.00-0.10 layer in vineyards under cultivation for 20 to 30 years and 34 to 52 mg kg⁻¹ in vineyards under cultivation for more than 90 years (Pietrzak and McPhail, 2004). In Spain, soils of an acidic nature, with less than 230 g kg⁻¹ of clay and with a history of more than 100 years of applications of fungicides, exhibited from 40 to 300 mg kg⁻¹ of total Cu in the 0.00-0.20 m layer (Arias et al., 2004).

The reference values established by government regulatory agencies are not specific for the use of fungicides and, in general, regulate the use of sewage sludge and other wastes. In Brazil, total contents of 60 and 200 mg kg⁻¹ are the prevention and intervention (maximum permitted) contents respectively for Cu in agricultural soils (CETESB, 2005; CONAMA, 2009). The USEPA in the USA establishes the maximum limit of 1,500 mg kg⁻¹ (USEPA, 1993), while the European Community allows from 50 to 140 mg kg⁻¹ of Cu for agricultural soils with pH 6.0 to 7.0 (Council Directive 86/278/EEC, 1986). In Australia and New Zealand, the content of 60 mg kg⁻¹ of Cu indicates the need for environmental studies for evaluation of the situation (ANZECC/NHMRC, 1992). Although they are still within the limits established by regulatory agencies and those reported in the literature, the accumulation of Cu in the vineyard soils of this study is already equal to that of older vineyards of other countries. In addition, some of the vineyards studied exhibit total Cu greater than or equal to 60 mg kg⁻¹, which indicates the need for preventive practices to avoid pollution of these soils (CETESB, 2005; CONAMA, 2009).

The accumulation of Cu in soils is directly connected with the quantity applied, the sorption capacity of the soils and losses through erosion or leaching. Studies show that in regions with greater mean rainfall, the accumulation of Cu in the soil planted to vineyards is greater (Vavoulidou et al., 2005; Komárek et al., 2010). This occurs because the greater the rainfall, the greater will be the incidence of fungal leaf diseases and, consequently, the greater the number of fungicide applications for control of diseases. In our investigation, we observed that the quantity of Cu applied ranges from less than five up to more than 20 kg ha⁻¹ year⁻¹

(Table 1). This is due to the different mean amounts of rainfall, especially in the spring summer period (la niña and el niño phenomena) which affect the need for fungicides. Nevertheless, the application rate is less than that reported in another important wine growing region in the south of Brazil, the Serra Gaúcha region, where up to 30 kg ha⁻¹ year⁻¹ is applied (Casali et al., 2008). In Australia, the annual application rate is from 2-14 kg Cu ha⁻¹ (Pietrzak and McPhail, 2004; Wightwick et al., 2008). As for Europe, rates from 0.5 to 8.7 kg Cu ha⁻¹ year⁻¹ are reported in Italy (García-Esparza et al., 2006; Toselli et al., 2009), and from five to eight applications from 0.5 to 2.5 kg of Cu per year in Greece (Vavoulidou et al., 2005). Nevertheless, in some regions such as Bordeaux in France, the application rate was much greater, arriving at 15 to 30 kg Cu ha⁻¹ year⁻¹ in past decades (Brun et al., 1998). Currently, in the organic production system, the European Community permits the application of up to 30 kg Cu ha⁻¹ every four years (mean value of 7.5 kg Cu ha⁻¹ year⁻¹) (Council Regulation 2092/91/EEC, 1991).

Our data show that Cu accumulation occurred mainly in the surface layers of the soil (Fig. 1a, 1b, 3a and 3b). This is often reported because, due to the high affinity of Cu with the soil constituents, it is normal that greater contents occur at the surface of the soils (McBride, 1994). Once in contact with the soil, the Cu binds to the functional groups of the diverse constituents of the crystalline phase and of the OM (Schramel et al., 2000). In the presence of a large quantity of high affinity functional groups, as for example, groups containing sulfur, nitrogen, carboxyls and phenols (Croué et al., 2003) which are present in OM, Cu will be complexed with high binding energy in not very soluble forms. The same occurs in limestone soils where the Cu binds to carbonates or precipitates in accordance with alkaline pH (Bradl, 2004). In these cases, the metal is less mobile in the soil profile.

The percolation values of Cu observed in our study (Fig. 1a, 1b, 3a and 3b), indicate that the soils studied have a low quantity of high binding energy functional groups. Our data show that the increase in Cu contents in the 0.10-0.20 m layer begins with 11 to 15 years under cultivation, being more intense after this period. In fact, the Cu content was greatest up to 0.60 m in a vineyard with only 31 years under cultivation (Fig. 3) but this does not yet represent a potential risk to groundwater because the contents at deeper layers are low. This movement of Cu in the soil profile is not the result of periodic soil turnover operations, as occurs in some tillage systems in the world. It occurs due to the sandy texture and the predominance of 1:1 clay minerals like kaolinite that results in a limited capacity of sorbing the Cu. In addition, the organic acids released by plants and in the decomposition process of plant residues may chelate the Cu and facilitate its percolation. Added to this is the movement

of particles in the soil profile due to the galleries formed by the biota and by decomposition of plant roots. A similar Cu distribution in the profile of sandy soils with an acidic nature planted to vineyards is reported in France and Australia (Brun et al., 1998; Pietrzak and McPhail, 2004). However, in the soils of the present study, percolation is occurring with lower total Cu contents than those reported by these authors.

The movement of Cu in the soil profile may be accelerated by soil turnover. The vineyards in the southern region of Brazil, like those of the present study, have a useful life of little more than 30 years. At the time of renewal of the vineyards, the plowing and harrowing operations undertaken to level the soil result in movement of Cu in the soil profile (Fig. 1). Inversion of the soil layers reduced the CuT and CuEDTA contents of the 0.00-0.10 m layers and raised them in the 0.10-0.20 m layer (Fig. 1), but not in the same proportion, indicating that the contents were also distributed to layers below 0.20 m. Nóvoa-Muñoz et al. (2007) report very similar total Cu contents (250 to 270 mg kg⁻¹) up to 0.30 m depth and they attribute this to frequent soil turnover in the vineyards. Turning over the soil to dilute the Cu in the profile has been presented as a remedial technique for soils with excessive accumulation of this metal (Pietrzak and Uren, 2011). Nevertheless, although the dilution effect seems to be interesting, increase of Cu contents in the layers below 0.10 m results in greater Cu exposure to plant roots, and may present greater potential for toxicity.

The total sum of CuEPA accumulated in the 0.00-0.60 m layer was calculated for three vineyards and compared with their histories of Cu applications. The total quantities accumulated in this soil layer were a little greater than the quantities applied via copper-based fungicides. The fungicides are not the only input that contains Cu in its composition. Frequent use of organic or chemical fertilizers, often enriched with micronutrients like Cu, may also carry this metal to the soil. In addition, other heavy metals are also found as contaminants in chemical fertilizers (Sharpley and Menzel, 1987) which are frequently used in vineyards. On the other hand, the quantity of Cu applied in the first 15 years of VN3 under cultivation (1978 to 1992) was not found in the 0.00-0.60 m layer, indicating transfer to the environment. Considering that leaching of Cu was not important, in view of its distribution in the soil profiles (Fig. 1a, 1b, 3a and 3b), it may be deduced that part of the Cu throughout the years may have been transferred by the solution in the soil surface, but also adsorbed to particles transported by erosion. The reason for this is that the soils studied have high fragility, due to their sandy texture, and are susceptible to water erosion, even though the topography is only lightly rolling (Streck et al., 2002). However, in the 1980s, the use of disking between the rows of the grapevines was common for weed control. The management technique ended up

exposing soil to the impact of raindrops, which leads to its degradation and surface runoff. In fact, sediments coming from vineyard areas may contain high Cu contents (Pietrzak and McPhail, 2004; Fernández-Calviño et al., 2008). Under other conditions, total accumulated quantity in the soil planted to vineyards may be much greater than that observed in the present study. Brun et al. (1998) report accumulation of up to 215 and 469 kg Cu ha⁻¹ in soils of an acidic and limestone nature, respectively, in the 0.00-0.15 m layer alone.

EDTA is frequently used to evaluate Cu availability to plants. In the soils evaluated in the present study, EDTA extracted nearly 77% of the total Cu and, in theory, this is available to plants. This indicates that the Cu accumulated in the soils of the vineyards is available and the contents are 120 or more times greater than the content necessary for the plants, which is 0.4 mg kg⁻¹ (CQFS-RS/SC, 2004), and this may be excessive for some species. Interestingly, the total content (CuEPA) and content extractable with EDTA exhibited proportional values in all the soils and layers evaluated (Fig. 1a, 1b, 3a and 3b). This shows proportional distribution of high and low binding energy forms of Cu, regardless of the layer and the total content. In addition, the differences of texture, quantity of OM, pH in water values and CEC_{pH7.0} cause little interference in extraction of Cu with EDTA, which may be seen by low dispersion of the points around the straight line of correlation (Fig. 2). The literature reports that EDTA has different extraction capacities in accordance with different soils and total contents. It is reported that, both for soils of an acidic nature and for limestone soils, EDTA may extract less than 10% (Brun et al., 1998), from 20 to 50% (Brun et al., 1998; Chaignon et al., 2003; Toselli et al., 2009) to up to 95% of total Cu (Schramel et al., 2000). The quantity extracted with EDTA generally presents strong correlation with the total quantity, but it is not always correlated with the availability evaluated for plants (Brun et al., 1998; Chaignon and Hinsinger, 2003). Due to the form of activity and time of contact with the soil, this extractor is not sufficiently strong to extract the most stable forms of Cu. Nevertheless, EDTA is a strong extractor, even capable of extracting Cu bound to OM (Arias et al., 2004), which is considered a quite stable form due to its high binding energy (McBride et al., 1998).

Regardless of the layer, the greatest quantity of Cu accumulated in the soils studied is connected with clay minerals (Table 3 and Fig. 4). This fraction is reported as the most important in Cu sorption in soils of an acidic nature and with not very high OM contents (Yu et al., 2004; Casali et al., 2008). Cu has high affinity to the functional groups present in Fe and Mn oxides (Bradl, 2004), but may also be adsorbed with less binding energy in the silanol and aluminol groups of the phyllosilicates (Sposito, 1989; McBride, 1994). However, soil pH alterations greatly affect sorption capacity of clay minerals, especially iron oxides (Sposito,

1989). In acidic pH values, sorption is less and desorption is facilitated (Bradl, 2004; Arias et al., 2005; Casali et al., 2008). For these reasons, the Cu fraction bound to minerals is considered available (Tessier et al., 1979). The large quantity of Cu sorbed in the mineral fraction explains the high availability to the plants evaluated in extraction with EDTA (Fig. 1b, 2 and 3b).

The quantity of Cu complexed in the OM was many times less than the quantity adsorbed in the clay minerals. The maximum quantity of Cu sorbed in the organic fraction in the soils of the vineyards ($<22 \text{ mg kg}^{-1}$) is within the lowest already reported in the literature. Important studies show that the OM of the soil may retain the majority of total Cu of contaminated soils and they report contents that often reach 40 to up to 400 mg kg^{-1} (Pietrzak and McPhail, 2004; Nóvoa-Muñoz et al., 2007; Fernández-Calviño et al., 2009). In percentage, these contents represent 20%, like those observed in the present study, and often 40% to even 80% of the total accumulated. This amplitude is evidently due to the differences in the OM and Cu contents, but is also due to the differences in the methodologies used (Kennedy et al., 1997). Nevertheless, from the fractionation data, it may be perceived that the OM of the soils of the present study exhibit small total capacity of sorption of Cu and may be saturated in the surface layers of the oldest vineyards. This hypothesis is based on the fact that even if there is greater affinity of Cu with the OM groups (McBride, 1994; Besnard et al., 2001; Croué et al., 2002), the greatest quantity of Cu is being accumulated in the CuMin fraction (Table 5 and Fig. 4).

The soluble and exchangeable fractions are considered to be mobile in the soil and easily available (Tessier et al., 1979). The exchangeable phase is in rapid equilibrium with the soil solution (Sposito, 1989). In solution, the Cu moves in accordance with and in the direction of water flow, and may, for example, be transported to the roots of plants and taken up (Marschner, 1995) or percolate into the soil profile. The soluble Cu contents observed in the present study represent little of the accumulated total ($<1.4\%$), which was expected due to the high affinity of Cu with the soil, which results in its low solubility (Sposito, 1989; McBride, 1994). Although the soluble content has increased of not detectable at $616.9 \text{ } \mu\text{g kg}^{-1}$ or 0.6 mg kg^{-1} (Table 5), it is within the range normally observed in soils of an acidic and sandy nature with accumulation of Cu. Pietrzak and McPhail (2004) report soluble Cu contents which are generally less $<1.0 \text{ mg kg}^{-1}$ to up to 1.4 when total Cu was <150 and 250 mg kg^{-1} , respectively. Nóvoa-Muñoz et al. (2007), showed that soluble Cu may vary from 0.5 to 3.8 mg kg^{-1} when the total contents are from 104 to 632 mg kg^{-1} .

The same behavior may be observed with the exchangeable Cu contents shown in Table 5 and Fig. 4 (all $<2.5 \text{ mg kg}^{-1}$). This occurs due to the fact that once certain maximum sorption capacity limits have been reached, which are different for each soil, there is a more pronounced increase of soluble and exchangeable forms. This may be seen in the surface layers of the vineyards with greater accumulation of Cu (Table 5). In the literature, exchangeable Cu contents extracted with different salts are reported (for example, CaCl_2 , $\text{NH}_4\text{CH}_3\text{COO}$, NH_4NO_3 or MgCl_2 , used in the present study) which generally are less than 10 mg kg^{-1} (Pietrzak and McPhail, 2004; Arias et al., 2004; Casali et al., 2008; Fernández-Calviño et al., 2009). However, when the total Cu contents are higher, the exchangeable Cu content may even be from 20 to 36 mg kg^{-1} (Nóvoa-Muñoz et al., 2007; Fernández-Calviño et al., 2009). The increase of the exchangeable and soluble content is connected with the equilibrium existing among the diverse forms of Cu and also partial saturation of the sites with greatest binding energy of the Fe, Mn and OM oxides (Sposito, 1989; McBride, 1994; Bradl, 2004).

Regardless of the fractionation scheme used, the residual fraction represents the most stable or unavailable forms of Cu. As may be observed in Table 5 and Fig. 4, under natural conditions, Cu was found predominantly in the residual fraction, a fact frequently reported in fractionations. On the other hand, under conditions of Cu accumulation, the behavior of the residual fraction differs among the soils. In the soils evaluated in the present study, the residual fraction is very small ($<4 \text{ mg kg}^{-1}$, Table 3) with differences of $\sim 1 \text{ mg kg}^{-1}$ among the layers and vineyards, showing the scarcity of high binding energy groups. The low residual Cu content shown in this study may be attributed to low OM and clay contents, in which there is the predominance of kaolinite. These characteristics determine the maintenance of nearly all the Cu in the available or potentially available forms. These data are among the lowest already reported and differ from most previous studies, which in general show that accumulation of Cu is followed by an increase of the residual fraction. In soils with clay contents less than 20%, the Cu residual fraction may contain less than 30 mg kg^{-1} (often $<10 \text{ mg kg}^{-1}$) (Pietrzak and McPhail, 2004; Arias et al., 2004) but in some soils and textures, it may reach values of 40 to up to 200 mg kg^{-1} (Nóvoa-Muñoz et al., 2007; Casali et al., 2008; Fernández-Calviño et al., 2009). These contents represent on average from 15 to 25 % of the accumulated total, but in some soils, this percentage is greater than 35%. This capacity of inactivating the Cu in the residual fraction is directly related to the greater presence of recalcitrant organic carbon, amorphous inorganic materials and high crystallinity Fe and Al

oxyhydroxides and also reaction time (Tessier et al., 1979; McBride, 1994; Fernández-Calviño et al., 2009).

Conclusions

1- Sandy soils of an acidic nature in vineyards show accumulation and high availability of copper. Approximately 77% of the total copper accumulated in these soils was extracted with EDTA and is potentially available to the plants. In the vineyards, the copper accumulation occurs mainly in the surface layers of the soil and is most notable up to the 0.05, 0.20 e 0.30 m depth layers in vineyards of 5, 11 and 31 years old, respectively. Percolation of copper below the 0.00-0.10 m layer could be perceived in vineyards of 11 years under cultivation and is most intense after 15 years of cultivation. The main form of copper accumulation in the soils is connected with clay minerals followed by organic matter. The exchangeable copper fraction did not increase in a proportional manner to total contents. On the other hand, soluble Cu content increased in a proportional manner to total contents, which makes its transfer to the environment or movement to plant roots possible. Alterations of the residual fraction were very small, which indicates the small number of high binding energy sites determining the predominance of more available forms.

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Table 1. Cultivars, time under cultivation and geographic location of the 19 vineyards of the southern region of Brazil.

Variety	Year Planted	Cultivation time	Class	Geographic coordinate	
				----- year -----	latitude
Tannat	2007	2	1-5	30° 47.872' S	55° 21.933' W
Tannat	2004	5	1-5	30° 46.807' S	55° 23.108' W
Cabernet Sauvignon	2004	5	1-5	30° 46.689' S	55° 22.576' W
Cabernet Sauvignon	2004	5	1-5	30° 46.853' S	55° 22.471' W
Cabernet Sauvignon	2003	6	6-10	30° 46.831' S	55° 21.618' W
Cabernet Sauvignon	2003	6	6-10	30° 47.524' S	55° 21.980' W
Cabernet Sauvignon	2000	9	6-10	30° 47.849' S	55° 21.793' W
Cabernet Sauvignon	1999	10	6-10	30° 47.722' S	55° 21.933' W
Tannat	1996	13	11-15	30° 46,957' S	55° 22.571' W
Tannat	1996	13	11-15	30° 46,863' S	55° 23.080' W
Cabernet Sauvignon	1981	28	>25	30° 46.890' S	55° 21.348' W
Tannat	1980	29	>25	30° 47.122' S	55° 21.648' W
Tannat	1980	29	>25	30° 47.134' S	55° 21.659' W
Cabernet Sauvignon	1980	29	>25	30° 47.170' S	55° 22.566' W
Cabernet Sauvignon	1978	31	>25	30° 47.616' S	55° 22.060' W
Tannat	1977	32	>25	30° 47.594' S	55° 22.659' W
Cabernet Sauvignon	1977	32	>25R†	30° 47.470' S	55° 23.455' W
Cabernet Sauvignon	1977	32	>25R†	30° 47.701' S	55° 23.396' W
Cabernet Sauvignon	1977	32	>25R†	30° 47.746' S	55° 23.398' W

†Vineyard whose soil was turned over through disking for replanting after 30 years under cultivation. This management procedure was performed in 2007, two years before soil sampling.

Table 2. Quantity of copper applied per annual cycle (crop season) and total quantity in three Cabernet Sauvignon vineyards cultivated in the southern region of Brazil.

Crop	Vineyard 1	Vineyard 2	Vineyard 3
	----- Cu, kg ha ⁻¹ -----		
93/94			9.62
94/95			19.16
95/96			12.17
96/97			6.82
97/98			8.80
98/99			12.36
99/00			9.25
00/01			17.24
01/02			13.14
02/03		7.43	13.68
03/04		12.33	21.05
04/05		4.16	8.63
05/06		4.31	3.36
06/07	3.17	9.54	2.18
07/08	2.01	5.66	0.25
08/09	1.16	1.60	-
Average	2.11	6.41	10.51
Total	6.34	45.05	157.72

Table 3. Chemical and physical attributes of the surface layer (0.00-0.10 and 0.10-0.20 m) of 19 soils collected from vineyards with different histories of application of copper-based fungicides and in a natural field area in the southern region of Brazil.

Cultivation time	n	Depth	Clay	Silt	Sand	OM‡	pH	CEC _{pH 7.0} §
years		m	----- g kg ⁻¹ -----					cmol _c kg ⁻¹
Natural field	1	0.00-0.10	54	47	899	9.0	5.2	3.2
		0.10-0.20	63	51	886	4.8	4.9	2.9
1-5	4	0.00-0.10	66 ±13	73 ±22	861 ±23	10.3 ±0.9	5.7 ±0.2	4.4 ±0.7
		0.10-0.20	70 ±36	79 ±18	850 ±18	7.0 ±2.0	5.3 ±0.3	3.9 ±0.9
6-10	4	0.00-0.10	45 ±23	72 ±7	883 ±21	7.9 ±0.9	6.2 ±0.5	3.8 ±0.5
		0.10-0.20	48 ±20	68 ±6	885 ±14	4.9 ±0.3	5.7 ±0.2	3.0 ±0.3
11-15	2	0.00-0.10	66 ±21	85 ±1	848 ±21	12.1 ±6.1	5.6 ±0.3	5.3 ±1.6
		0.10-0.20	75 ±19	71 ±9	854 ±11	7.2 ±2.4	5.5 ±0.3	4.4 ±1.0
>25	6	0.00-0.10	55 ±27	66 ±18	879 ±11	9.7 ±4.0	5.9 ±0.3	4.1 ±1.4
		0.10-0.20	63 ±51	73 ±22	863 ±30	6.6 ±2.1	5.4 ±0.3	3.4 ±0.8
>25 under cultivation†	3	0.00-0.10	59 ±29	95 ±15	846 ±33	12.5 ±2.6	6.2 ±0.1	5.2 ±0.8
		0.10-0.20	72 ±38	80 ±18	848 ±23	9.9 ±2.0	5.9 ±0.1	4.9 ±1.0

†Group of soils that were turned over with a disk for replanting of the vineyards after 30 years of cultivation. This management procedure was performed two years before soil sampling.

‡OM. organic matter = Organic carbon × 1.72.

§CEC_{pH 7.0}. cation exchange capacity at pH 7.0 = sum of exchangeable Ca²⁺, Mg²⁺, K⁺ and estimated H + Al.

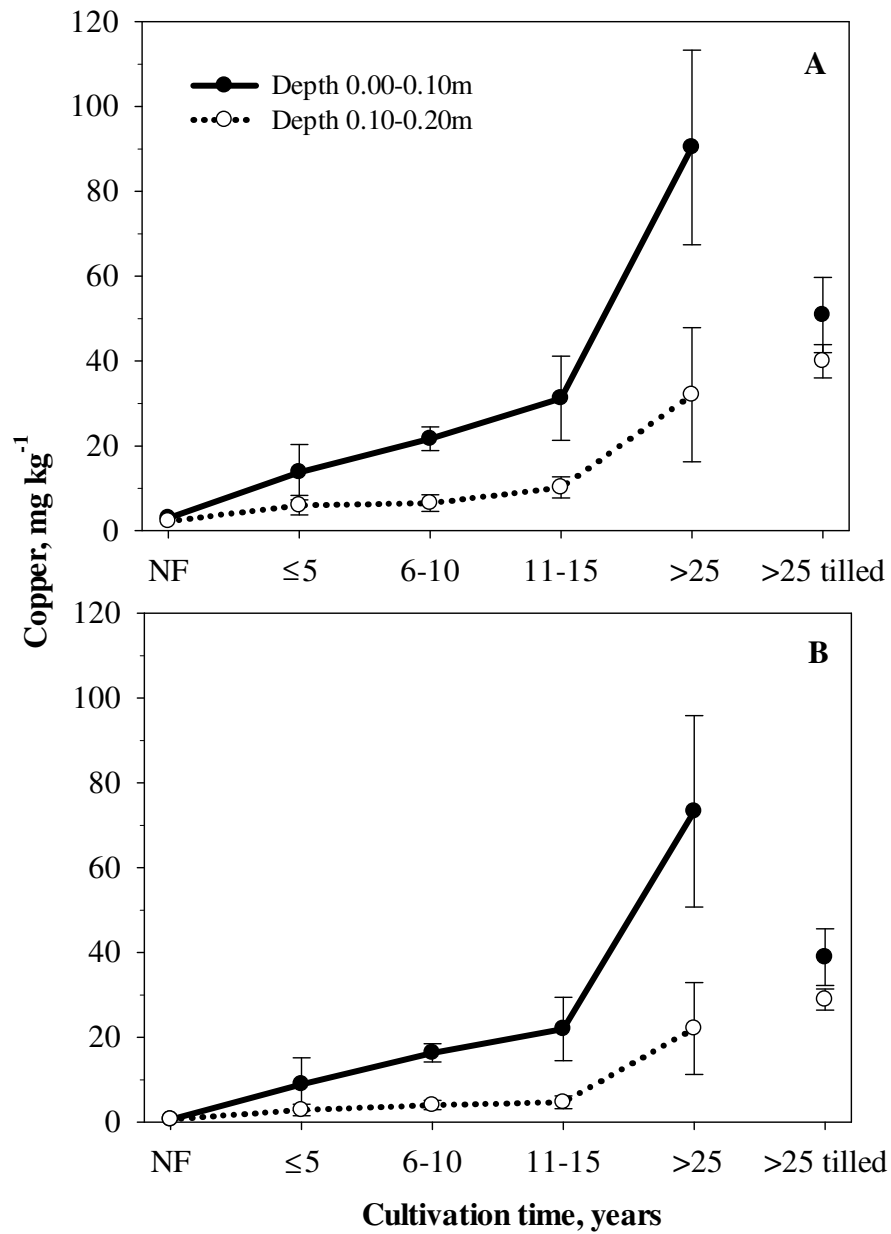


Fig. 1. Total copper “CuEPA” (A) and available copper “CuEDTA” (B) contents in two layers (0.00-0.10 and 0.10-0.20 m) of 19 soils collected in vineyards with different histories of application of copper-based fungicides and in a natural field area in the southern region of Brazil.

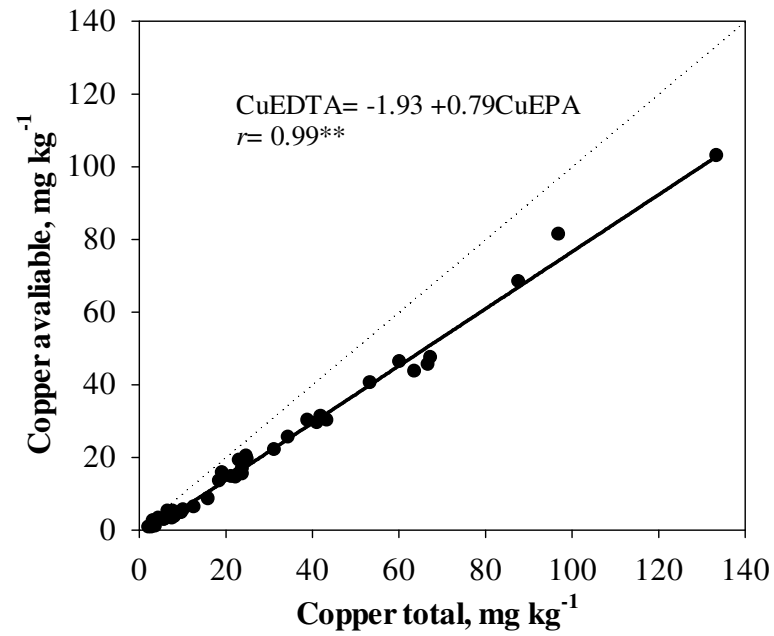


Fig. 2. Correlation between total “CuEPA” and available “CuEDTA” Cu of the layers (0.00-0.10 and 0.10-0.20m) of 19 soils collected in vineyards with different histories of application of copper-based fungicides and in a natural field area of the southern region of Brazil (n=40). ** Significant at P <0.01.

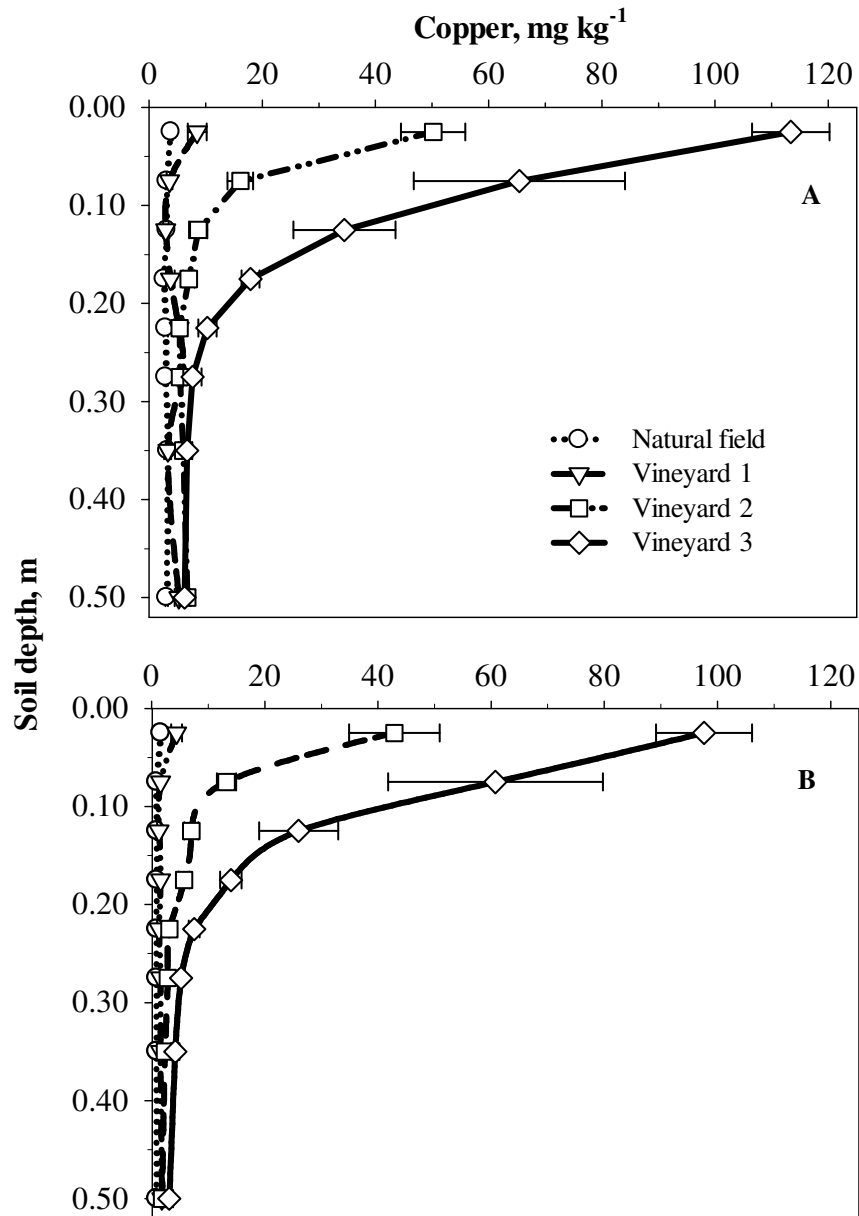


Fig. 3. Total “CuEPA” (A) and plant-available “CuEDTA” (B) copper content from eight soil layers collected in three vineyards with different histories of application of copper-based fungicides and in a natural field area in the southern region of Brazil.

Table 4. Quantity of total copper “CuEPA” accumulated per hectare in different layers of soil collected in three vineyards with different histories of application of copper-based fungicides and in a natural field area of the southern region of Brazil.

Depth	Natural field	Vineyard 1	Vineyard 2	Vineyard 2
m		Copper, kg ha ⁻¹		
0.00-0.05	2.59 ^{NS} C†	5.80 ^{NS} C	32.90 aB	76.11 aA
0.05-0.10	2.30 C	2.73 C	11.59 bB	46.72 bA
0.10-0.15	2.37 B	2.28 B	6.38 bB	25.84 cA
0.15-0.20	2.04 B	2.95 B	5.45 bB	13.54 dA
0.20-0.25	2.23	4.04	4.15 b ^{NS}	7.70 de
0.25-0.30	2.22	4.44	4.16 b	5.75 e
0.30-0.35	2.44	2.51	4.49 b	5.02 e
0.35-0.40	2.44	2.51	4.49 b	5.02 e
0.40-0.45	2.29	3.92	5.01 b	4.64 e
0.45-0.50	2.29	3.92	5.01 b	4.64 e
0.50-0.55	2.29	3.92	5.01 b	4.64 e
0.55-0.60	2.29	3.92	5.01 b	4.64 e
Sum	27.79	42.94	93.65	204.26
Net increase‡	-	15.15	65.86	176.47
Applied amount §	-	6.34	45.05	157.72
Difference		-8.81	-20.81	-18.75

** Significant at P < 0.01. ^{NS} Not significant.

† Mean values followed by the same small letter in the columns and capital letters in the lines do not differ among themselves by the Tukey test at P < 0.05.

‡ The existing quantity of Cu in the natural field was subtracted (27.79 kg).

§ Values obtained with the sum of the quantity applied from the year 1993 to 2009.

CV for layers = 34.17; CV for vineyards = 37.48.

Table 5. Copper content in individual fractions of soils collected in three vineyards with different histories of application of copper-based fungicides and in a natural field area in the southern region of Brazil.

Soil depth	Natural field	Vineyard 1	Vineyard 2	Vineyard 3	CV
m		Soluble (H ₂ O), µg kg ⁻¹			%
0.00-0.05	ND †	42.51	292.37	616.88	-
0.05-0.15	ND	ND	113.04	430.91	
0.15-0.30	ND	ND	54.73	188.64	
		Exchangeable (MgCl ₂), mg kg ⁻¹			
0.00-0.05	0.27 bC‡**	0.45 bC	1.51 aB	2.39 aA	11.46§
0.05-0.15	0.55 aB	0.70 aB	0.67 bB	2.50 aA	6.67¶
0.15-0.30	0.60 aBC	0.70 aAB	0.41 cC	0.85 bA	
		Mineral fraction (NH ₂ OHHCl), mg kg ⁻¹			
0.00-0.05	0.21 bD**	2.05 aC	37.84 aB	85.78 aA	4.62
0.05-0.15	0.67 abC	0.67 bC	7.86 bB	37.38 bA	3.92
0.15-0.30	1.48 aBC	0.32 bC	2.40 cB	7.08 cA	
		Organic fraction (HNO ₃ + H ₂ O ₂), mg kg ⁻¹			
0.00-0.05	1.61 ^{NS} C**	2.70 aC	10.83 aB	22.11 aA	12.73
0.05-0.15	0.95 C	1.50 abC	3.36 bB	6.89 bA	13.15
0.15-0.30	1.06 A	1.28 bA	1.61 cA	2.05 cA	
		Residue (HF), mg kg ⁻¹			
0.00-0.05	2.79 bC**	3.63 bA	3.21 aB	3.91 aA	5.35 ^V
0.05-0.15	2.79 bB	3.48 bA	3.07 aB	3.07 cB	3.34‡
0.15-0.30	3.07 aBC	5.87 aA	2.79 bC	3.35 bB	
		Sum fractions, mg kg ⁻¹			
0.00-0.05	4.91 ^{NS} D**	8.87 aC	53.69 aB	114.8 aA	5.74
0.05-0.15	5.00 C	6.39 bC	15.07 bB	50.28 bA	4.27
0.15-0.30	6.24 B	8.21 abB	7.26 cB	13.51 cA	
		Total (H ₂ O ₂ + HF), mg kg ⁻¹			
0.00-0.05	4.86 aC**	10.53 aC	51.09 aB	92.07 aA	10.82
0.05-0.15	4.18 aC	7.01 aC	14.9 bB	46.23 bA	15.61
0.15-0.30	4.65 aB	6.85 aAB	7.64 cAB	12.85 cA	
		Total EPA (H ₂ O ₂ + HNO ₃ + HCl), mg kg ⁻¹			
0.00-0.05	3.10 ^{NS} D**	7.92 aC	49.76 aB	117.20 aA	2.20
0.05-0.15	2.38 D	4.49 bC	12.08 bB	46.94 bA	4.63
0.15-0.30	2.86 C	4.25 bC	7.67 cB	12.33 cA	

† ND. Not detected (<40 µg kg⁻¹).

‡ Mean values followed by the same small letter in columns and capital letters in lines do not differ among themselves by the Tukey test at P <0.05.

** Significant at P <0.01. ^{NS} Not significant.

§ CV for vineyards.

¶ CV for soil layers.

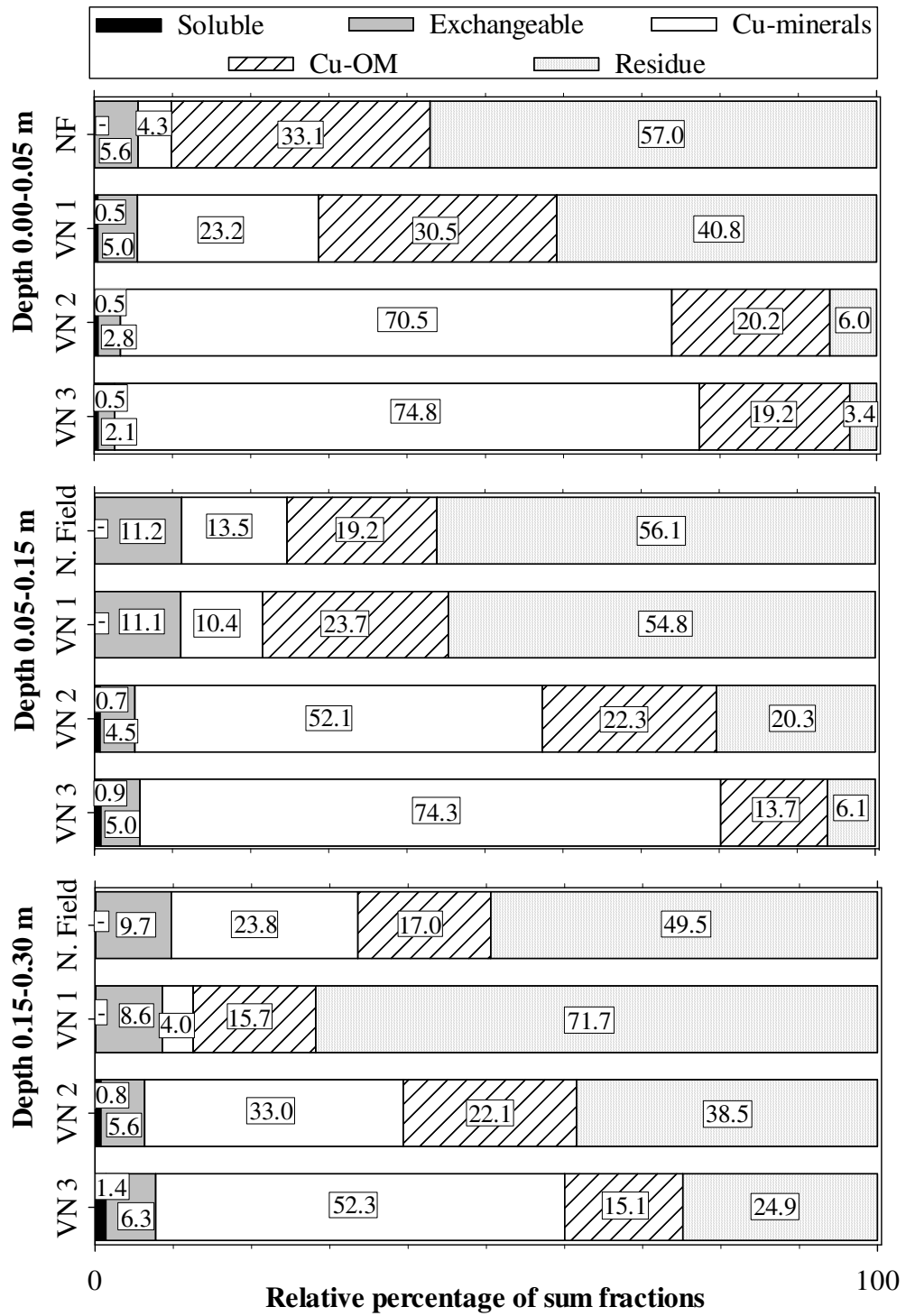


Fig. 4. Relative percentage of each fraction obtained in sequential extraction in relation to the sum of the quantity extracted. The numbers in boxes placed over the bars represent the percentage of the respective fraction. NF. Natural field. VN. Vineyard.

ARTIGO 2 – ABSORÇÃO, ACÚMULO E ALTERAÇÕES FISIOLÓGICAS EM VIDEIRAS ADULTAS EM RESPOSTA AO EXCESSO DE COBRE NOS SOLOS

Copper uptake and accumulation and physiological changes in adult grapevines in response to copper excess in soil²

Abstract

This study investigated Cu uptake and accumulation and physiological and biochemical changes in grapevines grown in soils with Cu excess. The grapevines were collected in two productive cycles, from three vineyards with increasing concentrations of Cu in the soil, and at various growth stages, before and after applications of Cu-based fungicides. Cu concentrations in the grapevines organs were analyzed; and macronutrients and biochemical parameters were analyzed in the leaf blades. The concentration and content of Cu in the leaves increased at stages close to the flowering of the grapevines. On the other hand, the Cu concentrations in roots, stem, shoots and bunches were not correlated with the metal concentrations in the soil. Applications of Cu-based fungicides on leaves increased Cu concentrations in the shoots, leaves and rachis, but their effect on the concentration in berries was not significant. Biochemical analyses of leaf blades showed oxidative stress symptoms correlated with Cu concentrations in soil. The increased availability of Cu in soils slightly affects the levels and accumulation of Cu in mature grapevines during the productive season and does not change their nutritional status. However, increased of Cu concentration were observed in leaves. Evidence of oxidative stress in leaves correlated with increased levels of Cu in soil was also found.

Keywords: Heavy metals, Plant nutrition, Oxidative stress, Copper-based fungicides, Vineyard.

Introduction

Copper(Cu)-based fungicides, such as Bordeaux mixture [$\text{CuSO}_4 \cdot 5\text{H}_2\text{O} + \text{Ca}(\text{OH})_2$] are applied in vineyards to control fungal diseases such as mildew (*Plasmopara viticola*). These fungicides are cheap, efficient in the control of diseases and present low toxicity to

² Artigo elaborado de acordo com as normas da revista Plant and Soil (Anexo B).

plants. Therefore, they are often used in vineyards, including organic farms (Council Regulation 2092/91/EEC, 1991). However, successive applications increase the total and available Cu in soils (Brun et al. 2001; Pietrzak and McPhail 2004; Fernández-Calviño et al. 2009), reaching high and even excessive concentrations for plants. The excessive accumulation of Cu in soils of vineyards is a major environmental problem, since it occurs in large areas of many countries (Komárek et al. 2010). When excessive in soil, Cu can leach in the soil profile, thus polluting groundwater (Robinson et al. 2006). It can enrich sediments and be transferred by run off to surface water (Fernández-Calviño et al. 2008), thus adversely affecting the biological activity of soils (Dumestre et al. 1999) and being toxic to plants (Brun et al. 2003; Chaignon and Hinsinger 2003).

Copper is a micronutrient to plants and operates in a large number of enzymes related to respiration and photosynthesis (Marschner 1995; Yruela et al. 2000). The main visual symptoms of excess of Cu are impaired growth of roots and shoots, nutrient deficiency, chlorosis, and in more severe cases, tissue necrosis and death of plants (Marschner 1995; Kopsell and Kopsell 2007). These symptoms are caused by direct and indirect action of Cu on plants. The first visible symptom of excess of Cu in the soil is reduced growth of plant roots (Marschner 1995). Besides decreasing the volume of soil explored, the toxic effect of Cu on plant roots also decreases the selectivity of the membranes (De Vos et al. 1989) and results in decreased absorption of water and nutrients (Kopsell and Kopsell 2007). Excess of Cu in the roots reduce branches growth and causes various visual symptoms, including chlorosis, which is caused by generalized deficiency of nutrients and water (Marchner 1995; Yruela et al. 2000). One of the direct effects of high concentrations of Cu at cell level is oxidative stress due to increased concentration of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}) (Apel and Hirt 2004). ROS can cause damage to all biomolecules, but lipid peroxidation of cell membranes is one of the most important effects observed. Damage to the membranes result in lower selectivity and their evolution results in breakage and leakage of cell content (De Vos et al. 1989; Yruela 2005).

Important studies were conducted to assess Cu availability and the effect of excessive Cu in soils of vineyards on plant nutrition. Studies using chemical extractors show that Cu availability is higher in acid and sandy soils with low concentrations of organic matter (Brun et al. 2001; Chaignon and Hinsinger 2003; Pietrzak and McPhail 2004; Arias et al. 2005). However, the total concentration, or those extractable by various methods, are ineffective in predicting the Cu absorption and its toxicity to plants (Brun et al. 1998). Thus, studies have

used other plants that co-inhabit vineyards or annual crops to assess the Cu availability in soils of vineyards (Brun et al. 2003; Chaignon et al. 2009), but studies on grapevines are still scarce in international literature.

The effects of excessive Cu in soils on the nutrition of grapevines are unclear. Young grapevines plants grown in soils newly contaminated with Cu show reduced growth in roots and shoots, leaf chlorosis and Cu accumulation in roots. However, a small amount of Cu is translocated to the branches (Toselli et al. 2009). On the other hand, adult grapevines in productive season do not exhibit visual symptoms of toxicity caused by Cu, but can uptake and accumulate Cu in perennial and annual organs (Lai et al. 2010).

Grapevines remain productive for decades in soils with high Cu concentrations and are often subjected to spraying with Cu-based fungicides. Under such conditions, excessive Cu levels may result in greater absorption, accumulation and toxicity to plants. In addition, Cu accumulation in roots may affect the uptake of other nutrients, causing nutritional problems to plants. The present study aimed to evaluate the concentration and content of Cu in plant organs and the physiological and biochemical changes in the leaf blades of mature grapevines during the productive season grown in soils with increasing Cu levels.

Material and methods

Study locality

The study was conducted in vineyards located in the municipality of Sant'Ana do Livramento, Rio Grande do Sul State, Brazil. The climate is subtropical, classified as Cfa (Köppen classification) with average rainfall of 1388 mm year⁻¹ (IPA, 1989). Three vineyards of the variety Cabernet Sauvignon (*Vitis vinifera*) grafted on rootstock SO4 (*Vitis berlandieri* x *Vitis riparia*) were used in the experiment. Vineyard 1 (VN1) was planted with a density of 2778 plant ha⁻¹ in 2004 (S 30°46'41", W 55°22'34"); Vineyard 2 (VN2) was planted with a density of 2525 plant ha⁻¹ in 1998 (S 30°47'44", W 55°21'56"); and Vineyard 3 (VN3) was planted in 1977 with a density of 1429 plant ha⁻¹ (S 30°46'36", W 55° 22'03"). The vineyards were conducted using the vertical shoot position training system with heavy pruning and fertilization with nitrogen (N), phosphorus (P) and potassium (K), which was performed equally. The control of fungal foliar diseases was carried out preventively and included the use of Cu fungicides only after the formation of the berries. In these vineyards, the 2009/2010

and 2010/2011 cycles were assessed. The phenological stages evaluated in the two cycles, the amounts and timing of application of Cu fungicides are shown in Table 1.

Design of grapevines sampling

Two sets of plants were delimited in each vineyard. For the evaluations carried out in the 2009/2010 cycle, three blocks consisting of three plots composed of six plants were delimited; one plot for each stage assessed. In the 2010/2011 cycle, four blocks were delimited, with plots composed of five plants. The two most representative plants from each plot were selected for many evaluation. In the second year, the sampling was performed always using the same plants, and three branches were removed at each stage assessed (Fig. 1).

Soil sampling and analysis

In each vineyard, on the occasion of the delimitation of the area and plants to be used in the study, three soil samples were randomly collected from the 0.00-0.20 m soil layer. Each soil sample was composed of three subsamples collected so as to represent the row, projection of the canopy of the plants and the middle of the between-row area. Soil samples were also collected in an area of natural field near the vineyards, which was used as background in the study. The samples were air dried and sieved to 2 mm mesh. The total concentration of Cu (Cu_T) was extracted according to the USEPA method 3050B (USEPA 1996) by oxidation and acidic attack using HNO_3 , H_2O_2 and HCl concentrated under heating. The available concentration of Cu (Cu_A), iron (Fe_A), manganese (Mn_A) and zinc (Zn_A) were extracted by EDTA (Chaignon et al. 2009). The exchangeable concentrations of calcium (Ca_E) and magnesium (Mg_E) were extracted by using KCl 1 mol L^{-1} . All determinations of the concentrations of Cu, Fe, Mn, Zn, Ca and Mg in the extracts obtained were performed in Atomic Absorption Spectrophotometer (AAS). The concentrations of available phosphorus (P_A) and exchangeable potassium (K_E) were extracted by using a Mehlich 1 solution, and P was determined by spectrophotometer and K, by flame photometer. The pH- H_2O was determined in soil:water suspension (1:1, m/v). The effective Cation Exchange Capacity (CEC_{eff}) was calculated by the sum of Ca_E , Mg_E and K_E (Tedesco et al. 1995; CQFS RS/SC 2004). The concentration of organic matter (OM) in the soil was determined according to the Walkley-Black method and the clay concentration by the pipette method (Embrapa 1997).

Sampling of grapevines and chemical and biochemical analyses

In the 2009/2010 cycle, the grapevines were cut and each organ was analyzed separately, as shown in Fig. 1. At each stage assessed (Table 1), the fresh matter of each organ was determined and a subsample was removed to determine the dry matter (DM) and the concentration of Cu. The root system was sampled by the opening of trenches of 0.50 m of depth, 1.50 to 1.75 m of length and width of 0.75 to 1.00 m, according to planting spacing, so as to include an area equivalent to one quarter of the area for one plant. The subsample of roots was immersed for 8 to 10 s in a solution of HCl 0.5 mol L⁻¹ for soil detachment, washed in running water, immersed in EDTA 0.02 mol L⁻¹ for 1 min and washed with distilled water three times. The subsamples of the stems were composed of five segments of 10 to 12 cm in length, which had their epidermis manually removed with the aid of a stylet and were subsequently washed three times in distilled water. The subsamples from leaves and shoots were also washed three times with distilled water.

In the 2010/2011 cycle, only the annual branches were evaluated. At four phenological stages (Table 1), three branches were removed from each plant and divided into the parts shown in Fig. 1. The leaves corresponding to the basal, middle and apical positions (Fig. 1) were collected only when present and fully expanded. All samples were washed three times with distilled water. All grapevines samples collected in the two harvests were dried at 60 °C until reaching constant weight and were ground for analysis.

Samples from leaf blades were removed for biochemical analyses at the phenological stages EL31 and EL37 of the 2010/2011 cycle (Table 1). After being washed three times with distilled water and dried with absorbent paper, the blades were fractionated in half longitudinally. One half was used as the samples for the biochemical analyses and was immediately frozen in liquid N₂ and stored in a freezer. The second half of the blades was oven dried at 60 °C and used to determine the total concentration of nutrients.

Chemical analyses of plant tissues

The determination of the total concentration of Cu in all samples, and of P, K, Ca and Mg in the leaf blades used in the biochemical analyses was performed after dry digestion of the tissues in muffle furnace. The samples were calcined at 500-550 °C for 3 h and the ashes obtained were diluted in HNO₃ 1 mol L⁻¹. It was determined the total Cu, Ca and Mg in the rates obtained, in AAS; K was determined in flame photometer; and P, in spectrophotometer

(Embrapa 1997). The concentrations of N were determined by the semi micro kjeldahl method (Tedesco et al. 1995).

Biochemical analyses of leaf blades

All biochemical analyses were performed after maceration of the leaf blades in a mortar using liquid N₂. The concentration of hydrogen peroxide (H₂O₂) was determined by spectrophotometer at 390 nm and calculated based on the standard calibration curve, according to Loreto and Velikova (2001). The level of lipid peroxidation was estimated by TBARS assay by measuring the concentration of malondialdehyde (MDA) in spectrophotometer at 532 nm (El-Moshaty et al. 1993). The effect of turbidity in the samples was reduced by subtracting the absorbances at 532 nm from those obtained at 600nm. The activity of superoxide dismutase (SOD) was measured according to the measurement of the formation of adrenochrome from epinephrine in alkaline medium, held in spectrophotometer at 480 nm at 4 min time (Misra and Fridovich 1972). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the epinephrine oxidation. The catalase activity (CAT) was determined in a spectrophotometer at 240 nm by monitoring the vanishing of the H₂O₂ added to the reaction mixture (Aebi 1984). The activity of ascorbate peroxidase (APX) was determined by the oxidation of H₂O₂ ascorbate-dependent measured by the decrease in absorbance in a spectrophotometer at 290 nm (Zhu et al. 2004).

Statistical analyses

The data were presented as average and standard deviations. The ANOVA significance test was used to identify significant differences between the variables. When significant differences were detected, the Tukey test was used at P <0.05 for comparison of the averages. A Pearson's linear correlation was established between DM, Cu_T and Cu_A in the soil and the concentration and content of Cu in plant tissues.

Results

Concentration of Cu and characteristics of the soils

The soils of the vineyards had very similar characteristics and increasing Cu concentrations (Table 2). All soils were classified as Ultisol (SSS 2010), with pH-H₂O

between 5.5 and 6.0, sandy texture and small amount of OM. In the VN2 and VN3 soils, the Cu_T concentration of 20.5 and 62.4 $mg\ kg^{-1}$ were observed, which are equivalent to, respectively, 6.4 and 19.5 times the concentration found in the natural field soil (background). Approximately 77% of the Cu_T in the soils of VN2 and VN3 is Cu_A and potentially available to plants. The P_A concentration was interpreted as "High" ($> 21.0\ mg\ kg^{-1}$) for VN1 and VN2 and "Very High" ($> 42.0\ mg\ kg^{-1}$) for VN3 (Table 2) (CQFS RS/SC 2004). The soils of the vineyards showed similar CEC_{eff} and concentrations of K_E , Ca_E , Mg_E , Fe_A , Zn_A and Mn_A , all at sufficient or high concentrations for plants (CQFS RS/SC 2004). A small increase in the concentrations of Zn_A was observed in the VN3 soil (Table 2).

Concentration, content and partitioning of Cu in grapevine (2009-2010 cycle)

The increased concentrations of Cu_T and Cu_A in the soils of the vineyards either did not increase or slightly altered the concentration of Cu in the roots, stems, shoots and bunches, but is correlated with leaves Cu concentrations. The Cu concentrations in roots, collected only at the stages EL09 and EL19, were equal for the vineyards and stages evaluated (Fig. 2). The Cu concentrations in stems in the VN3 were lower than in VN1 and VN2. However, a small change was observed for the three stages evaluated in which an average of 5.6, 5.1 and 3.0 $mg\ kg^{-1}$ was observed in stems of VN1, VN2 and VN3, respectively (Fig. 2). The application of Cu-based fungicide, carried out in VN2 and VN3, between stages EL19 and EL31, did not affect the Cu concentration in the stem. The Cu concentration in the shoots was not affected by the accumulation of Cu in the soil (Fig. 2 and Table 3). The Cu concentration in shoots decreased in the first two stages assessed. Their values were, on average, 11.7 $mg\ kg^{-1}$ in the VN1; 7.3 $mg\ kg^{-1}$ in VN2 and 8.0 $mg\ kg^{-1}$ in VN3. On the other hand, at stages EL31, in the VN2 and VN3, an increase in Cu concentration was observed, probably due to the residues of fungicide application. In bunches, Cu values at EL19 were similar for the grapevines of the three vineyards, all close to 7.0 $mg\ kg^{-1}$ DW. However, after the application of Cu-based fungicides, the Cu concentration increased many times and reached values above 50 $mg\ kg^{-1}$ in VN2 and 90 $mg\ kg^{-1}$ in VN3 (Fig. 2). The Cu concentration in the leaves of the three vineyards at EL09 was not altered by Cu in the soil. However, at the stage EL19, higher concentrations were observed in the leaves of the VN3, which coincided with the higher concentrations of Cu_T and Cu_A in the soil. At this stage, the concentration values of Cu in the leaves were on average 7.1, 8.0 and 10.5 $mg\ kg^{-1}$ in the VN1, VN2 and VN3, respectively. The higher leaves Cu concentration observed in VN3,

although numerically small, means an increase of 47% and 31% compared to VN1 and VN2, respectively. After the application of Cu-based fungicide, leaves Cu concentrations above 140 mg kg⁻¹ were observed in VN2 and VN3. However, in VN1 the Cu concentrations were 19.3 mg kg⁻¹, without fungicide application.

In general, Cu content in the roots of the VN3 was higher than in VN1 and VN2, which presented similar values (Fig. 2). Copper content in shoots at EL09 and EL19 was similar for the vineyards. In the VN3 it was equal or lower than that observed in VN1. The same occurred for the bunches. At stage EL19, bunches Cu content was slightly higher in VN2, in relation to VN1 and VN3. Moreover, Cu content in leaves was always higher in VN3 for the three stages assessed. However, it should be noted that, at EL31, the Cu content in leaves, shoots and bunches of VN2 and VN3 plants was considerably higher, probably due to the application of Cu-based fungicides (Fig. 2).

At stage EL19, when no fungicide was applied, the bunches Cu contents in VN1, VN2 and VN3 were, respectively, 14.8, 14.2 and 28.9 mg plant⁻¹. Roots and stems are the organs with most Cu accumulation, where they represent 91% in VN1, 98.7% in VN2 and 99.2% in VN3. Individually, roots and stems represent, respectively, 57.8 and 33.8% in VN1; 86.2 and 12.5% in VN2; and 75.1 and 24.1% in VN3. Copper content in leaves and shoots of VN1 equals 3.8% of the cumulative total for the plant and less than 1% for VN2 and VN3. Shoots represented 0.6% of the total accumulation in VN1; 0.1% in VN2 and only 0.03% in VN3.

The correlation analysis was performed at the stages EL09 and EL19, when no application of Cu-based fungicides was performed. The contents of Cu_T and Cu_A in the soil are weakly correlated to the concentrations of Cu in the roots, stems, shoots and bunches or do not explain them (Table 3). The concentrations of Cu in the soils do not affect the Cu concentration in leaves at EL09, but are positively correlated with the concentrations observed at EL19. The amount of Cu accumulated in roots, stems, bunches and leaves are positively correlated with the amount of DM, except at EL09 for shoots (Table 3, Fig. 2). The cumulative amount also correlated with Cu_T and Cu_A in the soil, but DM was responsible for most of the variance, whereas the values of total Cu concentrations are slightly altered by the concentrations in the soil.

Cu concentration in annual branches (2010/2011 cycles)

In the second evaluation cycle, the data obtained at the phenological stage of EL17 corroborate with those achieved at EL19 of the 2009/2010 cycle (Fig. 3). The data

demonstrate that the Cu concentrations in the leaf blades and shoots were higher in VN3, which showed the highest Cu accumulation in the soil. At EL17, shoots and basal leaf blades (BB) presented, respectively, Cu concentrations of 9.4 and 7.9 mg kg⁻¹ in the VN1; 9.7 and 9.4 mg kg⁻¹ in VN2; and 12.6 and 26.7 mg kg⁻¹ in VN3 (Fig. 3). These concentrations represent an increase on average, of 31% for shoots and 2 to 3 times the basal blades (BB) concentration in VN3, compared to VN1 and VN2. In these organs, a positive correlation was observed between the concentrations of Cu_T and Cu_A in the soil and Cu concentration in tissues (Table 3). On the other hand, shoots and basal petioles (BP) did not show the same trend and did not present correlation with Cu concentrations in the soil at this stage.

Copper concentrations in tissues were affected by residues of Cu-based fungicides at stages EL31, EL33 and EL37. The applications of Cu-based fungicides were conducted between stages EL17 and EL37 and increased Cu concentrations mainly in leaf blades, petioles and rachis (Fig. 3). In addition, they slightly affected the concentrations in shoots and berries. The Cu concentration in leaf blades was high, often above 100 mg kg⁻¹, and in some cases more than 400 mg kg⁻¹, as observed in the leaves blades at EL33 in VN1. Copper concentration in petioles was not proportional to its concentrations in the leaf blades. In petioles from the basal, middle and apical leaves at stage EL33, the maximum concentration of Cu was 80 mg kg⁻¹ in VN1, but lower values were often observed between 20 and 40 mg kg⁻¹ in the three vineyards assessed (Fig. 3). At EL37, the Cu concentration in the petioles collected at three positions in the branches were on average 33.6 mg kg⁻¹ in the VN1, 12.1 mg kg⁻¹ in VN2, and 16.3 mg kg⁻¹ in VN3. At the same stage, the leaf blades of three vineyards still showed high Cu concentration, ranging from 50 to over 300 mg kg⁻¹. Cu concentrations in the rachis were similar to those of petioles, but always with lower concentrations, compared to leaf blades. Cu concentrations in the shoots of grapevines from the three vineyards were not affected by the application of Cu-based fungicides. However, in the last harvest, the concentration of Cu in the shoots was 18.6 mg kg⁻¹ in the VN1; 10.4 mg kg⁻¹ in VN2; and 12.3 mg kg⁻¹ in VN3 (Fig. 3). These data, particularly those observed in VN2 and VN3, were very close to those observed at EL17, before the application of Cu-based fungicides. On the other hand, the Cu concentration in the berries was slightly affected by the concentrations in the soil and application of Cu-based fungicides. In general, the Cu concentration in berries of the three vineyards studied ranged between 8 and 20 mg kg⁻¹. At EL31 (not quite ripe berries) the grapevine Cu concentration was 12.4 mg kg⁻¹ in VN1; 3.14 mg kg⁻¹ in VN2; and 17.3 mg kg⁻¹ in VN3 (Fig. 3).

Concentration of macronutrients in leaf blades

The concentration of macronutrients in leaf blades was very similar for the three vineyards (Table 4). Based on the reference concentrations for full leaves (normal concentration = 16.0-24.0 mg kg⁻¹ for N; 1.2-4.0 mg kg⁻¹ for P; and 8.0-16.0 mg kg⁻¹ for K), the concentration of N, P, K were interpreted as normal and, in some cases, very high, for both stages evaluated (CQFS RS/SC, 2004). The Ca concentrations did not differ for the vineyards. On the other hand, and the Mg concentration at EL37 was slightly higher in VN1, but not different from VN3. Calcium concentrations between 16.0 and 24.0 mg kg⁻¹ and Mg concentration between 2.0 and 6.0 mg kg⁻¹ (CQFS RS/SC, 2004) are considered normal in full leaves. Between the stages EL31 and EL37, the concentration of N, P and K decreased, while the concentrations of Ca and Mg increased (Table 4).

Biochemical analyses of leaf blades

Biochemical analyses related to oxidative stress were performed at stages EL31 in basal (BB) and middle (MB) leaves blades; and at EL37, in BB, MB, and also in apical blades (AB). Increased concentration of H₂O₂ was observed between the three vineyards at the stage EL31 in BB, and especially at EL37 in MB, but it was statistically identical to the other assessments (Fig. 4). At EL31, a lipid peroxidation (MDA) value in BB was lower in VN2 than in VN1 and VN3. On the other hand, at EL37, lipid peroxidation did not differ in tree vineyards (Fig. 4). The SOD activity presented significant variation for the vineyards and stages assessed (Fig. 4). The data reveal higher activity of this enzyme in the leaf blades of VN2 and VN3 in MB at EL31 and in BB and AB at EL37. On the other hand, the SOD activity was lower in VN3 in BB at EL31 and equal to VN1 in MB at EL37. CAT activity at EL37 was higher in VN3 in MB and in VN2 and VN3 in AB, compared to VN1. In the other assessments, CAT presented slight variation for the different vineyards, except for EL31 in BB, where the enzyme activity was lower in VN3, compared to VN1 (Fig. 4). The activity of APX was always lower in the leaf blades of VN2 and VN3, except in BB at EL31, where VN2 showed the lowest activity (Fig. 4). Copper concentration in leaf blades, largely from residues of fungicides, and macronutrient concentration are not correlated with the concentration of H₂O₂ and MDA or the activity of SOD, CAT and APX (data not shown).

Discussion

Characteristics of the vineyards soils

Copper accumulation found mainly in soils of VN2 and VN3 can be attributed to foliar application of Cu-based fungicides over the years. Total Cu in topsoil (20.5 mg kg⁻¹ in VN2 and 62.4 mg kg⁻¹ in VN3, Table 2) is considered median or low, compared to those reported in the literature or by government regulatory agencies. Studies carried out in many wine regions of the world reported maximum concentrations of Cu in vineyard soils ranging from 200 to over 600 mg kg⁻¹ (Brun et al. 1998; Arias et al. 2004; Casali et al. 2008). On the other hand, according to government regulatory agencies in Brazil, the concentration of 60 mg Cu kg⁻¹ soil indicates the need for preventive measures, but the maximum concentration suggested is 200 mg kg⁻¹ (CETESB 2005; CONAMA 2009). In Australia and New Zealand, Cu concentration of 60 mg kg⁻¹ in soils suggests the need for studies on environmental impacts (ANZECC/NHMRC 1992), while the European community advocates that Cu concentrations should be maintained between 40 and 140 mg kg⁻¹ in soils with pH below 7.0 (Council Directive 86/278/EEC 1986). However, due to the low concentration of clay and organic matter, the soils studied in this work present low sorption capacity (Bradl 2004) and, hence, high Cu availability (Table 2). This was also reported in other studies conducted in acid and sandy soils of vineyards (Brun et al. 1998; Pietrzak and McPhail 2004). Acidic pH is one of the most important factors for increased Cu toxicity in soils (Brun et al. 1998; Pietrzak and McPhail 2004). Moreover, the addition of P fertile, due to the natural low P concentration in the soils under study, resulted in high P availability (Table 2). Phosphorus is a major factor that limits the growth of plants in soil contaminated by Cu (Nikolic et al. 2011) and has been used to mitigate toxicity (Cao et al. 2003). It must be said that, in recent years, Cu-based fungicides have been replaced by organic fungicides containing Zn salts, such as Mancozeb. The frequent use of these fungicides may increase the concentrations of Zn in the soils of vineyards (Fernández-Calviño et al. 2012), which explains the concentration observed in VN3 (Table 2).

Cu concentration and content in grapevines

Cu accumulation in roots of plants grown in soils is reported in the literature and it was observed in young grapevines cultivated in soils contaminated by Cu (Toselli et al. 2009).

In our study, the increasing Cu concentrations in soil did not affect the concentration in roots of the grapevines in productive season (Fig. 2, Table 2). This behavior may have occurred because, at the evaluation stage, the annual young roots, with small diameter, were present in smaller quantities, while thicker and lignified roots prevailed. Copper may have been accumulated in the fine roots emitted annually, which are more active in absorbing nutrients. However, these roots have short lifespan and are renewed every year (Anderson et al. 2003), which results in the elimination of the Cu accumulated in them. Moreover, the thicker and suberized roots contribute very little to the absorption of nutrients. On the other hand, they mainly allow the translocation of water and nutrients, besides storing carbohydrate and nutrients (Eissenstat 2007). However, the quantity of DM in roots was relatively high, compared to the DM found in stems, shoots and leaves (Fig. 2). Therefore, even with Cu concentrations of $\sim 15 \text{ mg kg}^{-1}$, roots are the grapevine organ containing the highest amount of Cu.

The main role of stems is supporting branches and serving as an organ that transports water and nutrients between roots and aerial parts. Our data show that the Cu concentration in the stems of grapevines was not affected by the increased availability of the metal in the soil. In addition, the lowest Cu concentrations were observed in the stems of the VN3 grapevines, which presented the highest Cu concentration in the soil. This data is explained by the dilution of the Cu accumulated in the DM of the stems, which is higher in VN2 and VN3, because they are older (Fig. 2, Table 3). On the other hand, stems, even with the lowest concentrations of Cu compared to other grapevine organs, have the second highest value for the cumulative amount, due to their higher DM (Fig. 2). Our data disagree with those presented by Lai et al. (2010), which show high Cu concentrations in perennial organs of grapevines, ranging from 200 to 600 mg kg^{-1} DM in response to total Cu concentrations in the soils, which are, on average, ~ 20 to $\sim 60 \text{ mg kg}^{-1}$ in the 0.00-0.20 m layer, similarly to the present study.

The Cu concentrations in the shoots of grapevines in both cycles studied were also little affected by the concentrations of Cu_T or Cu_A in the soil. A positive correlation between the Cu concentrations in the shoots and its concentrations in the soil was observed only at EL17 in the 2010/2010 cycle (Fig. 3, Table 3). However, the results obtained in the 2009/2010 cycle suggest that, at early stages after the start of sprouting, the Cu concentration in the shoots is related to the concentrations in the stems of grapevines, as observed at EL09 and EL19 (Fig. 2).

Shoots, similarly to stems, are organs that allow the translocation of water and nutrients, between the stem and leaves and bunches. The Cu concentration in shoots tends to

decrease during the vegetative cycle of grapevines because they grow quickly and turn from a tender structure, more metabolically active at early sprouting, into a semi hardwood structure at the end of the cycle. The applications of Cu-based fungicides increased the Cu concentration in the shoots of the grapevines and may be the main factor for the increase observed at EL31 in the 2009/2010 cycle and the variations observed from EL31 in the 2010/2011 cycle. However, increase in Cu concentrations in the shoots because of applications of Cu-based fungicides was lower than those observed in the leaves (Fig. 2 and 3). This can be attributed to the lower surface area of the shoots, compared to the leaves and also the dilution of the nutrient in DM. The data obtained in this study disagree with those obtained by Lai et al (2010), who reported concentrations in shoots of grapevines grown under conventional and organic system ranging between ~ 100 and $200 \text{ mg kg}^{-1} \text{ DM}$. Studies carried out in pots demonstrate that the Cu concentrations in the soil are not correlated with the concentrations in shoots of grapevines and pear trees (Toselli et al. 2008; Toselli et al. 2009).

The bunches of grapes are clearly visible from the stage EL12 (Five to six leaves unfolded), but the highest increase in dry matter and nutrient demand only begins with the emergence of berries (EL29, small berries) (Eichhorn and Lorenz 1977). The present work demonstrates that the Cu concentration in the bunches at phenological stages close to flowering was not correlated with the concentration in the soil and that the use of Cu-based fungicides increases the concentrations in bunches, particularly in the rachis (Fig. 2 and 3, Table 3). However, even after the growth of berries, with the consequent accumulation of nutrients from the soil and from various parts of the plant, Cu concentration remained close to $20 \text{ mg kg}^{-1} \text{ DM}$ (which is approximately 4 to $5 \text{ mg kg}^{-1} \text{ Fresh Weight (FW)}$), for the three vineyards. Li (1994) reports extremely high concentrations in leaves and petioles of grapevines (all $>118 \text{ mg kg}^{-1}$), while the concentrations in the pulp of the berries (skin and seeds removed) ranged from 0.33 to $1.7 \text{ mg kg}^{-1} \text{ FW}$. García-Esparza et al. (2006) showed that the amount of Cu applied via fungicides presented low correlation ($R^2=0.26$) with the metal residues in grapes. The same study reports Cu concentrations in grapes ranging from 2 to $40 \text{ mg kg}^{-1} \text{ FW}$, and, after 50 days of fungicide application, the concentrations decreased to values below $10 \text{ mg kg}^{-1} \text{ FW}$. These results can be attributed to the smaller surface area of the berries, compared to leaves, and their natural waxy coating that prevents the Cu accumulation from fungicides.

Studies carried out under field conditions generally report high Cu concentrations in the leaves of grapevines. Pinamonti et al. (1999) reported Cu concentration close to 270 mg

kg⁻¹ in leaves of grapevines cultivated in soils contaminated by the addition of organic compound. Higher concentrations were found by Lai et al. (2010), who studied the absorption of Cu by grapevines cultivated under organic or conventional systems and observed concentrations of 400-1600 mg kg⁻¹ in leaves of grapevines. Similarly, Li (1994) reported concentrations between 118 and 9845 mg kg⁻¹ in petioles and leaves of grapevines grown in soils with accumulation of Cu. On the other hand, Toselli et al. (2009) reported that, in a study using grapevines grown in pots containing soils with up to 1000 mg kg⁻¹, it was observed reduced plant growth and chlorosis of the leaves with Cu concentrations between 10 and 20 mg kg⁻¹. The data obtained in this study show that the concentrations of Cu in the leaves that effectively come from the soil range between 7 and 10 mg kg⁻¹, but they may reach up to 25 mg kg⁻¹ in the leaf blades in response to increased concentrations of Cu in the soil (Fig. 2 and 3). In spite of the correlation with Cu concentrations in the soil, the concentration and content of Cu in the leaves are weak and represent a small fraction of the Cu content in plants. On the other hand, after the application of Cu-based fungicides, the concentrations in the leaves increased several fold, reaching concentrations higher than those from soil. This is explained by the fact that the Cu applied via fungicide is absorbed by the apoplast of leaves, which is not removed by washing with water, as also reported by Li (1994). Due to their smaller surface area, the petioles of leaves of grapevines were less contaminated by spraying with Cu-based fungicides and showed Cu concentrations several times smaller than those of leaf blades. These data support the hypothesis that Cu applied via fungicide is not taken up, since the petiole are closely related to the concentrations of nutrients of the leaves and are also used to diagnose the nutritional status of grapevines (CQFS RS/SC 2004).

Nutritional status of the grapevines

The toxic effects of Cu on the root system of plants affects the absorption of water and nutrients. Reduced root growth mainly affects the uptake of nutrients supplied by diffusion, such as K and P (Marchner 1995), due to the reduced volume of soil explored. In studies on soils contaminated by Cu, P deficiency is the most limiting factor that hinders the growth of annual plants such as wheat, as demonstrated by Nikolic et al. (2011). Moreover, excess Cu also impairs nitrogen assimilation, especially in the root system, as observed in a study on young grapevines performed by Llorens et al. (2000). However, our data show that grapevine had similar concentrations of N, P, K, Ca and Mg in the leaf blades, indicating that excess of Cu in the soil did not affect the uptake of these nutrients. Grapevines during the season have a

broad and deep root system and might be up taking nutrients from soil layers with lower Cu levels. In addition, the constant fertilization performed to maintain the soil fertility will increase the availability of nutrients in the soil, thus mitigating the toxic effects of Cu through better plant nutrition (Marchner 1995). The toxic effects of Cu in the roots also reduce the absorption of micronutrients, mainly Fe, Zn and Mn (Marchner 1995; Toselli et al. 2008; Toselli et al. 2009). The concentrations of these nutrients were not investigated in this study, but visual symptoms of their deficiency were not observed.

Biochemical parameters in leaf blades

Excessive formation of ROS results from the imbalance in electron flow in oxidation-reduction reactions in the presence of excessive concentrations of Cu, Fe and Zn and other metals and factors (Apel and Hirt 2004). These oxidation-reduction reactions occur mostly in peroxisomes and mitochondria, and particularly in chloroplasts of photosynthetic organs (Dat et al. 2000). Under conditions of oxidative stress, increased concentrations of H₂O₂ and other ROS are observed, thus leading to lipid peroxidation, which results from the attack to hydrogens of fatty acid chains and consequent formation of lipid radical and aldehydes (Apel and Hirt 2004). Lipid peroxidation estimated by malondialdehyde (MDA) concentration is an important parameter to measure the damage due to the toxic effects of heavy metals (Islam et al. 2008; Yang et al. 2011). In this study, it was observed that the leaf blades exposed to direct insolation presented higher lipid peroxidation estimated by the concentration of MDA. Changes were also observed in the concentrations of H₂O₂ and in the activity of CAT and APX. This condition was observed mainly in BB and MB at EL31; and MB and AB at EL37 (Fig. 4). It is related to the growth of the shoots and emergence of new leaves, with the consequent shading of basal leaves. The changes in the cellular metabolism of leaf blades directly exposed to solar radiation are correlated with excessive Cu in soils (data not shown) and are not related to the Cu concentrations in leaves, which are mostly caused by the application of Cu-based fungicides Cu (Fig. 2 and 3). After fungicide applications, the highest concentrations of Cu in the leaves of the grapevines were observed in VN1, which has the lowest concentration of the metal in the soil and MDA in leaf blades. These results suggest that Cu applied via fungicide does not affect cellular metabolism. Therefore, it can remain in the apoplast of leaves or in other organs. On the other hand, increased concentrations of Cu in leaves due to excess of the metal in the soil (observed at EL19 in the 2009/2010 cycle; and at

EL17 in the 2010/2011 cycle) can be the main factor for the changes in metabolism and increased MDA.

In our study, the concentration of MDA in MB and AB was always higher in VN3 (Fig. 4), which presents the highest concentrations of Cu in the soil (Fig. 4, Table 2). However, H₂O₂ concentrations were higher in VN3 only in BB at EL31 and especially in MB at EL39. H₂O₂ is only one of the ROS formed by oxidative stress (Apel and Hirt 2004) and, at subtoxic concentrations, it works as intracellular signaling (Van Breusegem et al. 2001) and is related to the formation and modification of cell wall (Tamás et al. 2004). Cu present in cells can also catalyze the formation of OH⁻ from O²⁻ and H₂O₂, which is known as the Haber-Weiss reaction (Mithöfer et al. 2004), also working as a regulator of the H₂O₂ concentration.

SOD, CAT and APX are important key enzymes in the removal of ROS (Apel and Hirt 2004) and their activities are generally increased under conditions of high levels of metals (Zhang et al. 2010). However, in this study, the behavior of SOD activity was variable and poorly explained by the concentrations of H₂O₂ or Cu in soil and leaves. In addition, the concentration of H₂O₂ between stages EL31 and EL39 increased more than three times, probably due to older leaves, without a corresponding increase in SOD activity (Fig. 4). Yang et al. (2011) also found no changes in SOD activity in grapevines grown with excess of Zn in soil. Excessive Cu in cells may change differently the activity of the SOD isoforms present in the cytoplasm, chloroplasts, mitochondria and peroxisomes (Møller et al. 2007), which were not evaluated separately in this study. The formation of H₂O₂ from O²⁻ can also be catalyzed by transition metals, such as Fe³⁺ and Cu²⁺, by Fenton reaction (Møller et al. 2007), without requiring enzymes.

The increased CAT activity observed at EL39 in MB and AB also coincides with full exposure of the leaves to sun, but this effect was not observed at EL31, when the leaves were younger. CAT has high affinity with H₂O₂ (mM range) and can catalyze the direct conversion of H₂O₂ into H₂O and O₂. Because of these characteristics, increased CAT activity is observed under conditions of oxidative stress (Dat et al. 2000; Apel and Hirt 2004). Yang et al. 2011 observed that increased Zn in soil resulted first in increased CAT activity in leaves of grapevines, followed by a sharp decrease in its activity because of the high degree of toxicity. In the same study, increased concentrations of Zn in soil cause progressive reduction in peroxidase activity. This behavior was also observed in this study, where APX activity in leaf blades was lower in VN2 and VN3, where the highest concentrations of Cu in soil were found. The reduced APX activity can be a direct or indirect effect of excess of Cu on the functioning of this enzyme. APX is part of the glutathione-ascorbate cycle (Dat et al. 2000)

and is very sensitive to reduced concentration of oxidized ascorbate, which is one of its substrates (Shigeoka et al. 2002). Excessive Cu in cells leads to the formation of phytochelatins from glutathione and SH groups present in these molecules (De Vos et al. 1992). This process reduces the ascorbate-glutathione cycle activity, and the lower concentrations of oxidized ascorbate reduce APX activity.

Increased MDA, H₂O₂ concentrations and changes in CAT and APX enzymes observed in this work are signs of the effect of excess of Cu in mature leaves which are full photosynthetic activity. But, damage to the leaves tend to be small, since decreased vegetative vigor or chlorosis were not observed in the studied grapevines, which are classic symptoms of toxicity by Cu and other metals (Marchner 1995; Toselli et al. 2009; Yang et al. 2011). Besides, the excellent nutritional status of plants and the satisfactory production of grape in these vineyards indicate that the adult grapevines are tolerant to excess of Cu in soils.

Conclusion

Increased Cu concentrations were observed in leaves, as well as evidence of oxidative stress, which were correlated with increased Cu concentrations in the soil. However, the increased Cu availability in soils slightly affects the concentrations and accumulation of Cu in grapevines during the productive season and does not change their nutritional status. The use of Cu-based fungicides is the main determinant of increased Cu concentrations in annual organs of grapevines, especially in leaves and rachis, but the residue of Cu in berries is not related to the amount of Cu applied.

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Table 1. Phenological stages of the grapevines when it was conducted the sampling of tissues and amounts of Cu applied per hectare and equivalent per plant in the period between assessments for the 2009/2010 and 2010/2011 cycles.

Phenological stages		Copper applied					
		Vineyard 1		Vineyard 2		Vineyard 3	
Stage and description (Eichhorn & Lorenz, 1977)		g ha ⁻¹	mg plant ⁻¹	g ha ⁻¹	mg plant ⁻¹	g ha ⁻¹	g plant ⁻¹
2009/2010							
EL09	Two to three leaves	-	-	-	-	-	-
EL19	Beginning of flowering	-	-	-	-	-	-
EL31	Berries pea-sized	-	-	1250	495	1250	875
Total		-	-	1250	495	1250	875
2010/2011							
EL17	Twelve leaves	-	-	-	-	-	-
EL31	Berries pea-sized ^a	1190	428	1122	444	1122	785
EL33	Berry touch	1370	493	-	-	1123	786
EL37	Berries not quite ripe ^a	-	-	-	-	-	-
Total		2560	921	1122	444	2245	1571

^aPhenological stages when leaf blades were collected for biochemical analysis.

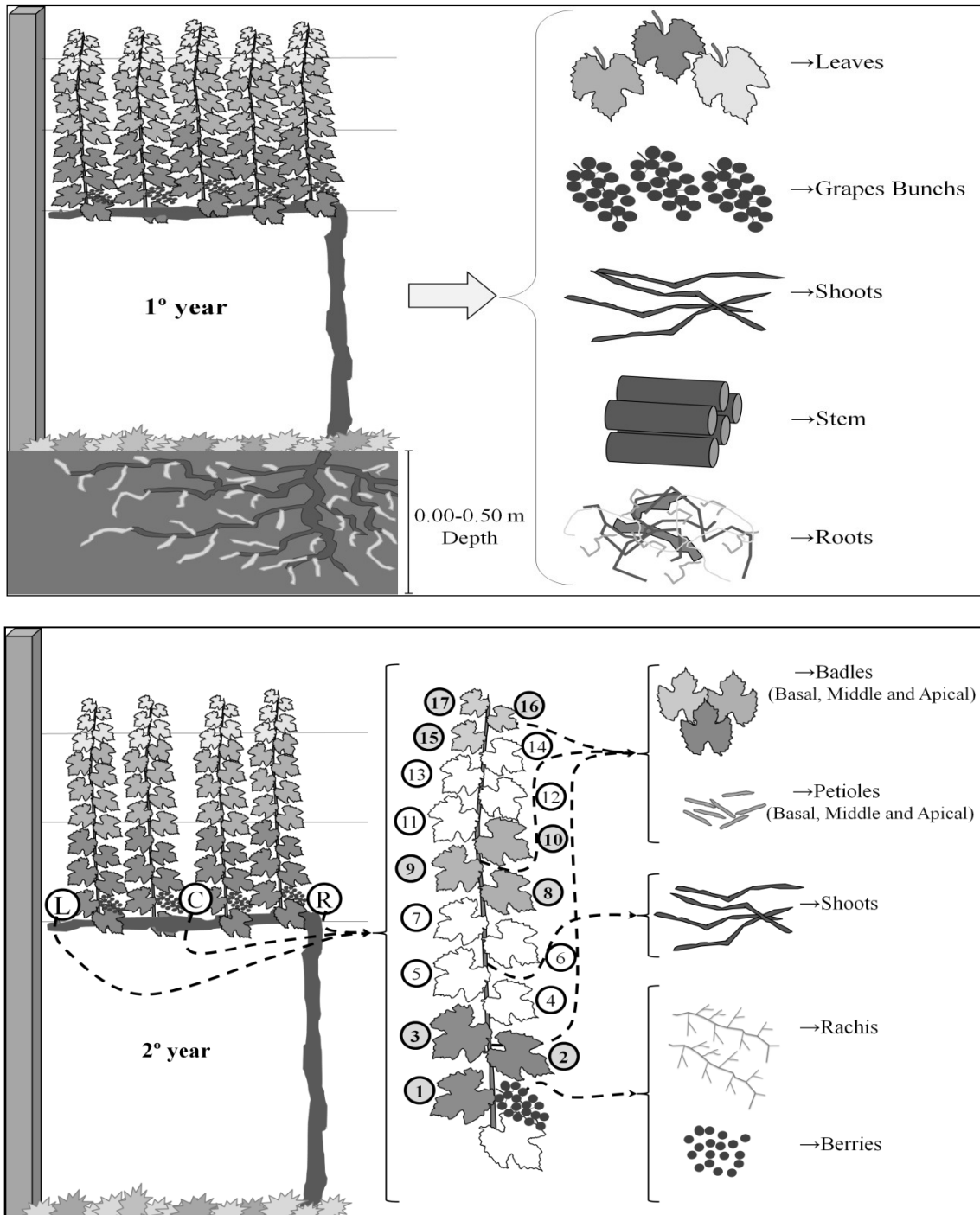


Fig. 1. The figures illustrate the organs and parts of grapevines evaluated at various phenological stages during the 2009/2010 cycle (1st year) and 2010/2011 cycle (2nd year). In the figure presenting the 2010/2011 cycle, the letters "L, C and R" indicate the left, central and right positions of the plants from which the branches were collected at each collection. In these branches, three groups of leaves were sampled. They were defined, according to their position, as basal (number 1, 2 and 3); middle (number 8, 9 and 10), and apical (numbers 15, 16 and 17).

Table 2. Chemical and physical attributes of the 0.00-0.20 m layer of the soils collected in three vineyards evaluated and in a natural field area.

Attribute	Natural field (background)	Vineyard 1	Vineyard 2	Vineyard 3
Cu _T , mg kg ⁻¹	3.2±0.1 ^c	4.7±0.7 ^c	20.5±2.4 ^b	62.4±6.7 ^a
Cu _A , mg kg ⁻¹	1.1±0.1 ^c	2.2±0.3 ^c	17.2±2.8 ^b	49.6±6.8 ^a
P _A , mg kg ⁻¹	5.9±1.0 ^c	28.4±2.5 ^b	30.0±2.2 ^b	48.2±4.3 ^a
K _E , mg kg ⁻¹	131.0±10.2 ^{ns}	148.0±15.5	149.0±8.2	165.0±18.7
Ca _E , cmol _c kg ⁻¹	0.4±0.1 ^b	1.1±0.2 ^a	0.9±0.1 ^a	0.8±0.1 ^{ab}
Mg _E , cmol _c kg ⁻¹	0.2±0.1 ^c	0.6±0.1 ^a	0.4±0.1 ^b	0.5±0.1 ^b
Zn _A , mg kg ⁻¹	3.0±0.1 ^{bc}	1.7±0.4 ^c	4.8±0.4 ^b	10.1±1.6 ^a
Fe _A , mg kg ⁻¹	32.8±1.9 ^{ns}	30.6±4.2	26.0±0.3	29.1±2.5
Mn _A , mg kg ⁻¹	49.0±2.8 ^{ns}	40.4±1.6	50.5±7.5	54.1±2.2
pH - H ₂ O	5.1±0.1 ^c	5.5±0.2 ^b	6.0±0.1 ^a	5.7±0.1 ^{ab}
CEC _{eff} , cmol _c kg ⁻¹	1.4±0.1 ^b	2.3±0.4 ^a	1.9±0.1 ^{ab}	1.9±0.1 ^{ab}
OM, g kg ⁻¹	6.9±0.2 ^b	8.8±0.4 ^a	7.0±0.3 ^b	8.2±0.3 ^a
Argila, g kg ⁻¹	58.2±5.2 ^a	63.4±4.5 ^a	48.2±0.2 ^b	43.3±0.1 ^b

Averages followed by the same letter in rows do not differ by Tukey test at P < 0.05. ns=Not significant.

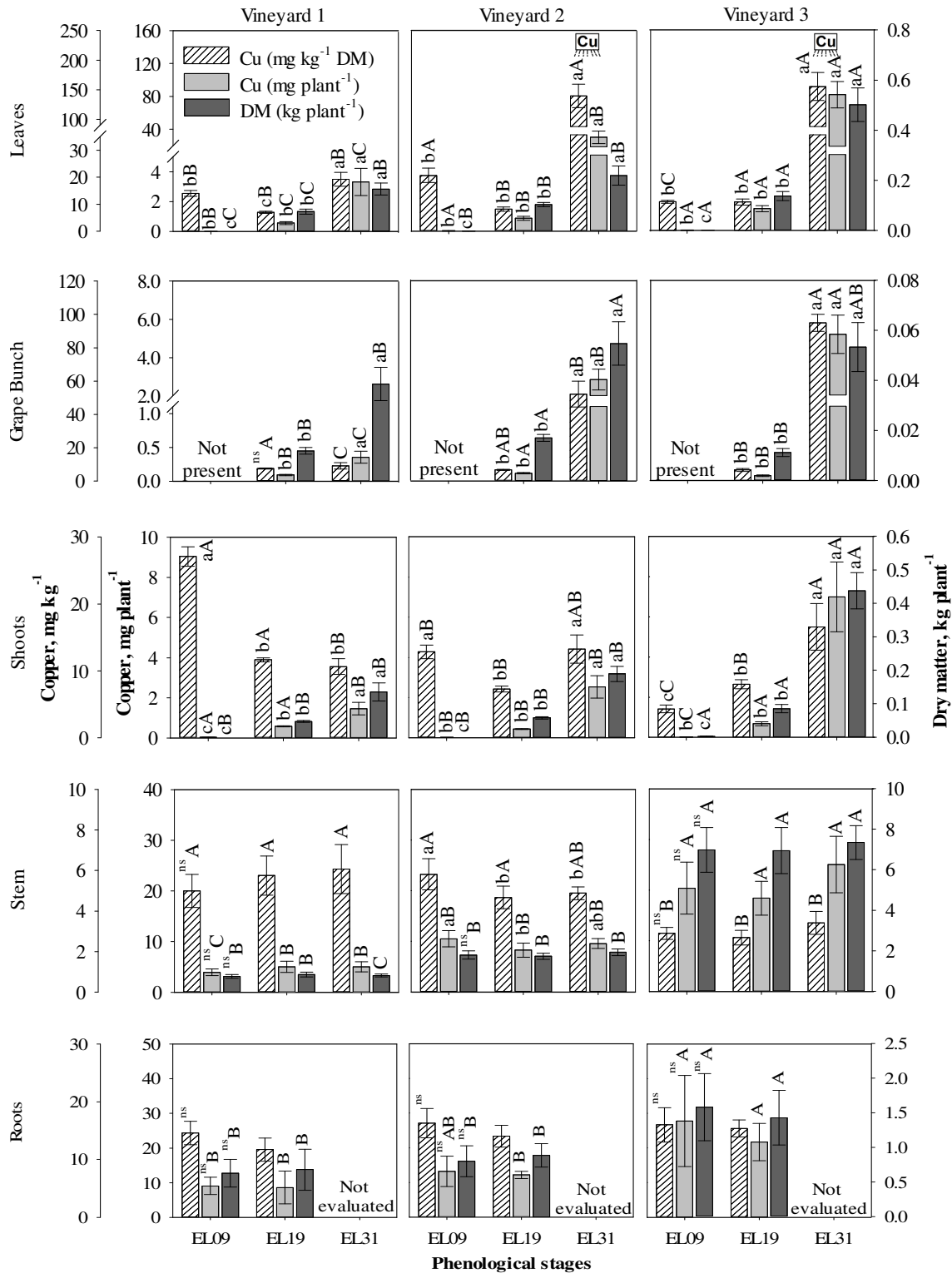


Fig. 2. Copper concentration and content in perennial and annual organs of grapevines grown on soils with increasing level of Cu, during the 2009/2010 cycle. Letters on the columns, lowercase for phenological stages of a single vineyard and uppercase for vineyards and a single phenological stage, do not differ by Tukey test at $P < 0.05$. ns= not significant. Cu Application of Cu-based fungicide in the period between the phenological stage indicated and the previous one (Table 1).

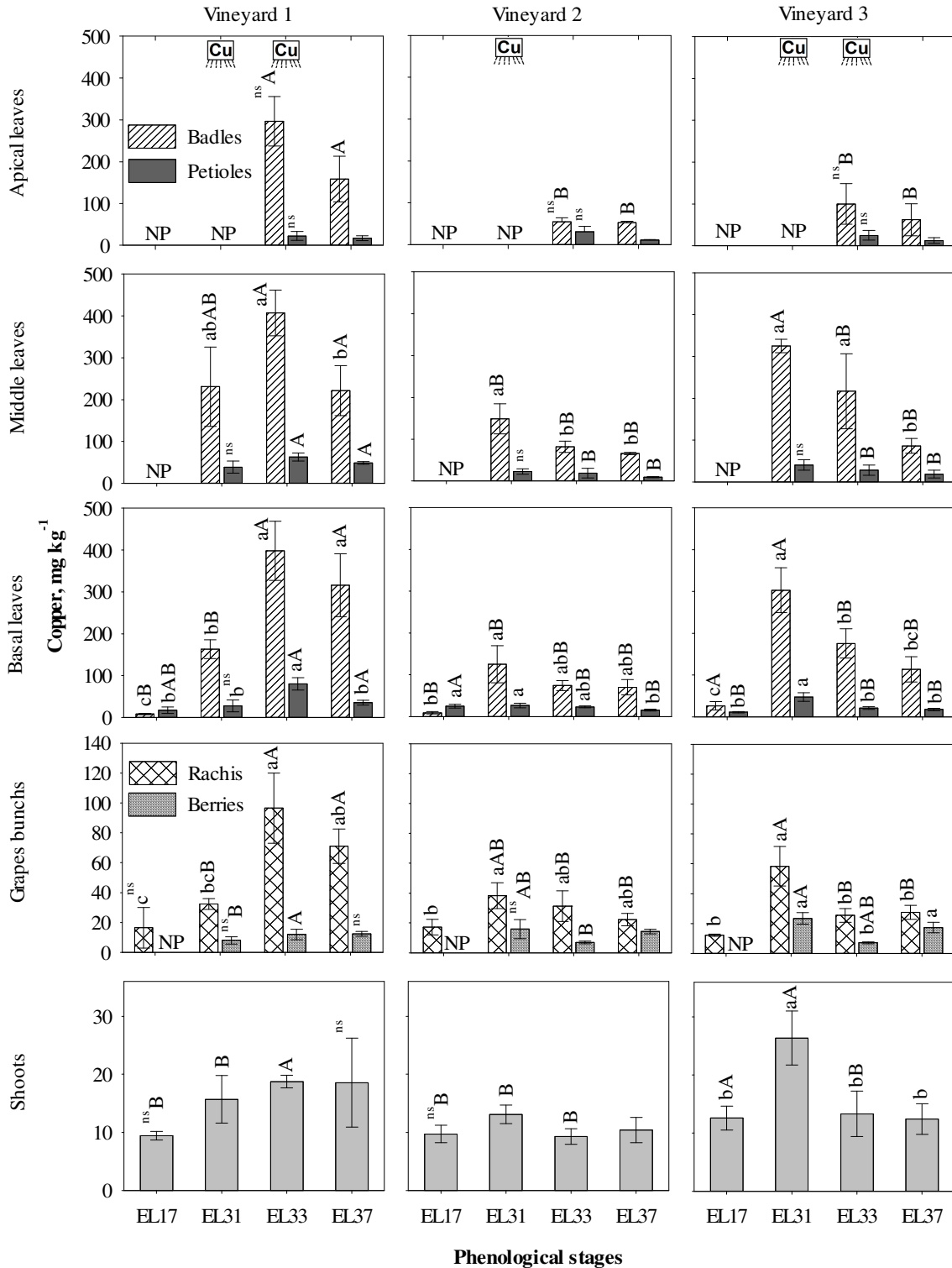



Fig. 3. Copper concentrations in annual organs of grapevines grown on soils with increasing level of Cu during the 2010/2011 cycle. Same letters on columns, lowercase letters for phenological stages of a single vineyard and uppercase letters for vineyards and a single phenological stage, do not differ by Tukey test at $P < 0.05$. ns= not significant. NP= not present.  Application of Cu-based fungicide in the period between the phenological stage indicated and the previous one (Table 1).

Table 3. Correlation between Cu concentrations and content in different organs of grapevines with dry matter, total concentration and available Cu in the soil. Data relating to stages EL09 and EL19 of the 2009/2010 cycle and EL17 of the 2010/2011 cycle, when no Cu-based fungicides were applied.

Variable	CuTSoil	CuASoil	Dry Matter
Cu in grapevine tissues			
EL09, two to three leaves (2009/2010)			
Leaves CC	-0.32*	-0,28*	-0.26*
Leaves CT	0.25*	0.29*	0.34*
Grape Bunch CC	-	-	-
Grape Bunch CT	-	-	-
Shoots CC	-0.83**	-0.86**	-0.64**
Shoots CT	-0.77**	-0.80**	-0.50**
Stem CC	-0.58**	-0.55**	-0.59**
Stem CT	0.80**	0.81**	0.89**
Roots CC	ns	ns	ns
Roots CT	0.50*	0.50*	0.95**
EL19, beginning of flowering (2009/2010)			
Leaves CC	0.77**	0.77**	0.54**
Leaves CT	0.86**	0.86**	0.89**
Grape Bunch CC	-0.25*	-0.27*	ns
Grape Bunch CT	-0.32*	-0.29*	0.83**
Shoots CC	-0.33*	-0.38**	-0.33*
Shoots CT	0.28*	0.24*	0.42**
Stem CC	-0.77**	-0.78**	-0.72**
Stem CT	0.87**	0.87**	0.90**
Roots CC	0.35*	0.36*	ns
Roots CT	0.63**	0.63**	0.93**
EL 17, twelve leaves (2010/2011)			
Basal Badles CC	0.70**	0.68**	-
Basal Petioles CC	ns	ns	-
Beries CC	-	-	-
Rachis CC	ns	ns	-
Shoot CC	0.45*	0.44*	-

CC= Cu concentration. CT= Cu content. *significant at P<0.05. **significant at P<0.01. ns= not significant.

Table 4. Total concentrations of nitrogen, phosphorus, potassium, calcium and magnesium in leaf blades collected at two phenological stages (EL 31 and EL 37) and located at the basal, middle and apical positions in branches of grapevines grown on soils with increasing Cu concentration.

Position	Nutrient g kg ⁻¹	Vineyard 1		Vineyard 2		Vineyard 3	
		EL 31	EL 37	EL 31	EL 37	EL 31	EL 37
Apical Badle (AB)	N	NP	24.2±2.0 ^{ns}	NP	23.9±2.1	NP	21.8±1.7
	P		2.6±0.1 ^B		3.1±0.2 ^A		2.3±0.1 ^C
	K		6.9±1.2 ^B		12.9±1.1 ^A		10.2±1.5 ^{AB}
	Ca		18.3±0.5 ^A		16.5±0.7 ^{AB}		15.1±2.4 ^B
	Mg		7.7±0.4 ^A		4.9±0.7 ^B		5.1±0.4 ^B
Middle Badle (MB)	N	24.4±1.4 ^{ns}	21.6±0.8 ^{ns}	24.8±1.3 ^{ns}	23.7±2.3	21.7±1.3 ^{ns}	21.6±0.8
	P	3.6±0.5 ^{ans}	2.5±0.2 ^{bB}	3.9±0.4 ^a	3.3±0.2 ^{bA}	3.6±0.2 ^a	2.2±0.1 ^{bB}
	K	11.5±1.2 ^{aB}	7.9±1.4 ^{bC}	16.1±1.6 ^{nsA}	14.4±0.8 ^A	13.2±1.5 ^{nsAB}	10.9±0.6 ^B
	Ca	8.8±0.6 ^{bns}	17.2±0.8 ^{ans}	10.1±1.4 ^b	17.3±0.8 ^a	9.7±1.4 ^b	15.9±1.5 ^a
	Mg	4.1±0.5 ^{bns}	6.7±0.5 ^{aA}	3.2±0.3 ^{ns}	4.7±1.1 ^B	3.6±0.5 ^b	5.0±0.4 ^{aAB}
Basal Badle (BB)	N	23.4±0.9 ^{ns}	23.9±2.8 ^A	22.6±1.5 ^{ns}	21.4±0.5 ^{AB}	22.2±0.6 ^{ns}	18.5±3.1 ^B
	P	3.1±0.1 ^{aB}	2.3±0.1 ^{bB}	3.9±0.4 ^{nsAB}	4.4±0.4 ^A	4.0±0.3 ^{aA}	2.8±0.5 ^{bB}
	K	10.9±1.1 ^{nsB}	9.6±1.2 ^B	14.4±0.8 ^{aA}	12.9±1.1 ^{bAB}	11.7±0.6 ^{nsB}	11.3±0.6 ^B
	Ca	11.9±1.4 ^{aB}	19.8±3.7 ^{bns}	13.1±1.2 ^{bB}	22.5±1.3 ^a	17.6±3.0 ^{bA}	25.3±2.4 ^a
	Mg	4.2±0.2 ^{bA}	6.0±0.6 ^{aA}	2.8±0.2 ^{aB}	3.9±0.2 ^{bB}	4.4±0.6 ^{bA}	5.7±0.4 ^{aA}

Averages followed by the same letter on the line, lowercase letters for phenological stages for a single vineyard and uppercase letter for vineyards and a single phenological stage do not differ by Tukey test at P < 0.05. ns= not significant. NP= not present.

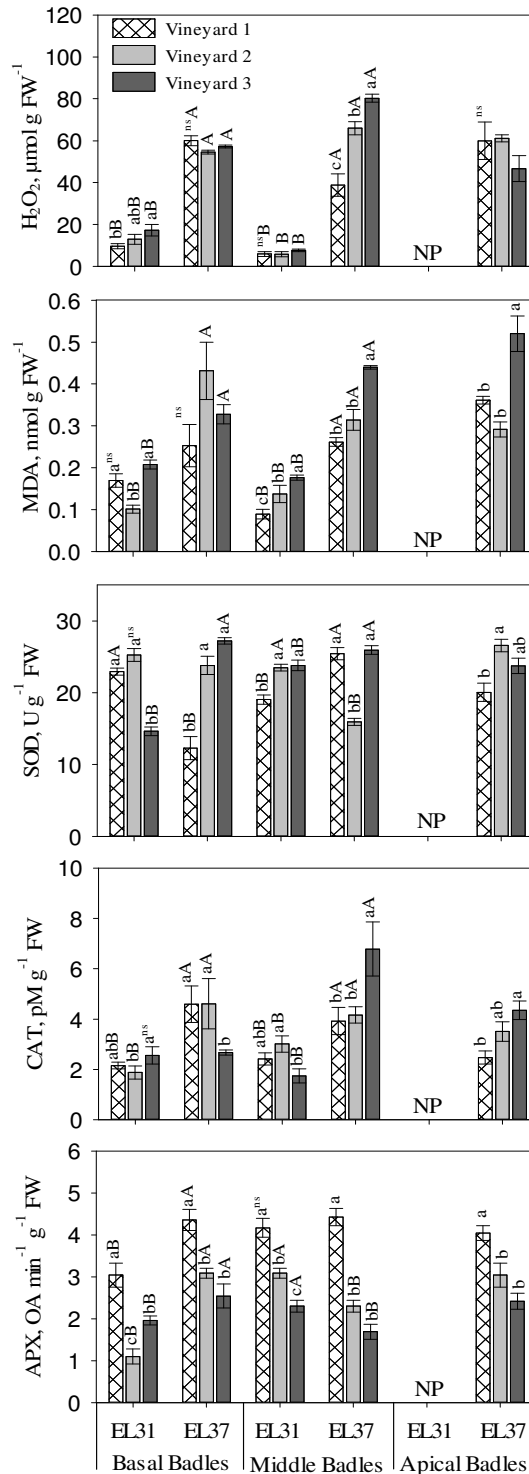


Fig. 4. Concentration of H₂O₂, level of lipid peroxidation (MDA), activities of enzymes SOD, CAT and APX in leaf blades collected in three positions (basal, middle and apical) in the branches and at two vegetative stages (EL31 and EL37) in three vineyards with increasing levels of Cu in the soil. Same letters on the columns, lowercase for vineyard and a single phenological stages and uppercase for phenological stages and a single vineyards, do not differ by Tukey test at P < 0.05. NP= not present. ns= not significant.

5 DISCUSSÃO GERAL

Neste trabalho buscou-se realizar um estudo detalhado do acúmulo de Cu nos solos sob vinhedos da região da Campanha Gaúcha e seus efeitos sobre a nutrição de videiras em produção. Primeiramente buscou-se fazer um levantamento das taxas de aplicações de fungicidas cúpricos, teores totais e disponíveis de Cu nos solos, sua distribuição nos perfis e formas de acúmulo. Em seguida, foram avaliados os teores e acúmulo de Cu em videiras, teores de macronutrientes e alguns parâmetros bioquímicos nos limbos foliares buscando avaliar o impacto do acúmulo de Cu nos solos na nutrição das plantas.

O cultivo da videira na região Campanha Gaúcha (RS) apresenta condições bastante particulares se comparada a outras regiões vitivinícolas no mundo. Embora seja uma região onde os verões são mais secos, em geral, na primavera e início de verão ocorrem diversas precipitações pluviométricas. A frequência dos eventos meteorológicos, principalmente em anos de El niño, resulta num prolongado tempo de molhamento foliar e, conseqüentemente, aumento da incidência de doenças fúngicas das videiras. Nestas condições, a produção de uvas viníferas só é possível com um intenso programa de defesa fitossanitário. No entanto, a taxa atual de aplicação fungicidas cúpricos, com exceção de anos muito chuvosos, não é muito maior das observadas em regiões vitícolas de menor precipitação pluviométrica da Europa e Austrália (Artigo I). A menor taxa de aplicação de Cu é o resultado do aumento do uso de outros fungicidas, como o Mancozeb e outras moléculas sintéticas modernas, que pode substituir os produtos cúpricos. Entretanto, a rotação de princípios ativos realizada para diminuir a resistência dos patógenos implica na necessidade de uso dos fungicidas cúpricos. Com isso, a taxa de aplicação de Cu tende a se manter entre 2,5 a 5,0 kg ha ano⁻¹ (2 a 4 pulverizações).

Contudo, se por um lado a taxa de aplicação de Cu atual é menor se comparada às quantidades aplicadas até poucos anos atrás (Artigo 1, Tabela 2), os solos estudados neste trabalho apresentam limitada capacidade de acumular este metal. Como pode ser visto no Artigo I, houve a percolação de Cu para camadas abaixo de 0,10 m quando os teores superficiais eram próximos a 100 mg kg⁻¹. Ainda neste mesmo estudo, o fracionamento químico mostrou que a MO, que possui a maior afinidade com o Cu dentre os componentes do solo, complexa pouco mais que 20 mg kg⁻¹ na camada 0,05 m (Estudo 1). A camada superficial dos solos estudados contém, em média, 15 g kg⁻¹ de MO (Figura 1) e pode ser composta em grande parte de material vegetal ainda em processo de decomposição (material

orgânico leve). Isto ocorre provavelmente porque na região da Campanha Gaúcha a precipitação pluviométrica e temperatura favorecem a decomposição da MO que recebe baixa proteção físico-química devido ao baixo teor de argila. Assim, os teores de MO dos solos são mantidos principalmente na camada superficial devido a maior quantidade de raízes e aporte de material orgânico proveniente principalmente das plantas que coabitam os vinhedos (Figura 1). Nas camadas abaixo de 0,10 m os teores de MO são próximos a $5,0 \text{ g kg}^{-1}$, 1/3 da concentração da camada superficial, o que limita ainda mais a capacidade do solo de sorver Cu em ligações de alta energia. Os teores de MO dos solos da Campanha Gaúcha são, em geral, menores dos observados em solos arenosos e ácidos cultivados com vinhedos na França, Espanha e Austrália; mas, os teores de Cu são em muitos casos similares (BRUN et al., 2001; ARIAS et al., 2004; PIETRZAK; MCPHAIL, 2004). Os dados apresentados no Artigo I fornecem indícios que as capacidades de sorção de Cu da camada superficial destes solos pode não ser muito maiores que os teores máximos observados nos vinhedos com mais de 25 anos de cultivo, aproximadamente, 100 mg kg^{-1} . Neste caso, mesmo que às aplicações de fungicidas cúpricos tenham diminuído, todo o Cu aplicado nos vinhedos mais antigos tenderá a aumentar as frações mais disponíveis, percolando no solo e aumentando a toxidez para as plantas.

Nos solos arenosos da Campanha Gaúcha a maior parte do Cu está ligado à fração mineral do solo independente da camada avaliada ou idade dos vinhedos. Esta condição não ocorre somente na Campanha Gaúcha, mas normalmente a fração orgânica dos solos assume maior importância, como observado nos solos da Serra Gaúcha (CASALI, 2008, GIROTTO, 2010). A sorção de Cu pela fração mineral ocorre por ligações do Cu aos grupos funcionais que contêm oxigênio presentes nos óxidos de ferro, manganês, alumínio e silicatos. Este tipo de ligação é mais sensível às alterações do pH do solo, sendo mais fracas em pHs ácidos (BRADL, 2004). A acidificação é um processo natural dos solos e também ocorre na rizosfera das plantas onde o pH pode ser diminuído em até uma unidade de pH (MARCHNER, 1995). Nestas condições, pode ocorrer a dessorção do Cu e problemas de toxidez para as plantas. De fato, teores de $50 \text{ a } 90 \text{ mg kg}^{-1}$ nos solos arenosos da Campanha Gaúcha já causam redução do crescimento de aveia preta e plantas de batata, como demonstraram os trabalhos realizados por Girotto (2010) e Farias (2012), respectivamente.

A alta disponibilidade do Cu acumulado nos solos da Campanha Gaúcha pode também ser percebida no aumento da concentração de Cu solúvel e trocável; comportamento frequentemente observado em estudo desta linha. Entretanto, a não alteração dos teores da fração residual, de alta energia de ligação, diferencia estes solos dos previamente reportados

pela literatura. Isto ocorre provavelmente porque nos solos arenosos da Campanha Gaúcha há baixa quantidade de óxidos de ferro, manganês e alumínio amorfos. Além disso, devido à baixa proteção físico-química, pouco do carbono adicionado ao solo é transformado em MO humificada. Com pequena quantidade destes componentes que podem adsorver especificamente Cu em ligações de alta energia, quase a totalidade do Cu sorvido no solo é potencialmente disponível.

Contudo, se os estudos do acúmulo de Cu nos solos da Campanha Gaúcha, com o uso de extratores químicos (Artigo I) e também pelo cultivo de plantas em vasos (GIROTTO, 2010; FARIAS, 2012), demonstram alta disponibilidade e teores tóxicos para as plantas coloca-se a seguinte pergunta: Por que as videiras em produção são pouco afetadas pelo excesso de Cu nos solos? Parte da resposta a esta pergunta está possivelmente relacionada à absorção e retenção do Cu pelas raízes finas anuais das videiras, como já discutido no Artigo II. Porém, outros fatores importantes podem também ajudar a responder a pergunta.

A textura arenosa confere aos solos da região da Campanha Gaúcha uma boa aeração e drenagem, o que permite o crescimento das raízes para camadas mais profundas. Durante a amostragem das videiras, analisadas no Artigo II, pode-se perceber que a maior parte das raízes das plantas estava localizada nas camadas 0,05 a 0,25 m (Observações de campo, dados não apresentados). Porém, algumas raízes, em geral mais grossas e lignificadas, cresciam abaixo desta camada, chegando a profundidades maiores que 0,60 m. Comparando a distribuição vertical das raízes com os teores de Cu no perfil dos solos (Artigo I, Figura 3) é possível constatar que as raízes encontram um gradiente decrescente de Cu em profundidade. Por isso, mesmo no VN3, onde há o maior acúmulo de Cu no solo, as raízes crescem em teores menores que 60 mg kg^{-1} quando atingem camadas abaixo de 0,20 a 0,25 m.

Da mesma forma que há um gradiente da concentração de Cu no perfil dos solos, também há uma variabilidade horizontal de sua concentração. Esta variação no teor de Cu pode ser percebida pelos desvios padrões das médias plotadas na Figura 3 do Artigo I. Pode-se perceber que, especialmente nas camadas acima de 0,15 m, os desvios padrões das observações podem ser de até ± 10 a $\pm 20 \text{ mg kg}^{-1}$. Estas diferenças na concentração são geradas provavelmente pela distribuição desuniforme de Cu, mas também pela movimentação de partículas na superfície do solo resultantes do escoamento da água ou pelo uso de implementos agrícolas, como a roçadeira. De forma similar ao gradiente de concentração vertical, a variabilidade horizontal dos teores de Cu resulta em zonas de menor toxidez para as raízes das plantas. Assim, as videiras em produção podem explorar camadas mais profundas ou zonas superficiais de menor teor de Cu, devido ao seu amplo sistema radicular.

Por outro lado, as operações de lavração e gradagem realizadas para nivelar o solo antes da reimplantação dos vinhedos também nivelam os teores de Cu na camada arável. Com estas operações os gradientes verticais e horizontais de Cu são desfeitos e há a formação de uma camada superficial de 0,20 a 0,25 m de profundidade, com teores de Cu próximos a 50 mg kg^{-1} (Artigo I, Figura 1). O revolvimento dos solos para a diluição dos teores de Cu é discutida como uma alternativa para diminuir a toxidez de Cu em vinhedos (PIETRZAK; UREN, 2011). Porém, nas condições apresentadas, o revolvimento do solo resulta no aumento dos teores de Cu na camada 0,05 a 0,25 m, que é mais explorada pelas raízes das videiras utilizadas na região. Cabe citar que teores totais maiores que 50 mg kg^{-1} nos solos da Campanha Gaúcha causam toxidez em plantas de aveia preta e batata (GIROTTI, 2010; FARIAS, 2012) e podem ser tóxicos para as mudas de videiras.

Outro fator importante a ser considerado na interpretação dos efeitos do excesso de Cu sobre a nutrição das videiras é a boa disponibilidade de nutrientes nos solos. Na figura 1 são mostrados alguns atributos da camada 0,00 a 0,60 m dos solos sob os vinhedos estudados no Artigo II. O pH dos solos foi corrigido para pH acima de 5,5 e, portanto, não apresentavam Al^{3+} que é tóxico para as plantas. As concentrações de K foram interpretadas como altas na camada 0,00 a 0,35 m (nível crítico = 60 mg dm^{-3}). Os teores de Ca e Mg foram interpretados como médios ou baixos (teor baixo: $\leq 2,0 \text{ cmol}_c \text{ dm}^{-3} \text{ Ca}$; $\leq 0,5 \text{ cmol}_c \text{ dm}^{-3} \text{ Mg}$), mas seus níveis são limitados pela pequena CTC efetiva dos solos. Os teores de P foram interpretados como altos ($> 21 \text{ mg kg}^{-1}$) até a profundidade 0,10 m no VN1 e 0,15 m no VN2. Porém, no VN3, as frequentes adubações ao longo dos anos resultaram em teores muito altos ($> 42 \text{ mg kg}^{-1}$) até a profundidade 0,15 m e altos até 0,60 m (CQFS RS/SC, 2004). Neste mesmo vinhedo observa-se também aumento nos teores de Zn até a profundidade de 0,15 m.

O aumento da disponibilidade dos nutrientes nos solos, até certos limites, melhora a nutrição das plantas e com isso sua tolerância a estresses (MARSCHNER, 1995). O efeito tóxico do Cu sobre as raízes provoca a redução do seu crescimento e também outros problemas, como já discutido no Artigo II, que resultam na menor absorção de nutrientes. A redução do crescimento das raízes afeta diretamente o volume de solo explorado, diminuindo a absorção de nutrientes, principalmente os que chegam até as raízes por difusão, como o P. O aumento dos teores de P disponíveis observado no VN3 pode resultar num melhor suprimento deste nutriente para as plantas, diminuindo os efeitos tóxicos do excesso de Cu. Estudos tem apontado relação entre a toxidez de Cu e a deficiência de P para as plantas. Nikolic et al. (2011), em um amplo estudo em condições de campo, concluem que o P é o principal fator limitante do crescimento de plantas de trigo em solos contaminados por Cu. Já Farias (2012)

observou que os danos oxidativos na parte aérea de batatas cultivadas em solos com excesso de Cu são similares aos observados em plantas sob deficiência de P, mostrando relação entre estes elementos.

Por outro lado, o aumento de P nos solos também pode diminuir a concentração de Cu disponível. Isto ocorre principalmente pela ação de dois mecanismos. No primeiro, a adsorção de P na superfície dos óxidos de Fe e Al resulta na redução de sua carga positiva e na formação de complexos ternários do tipo óxido-fosfato-metal (McBRIDE, 1994; GUILHERME; ANDERSON, 1998). Este mecanismo é importante em solos ácidos, pois a sorção de P pode inverter a carga positiva dos óxidos de Fe e Al e com isso aumenta a sorção de Cu, como demonstrado por (PÉREZ-NOVO et. al. 2009). Por outro lado, quando o fosfato é aplicado em maiores quantidades do que as necessárias para a adubação pode ocorrer a formação de precipitados de fosfato-metal. Isto foi demonstrado por Cao et al. (2003), em solos contaminados com Pb, Zn e Cu, onde a adição de fosfato promoveu a diminuição da quantidade de metais livres na solução do solo e no aumento de frações de alta estabilidade.

Cabe ressaltar que a adição de P com o intuito de diminuir a disponibilidade de metais é uma prática associada a condições de alta poluição do solo, como por exemplo, em áreas de descarte de resíduos de mineração. Estas condições não se assemelham aos atuais teores de Cu nos solos sob vinhedos, pois estes não representam condições de extrema toxidez para as plantas. Além disso, a disponibilidade de P nos solos sob vinhedos é em geral alta ou muito alta devido às adubações frequentes e maiores que as perdas e exportações. Assim, atualmente existem poucas justificativas para o uso de mais fósforo em vinhedos com o intuito de aliviar os efeitos tóxicos do Cu. Porém, mais estudos são necessários para melhor compreender os mecanismos e a magnitude dos efeitos do aumento dos teores de P sobre a toxidez de Cu.

Outro fato interessante observado nos solos sob vinhedo é o aumento nos teores de Zn, como pode ser observado no VN3 e figura 1. Isto está ocorrendo porque nos últimos anos houve o aumento da aplicação de fungicidas que contém sais de Zn na sua composição, como por exemplo, o Mancozeb. Além disso, o uso de caldas com ação fungicida enriquecidas com micronutrientes também tem se tornado uma prática frequente nos vinhedos. Um exemplo destes produtos é a Calda de Viçosa, que contém Cu, como principal agente fungicida, mas também contém Zn, Mg e B. Com o aumento da aplicação destes produtos também há o aumento da adição de Zn aos solos.

O Zn é um micronutriente para as plantas, mas quando em excesso pode provocar toxidez (MARCHNER, 1995). As videiras parecem tolerar teores relativamente altos de Zn, como demonstrado por Yongqing et al. (2011), em estudo com adição de Zn ao solo. Porém,

os efeitos do aumento de Zn em solos com teores excessivos de Cu não são conhecidos. No solo, o Cu apresenta maior afinidade que o Zn pelos grupos funcionais da MO e dos óxidos de Fe, Mn e Al (SPOSITO, 1989; ARIAS et al. 2006). Por este motivo, o Zn adicionado aos solos com acúmulo de Cu tende a ser mais disponível e, portanto, mais móvel. Este efeito já pode ser percebido no VN2 e VN3 onde os teores de Zn são um pouco maiores dos observados no VN1 até 0,60 m de profundidade.

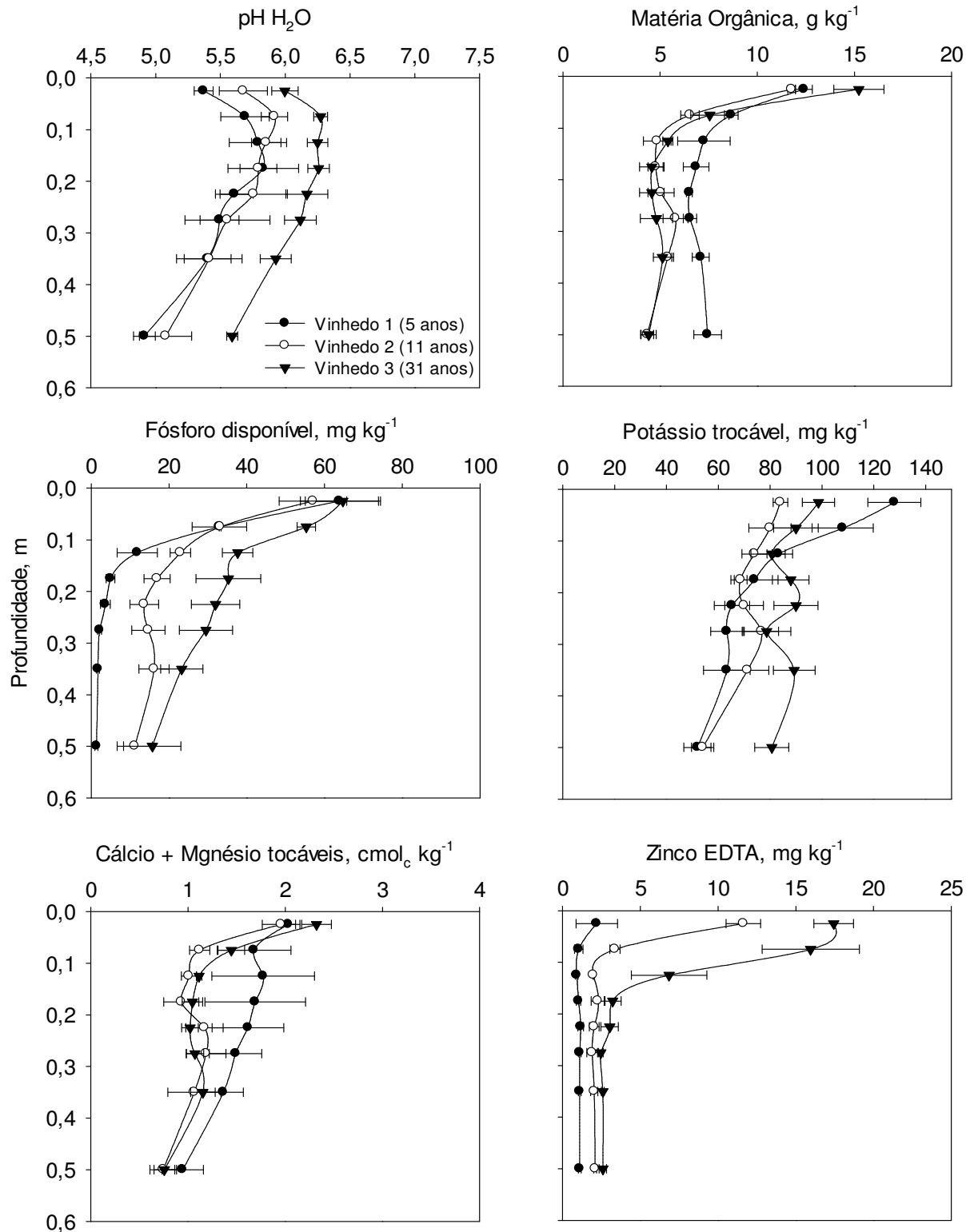


Figura 1. pH em água, teores de matéria orgânica, fósforo disponível, potássio trocável, cálcio mais magnésio trocáveis e zinco extraível (EDTA) em oito camadas até a profundidade de 0,60 m dos solos coletados sob os três vinhedos utilizados no Artigo I e II.

6 CONCLUSÃO

O uso de fungicidas cúpricos na cultura da videira resulta em acúmulo de Cu no solo. Nos solos arenosos de carácter ácidos cultivados com vinhedos na região da Campanha Gaúcha o Cu é acumulado em formas solúveis e trocáveis, principalmente, ligado aos minerais nos solo. Embora a maior concentração ocorra nas camadas superficiais do solo, nos vinhedos mais antigos a percolação de Cu atinge, aproximadamente, 0,25 m de profundidade.

O aumento dos teores de Cu nos solos sob vinhedos pouco afeta a absorção e acúmulo deste metal por videiras em produção e não interfere no seu estado nutricional. Contudo, foram observadas alterações bioquímicas nas folhas que indicando um possível estresse oxidativo resultante do excesso de Cu. A aplicação de fungicidas cúpricos resulta no aumento drástico dos teores de Cu nas folhas e ráquis, mas não determina a concentração deste metal nas bagas.

7 PERSPECTIVAS DE ESTUDOS FUTUROS

Os dados obtidos neste trabalho foram importantes para a melhor compreensão das condições de cultivo da videira na região da Campanha Gaúcha e servem como estímulo a novos estudos. Os vários fatores de solo e planta abordados ressaltam dúvidas e questões pertinentes:

- Nos solos dos vinhedos mais antigos observa-se o acúmulo de P e de Cu. O aumento da disponibilidade de P pode estar mitigando os efeitos do excesso de Cu? Se, sim. Qual é a magnitude dos efeitos e qual são os mecanismos envolvidos? Existem justificativas para a aplicação de mais P nos solos dos vinhedos?

- As videiras são plantas que fazem associação com fungos micorrízicos o que melhora sua eficiência na absorção de fósforo, mas também de outros nutrientes e água. Os fungos micorrízicos são importantes em condições de toxidez de metais, mas a sua associação com as raízes das plantas ocorre e é mais eficiente quando há baixa disponibilidade de P. Além disso, os teores excessivos de Cu podem também ser tóxicos para os fungos micorrízicos, prejudicando sua associação com as plantas. Neste contexto, o aumento dos teores de P e Cu nos solos sob vinhedos está interferindo na associação e eficiência da micorrização das viderias?

- Na renovação dos vinhedos as operações de lavração e gradagem dos solos diluem o Cu acumulado na camada arável. Por outro lado, a inversão de camadas resulta no aumento dos teores de Cu nas camadas mais exploradas pelas raízes. Assim, o revolvimento dos solos dos vinhedos diminui os efeitos tóxicos do Cu?

- O aumento do uso de fungicidas e caldas que contém zinco na sua formulação está resultando no aumento dos teores deste metal em solos sob vinhedos. A adição de Zn nestes solos pode aumentar a disponibilidade de Cu e sua toxidez para as plantas?

- A aplicação de calcário, com o intuito de aumentar a disponibilidade de Ca e Mg para as plantas, é uma prática frequente nos solos da Campanha Gaúcha. Por este motivo, o pH dos solos com maior tempo de cultivo são geralmente maiores que 6,0 até em profundidade abaixo da camada arável. Justifica-se a aplicação de mais calcário para elevar o pH para valores mais próximos da neutralidade e com isso diminuir a toxidez de Cu?

- A percolação de Cu no perfil depende das características físico-químicas do solo, da quantidade aplicada, do fluxo de água no perfil, entre outros fatores. Mantendo-se as taxas atuais de aplicação de Cu nos vinhedos na Campanha Gaúcha como será a progressão dos teores deste metal nos perfil dos solos? Quais são os efeitos do revolvimento dos solos na distribuição de Cu nos perfil dos solos? Quais as projeções resultantes da adição de Zn aos solos com teores excessivos de Cu?

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ANEXOS

Anexo A

Estilos e normas de citações e referências bibliográficas conforme as normas da revista Soil Science Society of America Journal

Style of the Reference List

Some common types of references are shown below. Extensive rules and examples for references of all kinds are given in the Chicago Manual of Style (UCP, 2010, Chapters 14 and 15), Scientific Style and Format (CSE, 2006, Chapter 30), and the ACS Style Guide (Coghill and Garson, 2006, Chapter). Although the examples given do not always conform to the details of ASA, CSSA, SSSA style, they can be used to prepare reference entries that contain all the required elements.

Journal Articles

Each reference to a periodical publication must include, in order, the author(s), year of publication, full title of the article, publication in which it appears, and volume and inclusive page numbers. For publications without consecutive pagination (i.e., each issue within the volume begins with page 1), include the issue number. Example: 11(2):5–10.

First author, second author, and third author. Year. Title of article. Journal Title Vol.:pages.

Bordoli, J.M., and A.P. Mallarino. 1998. Deep and shallow banding of phosphorous and potassium as alternatives to broadcast fertilization for no-till corn. *Agron. J.* 90:27–33. Lemmon, H. 1986. Comax: An expert system for cotton crop management. *Science* 233:29–32.

Lyle, W.M., and J.P. Bordovsky. 1995. LEPA corn irrigation with limited water supplies. *Trans. ASAE* 38:455–462.

Article in serial publication

Brown, P.D., and M.J. Morra. 1997. Control of soil-borne plant pests using glucosinolate-containing plants. *Adv. Agron.* 61:167–231.

Edwards, A.C., and M.S. Cresser. 1992. Freezing and its effect on chemical and biological properties of the soil. *Adv. Soil Sci.* 18:59–79. [After Vol. 20, *Advances in Soil*

Science is no longer published as a serial with volume numbers. Treat listings in later editions as you would a chapter in a book.]

Article not in English with English abstract

Title translated into English

Rosolem, C.A., J.C.O. Silverio, and O. Primaves. 1982. Foliar fertilization of soybean: II. Effects of NPK and micronutrients. (In Portuguese, with English abstract.) *Pesq. Agropec. Bras.* 17:1559–1562.

Title in original language

Rosolem, C.A., J.C.O. Silverio, and O. Primaves. 1982. Adubação foliar de soja: II. Efeitos de NPK e micronutrientes. (In Portuguese, with English abstract.) *Pesq. Agropec. Bras.* 17:1559–1562.

Article not in English and without English abstract (translated title).

Vigerust, E., and A.R. Selmer-Olsen. 1981. Uptake of heavy metals by some plants from sewage sludge. (In Norwegian.) *Fast Avfall.* 2:26–29.

Article with known erratum follow-up. Baker, J.M., E.J.A. Spaans, and C.F. Reece. 1996. Conductimetric measurement of CO₂ concentration: Theoretical basis and its verification. *Agron. J.* 88:675–682 [erratum: 88(6):vi].

Articles in press

For an in-press article, use the current year as the date. If the manuscript has been posted online ahead of publication, include the DOI (digital object identifier).

Author. Year. Article title. Journal Title DOI number (in press).

Magazine Article

Davenport, C.H. 1981. Sowing the seeds. *Barron's*, 2 March, p. 10.

Mulvaney, D.L., and L. Paul. 1984. Rotating crops and tillage. *Crops Soils* 36(7):18–19.

Books (including bulletins, reports, multivolume works, series)

Brown, J. 1966. Soils of the Okpilak River region, Alaska. CRREL Res. Rep. 188. U.S. Army Cold Reg. Res. Eng. Lab, Hanover, NH.

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Doty, W.T., M. Amacher, and D.E. Baker. 1982. Manual of methods: Soil and environmental chemistry.

Chapter in a Book

- Author. Year. Chapter title. In: Editor name(s), editor(s), Book title. Publisher, Place of publication. page range. Achorn, F.P., and H.L. Balay. 1985. Developments in potassium fertilizer technology. In: R.D. Munson, editor, Potassium in agriculture. ASA, CSSA, and SSSA, Madison, WI. p. 49–66.
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Anexo B

Estilos e normas de citações e referências bibliográficas conforme as normas da revista Plant and Soil Journal

Cite references in the text by name and year in parentheses. Some examples: Negotiation research spans many disciplines (Thompson 1990). This result was later contradicted by Becker and Seligman (1996). This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list. Reference list entries should be alphabetized by the last names of the first author of each work.

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol* 105:731-738. doi: 10.1007/s00421-008-0955-8.

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. *N Engl J Med* 341:325–329.

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med*. doi:10.1007/s001090000086.

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London Book chapter.

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257.

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. <http://physicsweb.org/articles/news/11/6/16/1>. Accessed 26 June 2007.

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California.

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations.