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Research article

Reduction of copper phytotoxicity by liming: A study of the root anatomy of young vines (*Vitis labrusca* L.)

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ABSTRACT

Frequent applications of copper (Cu)-based fungicides on vines causes the accumulation of this metal in vineyard soils, which can cause toxicity in young vines. However, liming may reduce these toxic effects. The present study aimed to evaluate the effects of Cu toxicity on the root anatomy of young vines and the alleviation of Cu toxicity by lime applications to contaminated sandy soil. The treatments consisted of the addition of lime (0.0, 1.5 and 3.0 Mg ha^{-1}) and two Cu concentrations (0 and 50 mg kg^{-1}) to Typic Hapludalf soil. Young vines 'Niágara Branca' (*Vitis labrusca* L.) were obtained by micropropagation and cultivated for 70 days. The young vines grown with Cu and without liming presented a disorganized root structure; reduced root cap size; increased diameter (47%), cortex area (128%), vascular cylinder area (93%), and number of cortical layers and cells containing phenolic compounds (132%); and reduced root (41%), stem (44%) and leaf dry mass (21%) and height increase (55%). Moreover, Cu exposure reduced Ca concentrations (13%) and increased Cu concentrations (371%) in the roots. Liming, primarily with the highest tested dose, increased the soil pH (from 4.4 to 5.4–6.1), decreased the Cu concentration in the soil (extracted by $CaCl_2$), increased the calcium (Ca) and magnesium (Mg) uptake by plants, prevented root anatomical changes and benefited young vine growth in soil with higher Cu concentrations.

1. Introduction

Frequent applications of copper (Cu)-based fungicides for the control of foliar disease in vines (*Vitis* sp.) causes this heavy metal to accumulate in the soil, especially in the upper soil layers (Brunetto et al., 2014). Over time as the grape yield decreases, older vines are removed, the soil is tilled, and young vines are transplanted, and this process causes organic matter oxidation, which increases the availability of Cu in the soil (Toselli et al., 2009).

Copper is an essential nutrient for plants; however, excessive amounts can lead to anatomical, morphological and physiological changes, such as damage to the roots, inhibited nutrient uptake and reduced photosynthesis rates and plant growth (Michaud et al., 2008; Toselli et al., 2009; Lequeux et al., 2010; Cambrollé et al., 2015). Nevertheless, toxicity symptoms can be observed in

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various regions of the roots: in the root apex, changes to the cell walls and tissue arrangement cause the root to shorten and thicken and lateral roots to increase, and in the epidermis, plasmolysis has been observed in certain cells, which can reduce the density of the root (Arduini et al., 1995; Sheldon and Menzies, 2005; Michaud et al., 2008; Juang et al., 2014; Chen et al., 2013; Zhang et al., 2014).

Changes in the root structure may be reflected in a reduced uptake of nutrients and water that may inhibit plant growth and root and shoot biomass increases (Kopittke et al., 2009; Toselli et al., 2009). The reduced uptake and accumulation of calcium (Ca), magnesium (Mg) and potassium (K) may occur because of competition for adsorption on the root surface between these elements and Cu, thus obstructing the passage of these nutrients into the plant (Luo et al., 2008; Kopittke et al., 2011).

Excessive quantities of heavy metals, such as Cu, in the soil can also stimulate the production of phenolic compounds within plants, which can accumulate in different organs, such as the roots (Kováčik and Bačkor, 2007; Bouazizi et al., 2010). This accumulation occurs in response to oxidative stress caused by heavy metals (Michalak, 2006), which can reduce the concentration of pigments and rate of photosynthesis, inhibiting the growth and development of the shoots (Cambrollé et al., 2015). Because phenolic compounds present high reactivity to heavy metals, their actions may be similar to chelating agents within the plant; combined with their antioxidant properties, this pattern makes the accumulation of these compounds an important tool for combating oxidative stress (Michalak, 2006).

Liming is usually performed before vineyard implantation, and it increases the soil pH and cation exchange capacity (CEC) values, which may reduce Cu availability and decrease Cu uptake by the plants (Agbenin and Olojo, 2004; Joris et al., 2012). Furthermore, liming may also promote Ca and Mg uptake by plants and reduce the toxic effects of Cu. The effect of Ca and Mg on Cu toxicity in young vines was observed by Chen et al. (2013) and Juang et al. (2014), respectively, who found that an increase of these nutrients in solution reduces the effects of Cu on the root structure by decreasing Cu transportation to the shoots and promoting increased plant biomass. Therefore, the application of lime to the soil before vineyard planting has the potential for use as a strategy to minimize the toxic effects of Cu in young vines.

Because lime doses are dependent on the potential acidity of the soil, which is associated with soil clay content and organic matter, in particular (Kaminski et al., 2007), the appropriate dose of lime must be established for each type of soil to reduce Cu toxicity in young vines. This process is important because if a higher than optimal dose is applied, the nutritional balance of the plant can be changed, which may diminish its resistance to stresses and reduce its growth (Spann and Schumann, 2010). Moreover, in most studies on plant heavy metal toxicity, the plants are grown in a nutrient solution, thus requiring further studies in soil. The results obtained in experiments with plants grown in soil are more closely related to the actual conditions in a production field. These limitations demonstrate the importance of conducting studies to evaluate the structural, nutritional and growth responses of young vines planted in soil with high amounts of Cu.

The present study therefore aimed to evaluate the effects of Cu toxicity on the root anatomy of young vines 'Niágara Branca' (*Vitis labrusca* L.) and the alleviating effect of lime on contaminated sandy soil.

2. Materials and methods

2.1. Soil collection and preparation

The soil used in the experiment was collected in the 0–20 cm layer in a native grass area adjacent to vineyards in the city of Santana do Livramento, Campanha Gaúcha region, Rio Grande do Sul State, Brazil. The soil was classified as Typic Hapludalf (Soil Survey Staff, 2006). After collection, the soil was air dried, passed through a sieve with a 2 mm mesh and homogenized.

The soil was divided into three parts, to which doses of lime equivalent to 0.0, 1.5 and 3.0 Mg ha^{-1} (megagrams per hectare) $CaCO_3$ (equivalent to 0.0, 0.5 and 1.0 g kg^{-1} soil, respectively) were applied (relative power of the total neutralization [RPTN]: 100%). The soil was subsequently incubated for 40 days. The lime used in the experiment was composed of a mixture of $CaCO_3$ and $MgCO_3$ (PA reagents, Synth brand) in a Ca:Mg ratio of 2:1. The lime doses were defined by a titration curve previously generated in a pre-test, which was based on a 0–20 cm soil depth with a density of 1.5 g cm^{-3}.

Each of the three soil parts was again separated, with the first portion maintained without the application of Cu and the second portion exposed to 50 mg kg^{-1} Cu in the form of $CuSO_4 \cdot 5H_2O$ (PA reagent, Synth brand). The soil samples were incubated again for 30 days. The Cu concentrations (0 and 50 mg kg^{-1}) were based on a preliminary study (unpublished) with the same soil.

In both incubations, the soil was maintained with soil pores filled with 70% water. After preparation, the soil was divided into two portions: one portion for the experiment and another for the analysis of chemical and particle size characteristics as presented in Table 1.

2.2. Plant material

Young vines 'Niágara Branca' were obtained by micropropagation and acclimatized in sterile substrate. Explants were cultivated for 30 days in test tubes (110 × 23 mm) containing 10 mL of Galzy culture medium (Galzy, 1964) in a growth room with a temperature of 23 ± 2 °C, a photoperiod of 16 h daylight, and photosynthetically active radiation (PAR) of 75 μmol photons m^{-2} s^{-1}. The plants were subsequently transferred into 200 mL plastic pots containing horticultural substrate and thin vermiculite (1:1 ratio) and cultivated for another 30 days in a growth room with a temperature of 25 ± 4 °C, a photoperiod of 12 h daylight, and PAR of 100 μmol photons m^{-2} s^{-1}. The average plant height was 5.0 cm at the end of acclimatization and at the beginning of the experiment.

2.3. Experimental procedure

The experiment was conducted in a controlled environment (phytotron) with a temperature of 25 ± 2 °C, a photoperiod of 16 h daylight, and PAR of 200 μmol photons m^{-2} s^{-1}. The experimental units were composed of rhizobox-type containers (dimensions 20 × 32 × 4 cm – width × height × depth, respectively) composed of wood with the inner surfaces coated with acrylic plates to prevent soil and plant root contact with the wood. The upward-facing rhizobox acrylic surface was covered with aluminum foil, and all sides of the box were wrapped in black plastic to prevent light from reaching the soil. The rhizoboxes were arranged on a bench with a slope of approximately 30°, which permitted root growth toward the acrylic surface that could be viewed by withdrawing the plastic covering the box. Soil (1.2 kg) was added to each experimental unit, and a young vine was then transplanted.

The experimental design was a randomized block design with six replications in a 3 × 2 factorial arrangement, which included three doses of lime and two Cu concentrations applied to the soil, for a total of six treatments. The plants were grown for a period of 70 days. During cultivation, Hoagland and Arnon (1950)

Table 1
Chemical and granulometric attributes of the Typic Hapludalf used in the cultivation of young vines 'Niágara Branca' (*Vitis labrusca* L.) with and without addition of Cu and with increasing doses of lime.

Attributes	Cu0 Lim0.0	Cu0 Lim1.5	Cu0 Lim3.0	Cu50 Lim0.0	Cu50 Lim1.5	Cu50 Lim3.0
Before cultivation						
Sand (g kg^{-1}) [a]	909	909	909	909	909	909
Silt (g kg^{-1}) [a]	30	30	30	30	30	30
Clay (g kg^{-1}) [a]	61	61	61	61	61	61
Total organic C (g kg^{-1}) [b]	5.1	4.5	4.9	5.6	5.6	5.4
Soil organic matter (g kg^{-1}) [b]	8.8	7.7	8.5	9.6	9.6	9.4
pH in water [b]	4.5	5.6	6.4	4.4	5.4	6.1
Available P (mg kg^{-1}) [c]	4.8	4.3	5.7	5.1	4.6	6.4
Exchangeable K (mg kg^{-1}) [c]	30.7	26.9	29.2	34.5	32.1	33.3
Exchangeable Al ($cmol_c$ kg^{-1}) [d]	3.1	0.0	0.0	4.1	1.1	0.0
Exchangeable Ca ($cmol_c$ kg^{-1}) [d]	2.0	2.5	4.4	2.3	2.6	4.2
Exchangeable Mg ($cmol_c$ kg^{-1}) [d]	1.8	1.8	2.3	1.8	2.6	2.7
Available Cu (extracted by EDTA) (mg kg^{-1}) [e]	2.4	2.4	2.2	23.0	23.2	21.5
Available Cu (extracted by $CaCl_2$) [f]	0.1	0.1	0.1	0.2	0.1	0.1
After cultivation						
Available Cu (extracted by EDTA) (mg kg^{-1}) [e]	2.6	2.5	2.8	39.5	39.2	39.7
Available Cu (extracted by $CaCl_2$) (mg kg^{-1}) [f]	0.1	0.1	0.1	6.6	2.7	0.3

Cu0 Lim0.0 – without addition of Cu and without lime; Cu0 Lim1.5 – without addition of Cu and with 1.5 Mg ha^{-1} lime; Cu0 Lim3.0 – without addition of Cu and with 3.0 Mg ha^{-1} lime; Cu50 Lim0.0 – with addition of 50 mg kg^{-1} Cu and without lime; Cu50 Lim1.5 – with addition of 50 mg kg^{-1} Cu and with 1.5 Mg ha^{-1} lime; Cu50 Lim3.0 – with addition of 50 mg kg^{-1} Cu and with 3.0 Mg ha^{-1} lime.

[a] Pipette method (Embrapa, 1997).
[b] Determined according to Tedesco et al. (1995).
[c] Extracted by Mehlich 1 extractor (Tedesco et al., 1995).
[d] Extracted by KCl 1 mol L^{-1} extractor (Tedesco et al., 1995).
[e] Extracted by disodium ethylenediaminetetraacetate (Na_2-EDTA) 0.05 mol L^{-1}/ammonium acetate 1.0 mol L^{-1}, pH 6.0 (Chaignon and Hinsinger, 2003).
[f] Extracted by $CaCl_2$ 0.01 mol L^{-1} (Novozamsky et al., 1993).

modified solution (without Cu, Ca and Mg, and pH adjusted to 6.0 with NaOH 0.1 mol L^{-1}) was applied in increments every week, with the following added (per kg of soil): 15.8 mg N; 2.4 mg P; 17.6 mg K; 5.0 mg S; 5.4 mg Cl; 0.4 mg Fe; 0.2 mg Na; 37.0 μg B; 37.6 μg Mn; 3.8 μg Zn; and 0.2 μg Mo. In addition, the soil moisture was monitored daily, and irrigation was performed when necessary.

2.4. Anatomical analysis of the roots

At the end of the experiment, five roots in the lower third of each rhizobox were randomly selected for anatomical analysis, and 1.5 cm samples were sectioned from the root apex. Each sample was divided into two segments: a 0.5 cm long segment from the apex for longitudinal sections and a 1.0 cm long segment for cross sections.

The samples were fixed in 2.5% paraformaldehyde in a sodium phosphate buffer 0.1 mol L^{-1} (1/1, v/v) at pH 7.2 for 24 h (Schmidt et al., 2009). After fixation, the samples were washed three times with 0.1 mol L^{-1} phosphate buffer and then dehydrated in an increasing ethanol series. The soil was subsequently removed from the root surface using an ultrasonic washer (Unique® MaxiClean 750 model). The samples were then subjected to a glycol methacrylate inclusion process (Historesin, Leica®) according to the manufacturer's instructions. After drying, 5 μm sections were cut with a manual microtome (model RM 2135, Leica®) using a steel blade. The sections were placed on slides, stained with toluidine blue 0.05% in 0.1 mol L^{-1} phosphate buffer (pH 6.8; O'Brien et al., 1964), and mounted with Canada balsam. Subsequently, the slides were observed under an optical microscope (Olympus® BX40 model), and images were captured using a camera (Olympus® model DP71) attached to the microscope.

The root diameter and areas of the cortex and vascular cylinder were determined by analyzing the cross-section images using ImageJ® software. The number of cortical layers and the number of cortical cells containing phenolic compounds were counted using the root cross-section images.

2.5. Sampling of plant material

At the beginning and end of cultivation, the plant height was determined using a graduated ruler, with the initial height subtracted from the final height to obtain the plant height increase.

At the end of cultivation, the shoot was cut at the soil surface, the leaves were detached from the stem, and the roots were separated from the soil by hand. Subsequently, the fresh mass of each part of the plant was dried in a forced-air oven at 65 °C until a constant mass was obtained to determine the dry matter (DM) content using a precision scale. The tissue was ground and set aside for later analysis of the total amount of Cu, Ca and Mg.

2.6. Plant tissue nutrient analysis

To determine the Cu, Ca and Mg concentration, digestion was conducted in a muffle furnace at 500–550 °C for 3 h followed by dilution in HNO_3 (1 mol L^{-1}) (Embrapa, 1997). The concentration of Cu, Ca and Mg in the extracts was then quantified using atomic uptake spectrophotometry.

The accumulation of nutrients in each organ of the plant (expressed in g $plant^{-1}$) was calculated by multiplying the concentration of each nutrient by the DM of each part. The relative nutrient accumulation percentage in each organ of the plant was then calculated by adopting the total nutrient accumulation throughout the plant as a reference (value of 100%).

2.7. Statistical analysis

The data for the relative percentage of accumulated Cu, Ca and Mg in each organ were transformed by applying the arcsine of the square root of the percentage for variance homogenization. All the data were then subjected to the Cochran test to verify the homoscedasticity. An analysis of variance was then performed, and Tukey's mean separation test ($P < 0.05$) was performed for significant results (Table 2).

The data were analyzed using a factorial model (doses of Cu vs.

doses of lime). The presentation and discussion of interaction effects were prioritized, and the means of each factor are presented and discussed in the text independently when interaction effects were not observed.

3. Results

3.1. Anatomical root structure

The root apex showed changes in cell organization between treatments (Fig. 1a–f). When 50 mg kg^{-1} Cu was added to the soil, without liming and with the addition of 1.5 Mg ha^{-1} lime, there was a shortening of the cell differentiation region, with more elongated and well-differentiated cells near the apex, and a reduction in root cap size (Fig. 1b and d). In the treatments without the addition of Cu (Fig. 1a, c and e) and with the addition of 50 mg kg^{-1} Cu and 3.0 Mg ha^{-1} of lime (Fig. 1f), the root apex showed well-defined regions of division, expansion and differentiation.

An analysis of the root cross-sections under different treatments (Fig. 2a–f) revealed structural changes only when 50 mg kg^{-1} Cu was added without liming (Fig. 2b). Under this treatment, the diameter increased by 47%, the cortex area increased by 128% and the vascular cylinder root area increased by 93% on average (Fig. 2b; Table 3). With the addition of 1.5 and 3.0 Mg ha^{-1} lime (Fig. 2d and f), the roots decreased and exhibited measurements similar to the roots of the plants grown in soil without the addition of Cu (Fig. 2a, c and e; Table 3).

Adding 50 mg kg^{-1} Cu to the soil promoted an increase in the number of cortical layers but only without the addition of 1.5–3.0 Mg ha^{-1} lime (Fig. 2a–f; Table 3). It should be noted that the magnitude of the changes caused by Cu and liming was only on the scale of one cell layer.

The roots of young vines grown in all the treatments showed cells containing phenolic compounds (Fig. 2a–f). However, the addition of Cu to the soil without liming resulted in an average increase of 132% in the number of cortical cells with these compounds (Fig. 2b). However, when 1.5 or 3.0 Mg ha^{-1} lime was added, fewer cortical cells containing phenolic compounds were observed (Fig. 2d and f; Table 3). Liming had no effect on this variable in treatments without the addition of Cu to the soil (Fig. 2a, c and f; Table 3).

The cross-sections showed an accumulation of phenolic compounds in the endoderm cells (Fig. 2a–f), and similar to the other cortical layers, the most intense accumulation of these compounds occurred in the roots of the plants grown in soil with the addition of Cu and without liming (Fig. 2b).

3.2. Dry matter and plant height

The addition of Cu to the soil without liming reduced the root DM on average by 41%; however, the addition of 1.5 or 3.0 Mg ha^{-1} lime to the soil together with Cu caused no effect on this variable (Fig. 3a). When Cu was not added to the soil, liming did not affect the root DM.

Stem DM and plant height increase were reduced on average by 44 and 55% in the soil with addition of Cu and without liming, respectively (Fig. 3b and d). The addition of 1.5 Mg ha^{-1} lime to the soil decreased the effect of Cu on these variables. On average, the stem DM was reduced by 31% and the height was increased by 44% with the addition of Cu; when 3.0 Mg ha^{-1} lime was added, the Cu factor in the soil had no effect on these variables.

The leaf DM only suffered from effects of Cu (Table 2) and was reduced on average by 21% with the addition of 50 mg kg^{-1} Cu (Fig. 3c).

3.3. Concentration and accumulation of nutrients in plants

In the roots, the Cu concentrations were increased by 371% on

Table 2
Summary of analysis of variance (P values) and coefficients of variation for the effects of the analyzed variables in the young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated in soil with and without addition of Cu and with increasing doses of lime.

Variable	P values			CV (%)
	Cu (A)	Liming (B)	A × B	
Root diameter (mm)	0.001**	<0.001***	0.030*	14.11
Cortex area (mm^2)	0.002**	<0.001***	0.012*	30.58
Vascular cylinder area (mm^2)	<0.001***	<0.001***	0.025*	27.31
Number of cortex layers	0.004**	<0.001***	0.647 ns	13.93
Cortex cells containing phenolic compounds	0.005**	<0.001***	0.008**	40.89
Root DM (g)	0.036*	0.511 ns	0.039*	24.89
Stem DM (g)	<0.001***	0.429 ns	0.044*	16.37
Leaf DM (g)	<0.001***	0.288 ns	0.832 ns	13.81
Height increase (cm)	<0.001***	0.049*	0.004**	16.96
Root Cu concentration (mg kg^{-1})	<0.001***	<0.001***	<0.001***	14.19
Root Ca concentration (g kg^{-1})	0.040*	0.003**	0.369ns	11.28
Root Mg concentration (g kg^{-1})	0.109 ns	0.139 ns	0.088 ns	14.87
Stem Cu concentration (mg kg^{-1})	0.330 ns	0.001**	0.047*	10.22
Stem Ca concentration (g kg^{-1})	0.337 ns	<0.001***	0.215 ns	7.55
Stem Mg concentration (g kg^{-1})	0.998 ns	0.012*	0.397 ns	11.59
Leaf Cu concentration (mg kg^{-1})	0.415 ns	0.013*	0.176 ns	20.13
Leaf Ca concentration (g kg^{-1})	0.322 ns	0.001**	0.103 ns	12.33
Leaf Mg concentration (g kg^{-1})	0.054 ns	<0.001***	0.053 ns	11.66
Cu accumulation in roots (%)	<0.001***	0.040*	0.009**	3.15
Ca accumulation in roots (%)	0.213 ns	0.403 ns	0.144 ns	5.91
Mg accumulation in roots (%)	0.045*	0.046*	0.292 ns	7.83
Cu accumulation in stems (%)	<0.001***	0.002**	0.008**	10.10
Ca accumulation in stems (%)	0.496 ns	0.227 ns	0.694 ns	9.24
Mg accumulation in stems (%)	0.269 ns	0.116 ns	0.378 ns	8.21
Cu accumulation in leaves (%)	<0.001***	0.212 ns	0.049*	11.77
Ca accumulation in leaves (%)	0.302 ns	0.242 ns	0.274 ns	4.95
Mg accumulation in leaves (%)	0.013*	0.065 ns	0.202 ns	5.93

* = Significant according to F test ($P < 0.05$); ** = Significant according to F test ($P < 0.01$); ns: not significant.

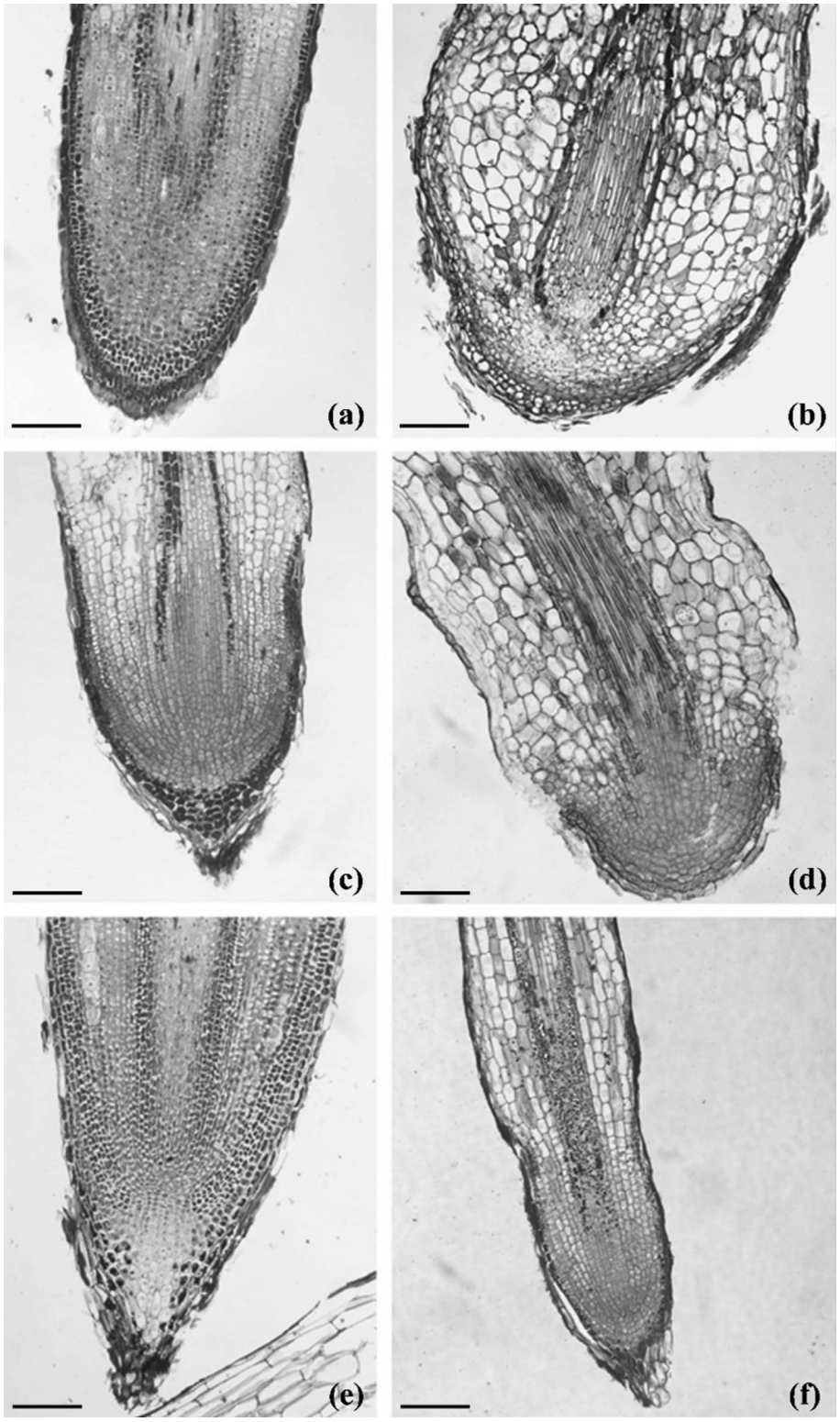

Fig. 1. Longitudinal sections of the root apex of young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated with and without addition of 50 mg kg^{-1} Cu and with addition of 0.0, 1.5 or 3.0 Mg ha^{-1} lime. (a) – without addition of Cu and without lime; (b) – with addition of 50 mg kg^{-1} Cu and without lime; (c) – without addition of Cu and with 1.5 Mg ha^{-1} lime; (d) – with addition of 50 mg kg^{-1} Cu and with 1.5 Mg ha^{-1} lime; (e) – without addition of Cu and with 3.0 Mg ha^{-1} lime; (f) – with addition of 50 mg kg^{-1} de Cu and with 3.0 Mg ha^{-1} lime. Bars = 100 μm.

average in the plants cultivated with the addition of Cu, regardless of the lime dose (Fig. 4a). A comparison of the doses of lime and Cu only showed an effect with the addition of 50 mg kg^{-1} Cu. In this case, the metal concentration in the roots remained similar for treatments without liming and with the addition of 1.5 Mg ha^{-1} lime. However, when 3.0 Mg ha^{-1} lime was added, the Cu concentration increased by 58% in the root compared with the treatments without liming.

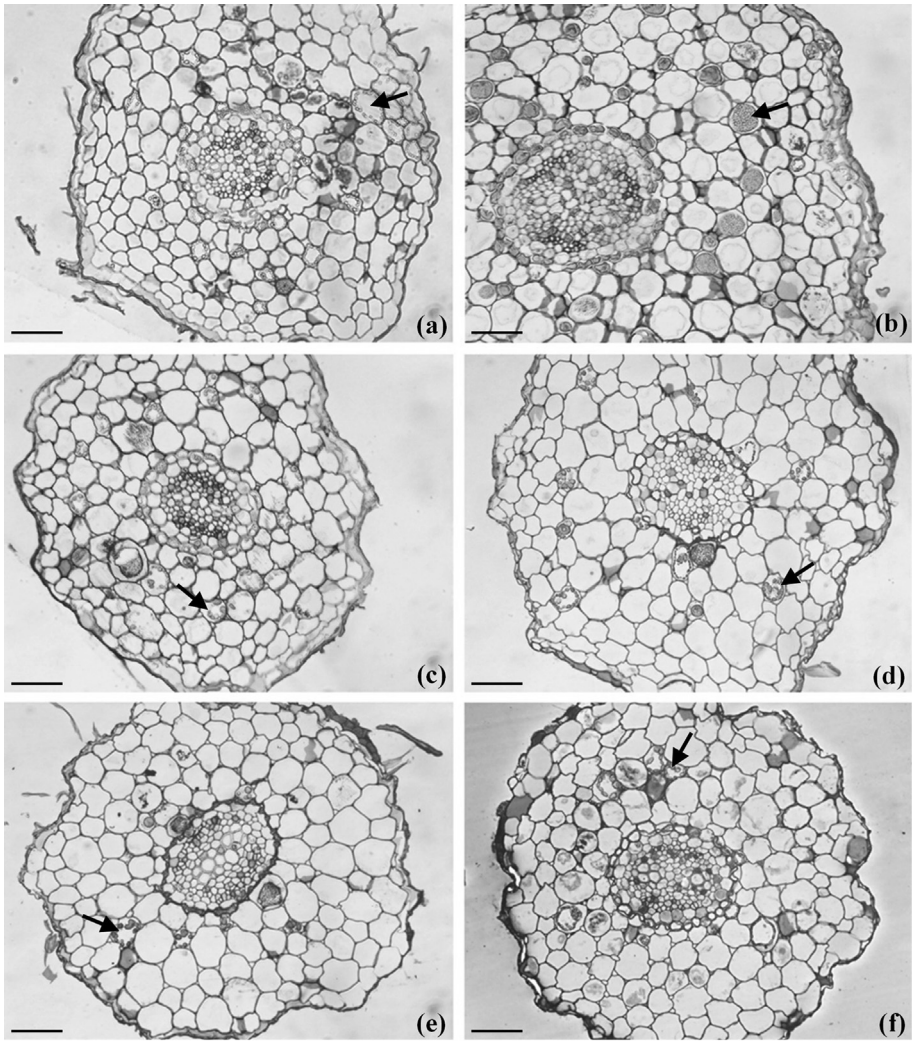

Fig. 2. Cross-sections (0.5–2.0 cm) of the root apex of young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated in soil with and without addition of 50 mg kg^{-1} Cu and with addition of 0.0, 1.5 or 3.0 Mg ha^{-1} lime. (a) – without addition of Cu and without lime; (b) – with addition of 50 mg kg^{-1} Cu and without lime; (c) – without addition of Cu and with 1.5 Mg ha^{-1} lime; (d) – with addition of 50 mg kg^{-1} Cu and with 1.5 Mg ha^{-1} lime; (e) – without addition of Cu and with 3.0 Mg ha^{-1} lime; (f) – with addition of 50 mg kg^{-1} Cu and with 3.0 Mg ha^{-1} lime. Arrows: cortical cells with phenolic compound accumulation. Bar = 50 μm.

Cu contamination to the soil did not influence the levels of this element in the vine stem (Fig. 4b). However, the addition of lime increased the Cu concentration in the roots, and in the treatments without the addition of Cu, the addition of 1.5 Mg ha^{-1} lime did not have an effect on the heavy metal concentration, whereas the addition of 3.0 Mg ha^{-1} caused the Cu concentration to increase by 62% in the stem relative to that of young vines grown in soil without the addition of Cu and lime. When the plants were grown in soil with the addition of 50 mg kg^{-1} Cu, the dose of 1.5 Mg ha^{-1} lime caused the Cu concentration to increase by 30% in the stem compared with the treatment without liming. With the dose of 3.0 Mg ha^{-1} lime, the stem Cu concentration did not differ between treatments with and without the addition of Cu to the soil.

In the leaves, the Cu concentration was only affected by the liming factor (Fig. 4c). With the addition of 1.5 Mg ha^{-1} lime, the mean Cu concentration was reduced by 31% in the leaves compared with the treatment without liming. However, when 3.0 Mg ha^{-1} lime was added to the soil, the Cu concentration in the leaves returned to the condition observed for the treatment without liming.

The addition of 50 mg kg^{-1} Cu to the soil increased the percentage of Cu accumulation in the roots and reduced the percentage of Cu accumulation in the stems and leaves compared with the treatments without the addition of Cu, independent of the applied dose of lime (Fig. 5a). A comparison of the effects of lime doses according to the Cu doses showed that an increase in lime doses to plants grown in soil without the addition of Cu reduced the percentage of heavy metal accumulation in the roots and increased the percentage of accumulation in the stems and leaves. However, regardless of the treatment, the highest percentage of Cu accumulation was in the roots. In the plants exposed to 50 mg kg^{-1} Cu, liming had no effect on the percentage of Cu accumulated in each organ.

The addition of 50 mg kg^{-1} Cu to the soil decreased the Ca concentration in the roots on average by 13% (Fig. 4d). However, the addition of 3.0 Mg ha^{-1} lime to the soil caused the Ca concentration to increase by 43% in the roots compared with the treatments without liming. Only liming had an effect on the stem and leaf Ca concentrations. In the stem, the addition of 1.5 and 3.0 Mg ha^{-1} lime caused the Ca concentration to increases by 33 and 20% on average, respectively (Fig. 4e). In the leaves, only the addition of 3.0 Mg ha^{-1} had an effect on the Ca concentration, which increased by 38% on average compared with the treatments without liming (Fig. 4f).

Table 3
Diameter, cortex and vascular cylinder areas and cortical layers with phenolic compounds in the roots of young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated in soil with and without addition of Cu and with increasing doses of lime.

Cu (mg kg^{-1})	Lime dose (mg ha^{-1})			Mean
	0.0	1.5	3.0	
	Root diameter (mm)			
0	389.3 aB	323.3 aA	320.7 aA	344.4 B
50	573.2 aA	396.0 bA	332.2 bA	433.8 A
Mean	481.2 a	359.6 b	326.5 b	
	Cortex area (mm^2)			
0	97.0 aB	73.9 aA	72.4 aA	81.1 B
50	220.7 aA	103.6 bA	79.7 bA	134.7 A
Mean	158.8 a	88.8 b	76.1 b	
	Vascular cylinder area (mm^2)			
0	8.7 aB	5.8 aA	6.9 aA	7.1 B
50	16.8 aA	8.6 bA	8.1 bA	11.1 A
Mean	12.7 a	7.2 b	7.5 b	
	Number of cortical layers			
0	7 ^{nsi}	5	5	6 B
50	8	6	6	7 A
Mean	7 a	6 b	5 b	
	Number of cortical cells with phenolic compounds			
0	19 aB	12 aA	9 aA	14 B
50	44 aA	20 bA	7 bA	23 A
Mean	31 a	16 b	8 b	

Means ± standard deviation followed by the same uppercase letter in the column and by the same lowercase letter on the line do not differ from each other according to Tukey's test ($P < 0.05$); nsi: non-significant interaction.

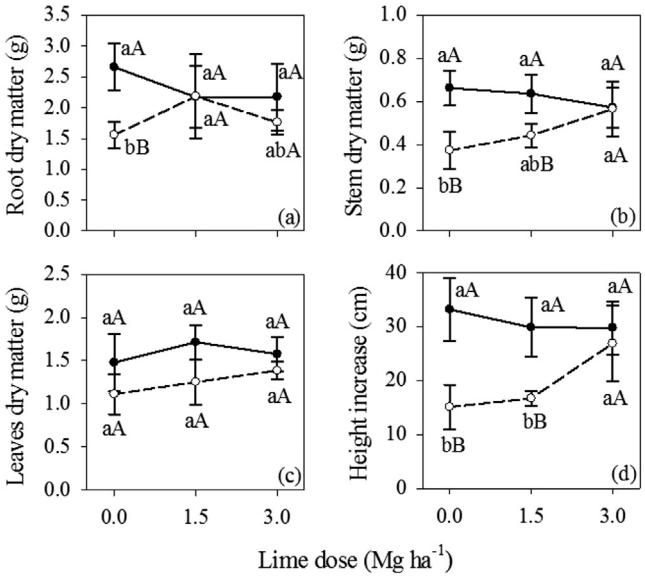

Fig. 3. Dry matter of the roots (a), stems (b) and leaves (c) and height increase (d) of the young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated in soil with and without addition of Cu and with increasing doses of lime. Continuous line: without addition of Cu; dotted line: with addition of Cu. Means followed by the same uppercase letter (Cu factor) and by the same lowercase letter (liming factor) do not differ from each other according to Tukey's test ($P < 0.05$); vertical bars at each point correspond to the standard deviation.

The concentration of Mg in the roots was not affected by the treatments (Table 2, Fig. 4g). However, in the stems, the concentration of this nutrient was increased by 30 and 35% on average with the addition of 1.5 and 3.0 Mg ha^{-1} lime, respectively, compared with the treatments without liming (Fig. 4h). The concentration of Mg in the leaf was also increased by 35% on average with the addition of 3.0 Mg ha^{-1} lime compared with the treatment without the addition of lime (Fig. 4i).

The addition of 50 mg kg^{-1} Cu to the soil decreased the relative percentage of Mg accumulation in the roots from 42 to 36% and increased the percentage of Mg accumulation in leaf from 48 to 55% compared with the treatment without the addition of Cu (Fig. 5c). Furthermore, increased additions of lime decreased the relative percentage of Mg accumulation in the roots from an average of 44% without liming treatment to 36% for the treatment with the addition of 3.0 Mg ha^{-1} lime. Nevertheless, the lime dose did not have an effect on the relative percentage of Mg accumulation in the stem and leaf.

4. Discussion

4.1. Anatomical root structure and plant growth

In the present study, the cell organization of the young vine root apex showed changes when grown in soil with the addition of Cu, especially when liming was not applied. One of the symptoms of Cu toxicity observed in these treatments was a shortening of the cell differentiation region. Similar results were found in maize (*Zea mays* L.) by Jiang et al. (2001), who observed that excessive Cu changed the mitotic index and reduced the cell division frequency at the root apex, thus inhibiting root growth.

A reduction in root cap size at the root apex of young vines under Cu exposure without liming and with the addition of 1.5 Mg ha^{-1} lime, in this study, is a known symptom of Cu toxicity and is most likely caused by a reduction in cell division, which is also caused by excessive Cu (Ouzounidou et al., 1992). A thinner root cap can be harmful to the plant because this structure protects the root apical meristem and facilitates root penetration into the soil (Lynch et al., 2012).

Excessive Cu in the soil also causes changes in more distal regions of the root apex, including increases in the diameter and in the cortical and vascular cylinder areas, how noted in young vines of the present study. These symptoms are also observed in other species, such as *Pinus* (Arduini et al., 1995), wheat (*Triticum turgidum durum* L.) (Michaud et al., 2008) and *Kummerowia stipulacea* (Maxim.) Makino (Zhang et al., 2014). The increased cortical area, and hence increased diameter of the roots in young vines, may be caused by a disruption in the arrangement of cortical cells. However, the increased diameter of roots exposed to high levels of heavy metals is typically related to an inhibition of root length (Arduini et al., 1995; Rucinska et al., 1999), which is associated with disturbances in cell division (Rucinska et al., 1999; Jiang et al., 2001). In the present study, this result may be related to a shortening of the cell differentiation region in the roots exposed to excessive Cu in the soil.

An intense accumulation of phenolic compounds was observed in the endoderm cells and cells of other layers of the cortex in the roots of vines grown in soil with the addition of Cu and without liming in this study. The accumulation of phenolic compounds in the plant roots exposed to excessive Cu and other metals has been observed in other species (Kováčik and Bačkor, 2007; Bouazizi et al., 2010). According to Michalak (2006), the accumulation of these compounds in plants with heavy metal stress is a defense strategy against oxidative stress caused by reactive oxygen species (ROS), which present more intense production in such situations. Also according to these authors, these compounds present different methods of protecting plants against ROS: 1. they act as chelating agents for the metals inside the plant, which reduces the reaction power of metals during plant metabolism; 2. they inhibit lipid peroxidation by metal ions; and 3. they directly eliminate ROS.

It is important to emphasize the results obtained by Ofei-Manu et al. (2001), who found that woody plants that are more tolerant to

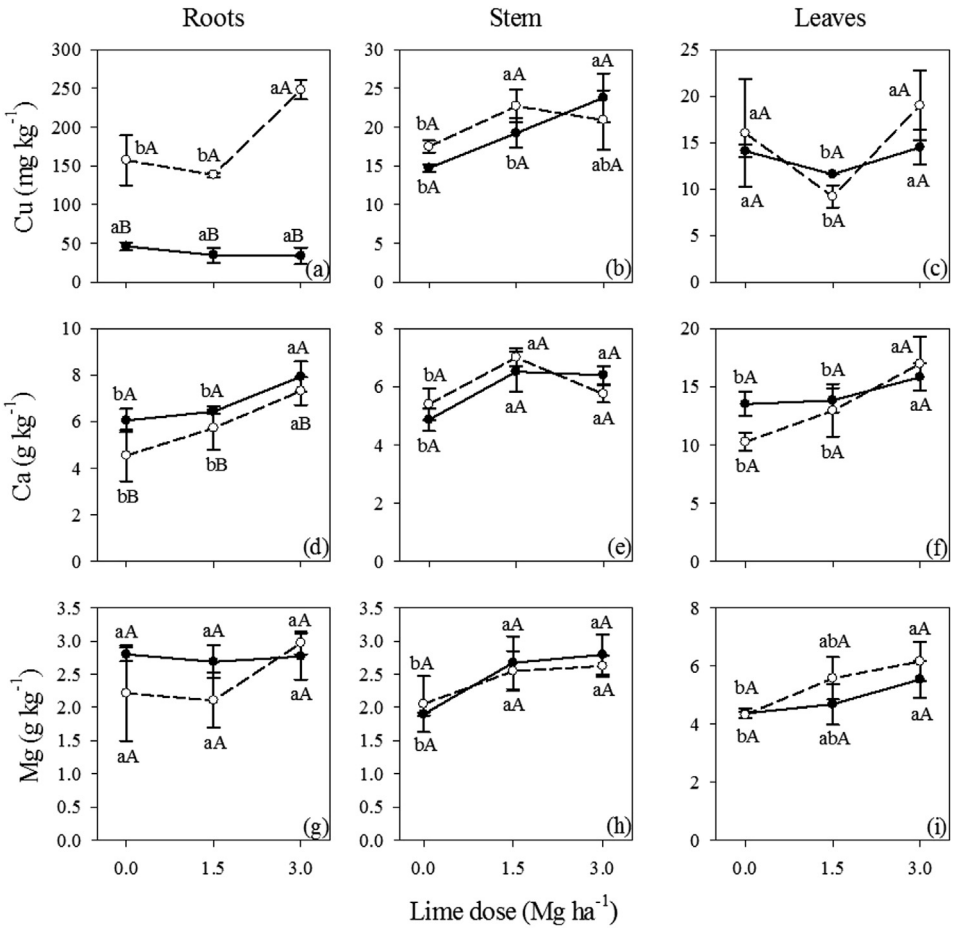

Fig. 4. Cu (a, b, c), Ca (d, e, f) and Mg (g, h, i) concentration in roots, stems and leaves of young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated in soil with and without addition of Cu and with doses of lime. Continuous line: without addition of Cu; dotted line: with addition of Cu. Means followed by the same uppercase letter (Cu factor) and by the same lowercase letter (liming factor) do not differ from each other according to Tukey's test ($P < 0.05$); vertical bars at each point correspond to the standard deviation.

aluminum (Al) take up greater amounts of phenolic compounds. Furthermore, certain phenolic compounds also act on lignin synthesis in plants, and their accumulation in the plant could promote stronger cell walls and form physical barriers against the entry and distribution of heavy metals (Michalak, 2006). Therefore, the increased accumulation of phenolic compounds in the roots of young vines grown in soil with the addition of Cu and without liming is a defense strategy against stress caused by this heavy metal.

The exposure to excessive Cu also reduced the root, stem and leaf DM and affected increases in height in the young vines of this study. These symptoms are characteristic of Cu toxicity and have been reported in studies with several species, such as wheat (Michaud et al., 2008), *Urochloa mosambicensis* (Hack.) Dandy (Kopittke et al., 2009), *Arabidopsis thaliana* (Lequeux et al., 2010), *K. stipulacea* (Maxim.) Makino (Zhang et al., 2014) and even vines (Toselli et al., 2009; Juang et al., 2014; Chen et al., 2013; Cambrollé et al., 2015).

The reduced biomass of plants exposed to excessive Cu may have resulted from other symptoms. For example, contact between the root system and soil with a high Cu concentration may present toxicity symptoms that include damage to the cuticle, cracks in the roots, and the above-mentioned structural changes, thus inhibiting growth and biomass production in the roots (Sheldon and Menzies, 2005; Michaud et al., 2008). As a result, the root system presents decreased soil exploration and water and nutrient uptake, which is reflected in lower biomass and plant growth (Kopittke et al., 2009; Toselli et al., 2009).

In the present study, the symptoms of excessive Cu in the plants' root structure and growth were alleviated by addition of lime, mainly with the highest tested dose, and this result was likely caused by the increased soil pH from 4.4 (without liming) to 5.4 (1.5 Mg ha^{-1} of lime) and 6.1 (3.0 Mg ha^{-1} of lime), which was reflected in the decreased availability of Cu (extracted by $CaCl_2$) from 6.6 to 2.7 and 0.3 mg kg^{-1}. Moreover, the Ca and Mg concentrations in the soil also increased with liming, which had an alleviating effect on Cu toxicity in the plants. For example, the studies of young vines placed in a solution containing concentrations of Cu and Ca (Chen et al., 2013) and with the addition of Cu and Mg (Juang et al., 2014) revealed that Cu toxicity symptoms observed in the roots were alleviated by Ca and Mg.

Increases in the concentrations of Ca and Mg within the plant, such as those observed in the present study, are important because these nutrients constitute the pectin in the middle lamella, and increased concentrations in the plant can strengthen the cell wall (Hawkesford et al., 2012) and reduce the toxic effects of Cu on the root tissue (Chen et al., 2013; Juang et al., 2014). Furthermore, increases of Ca content within the plant may promote the formation of Ca-oxalate crystals that can incorporate heavy metals, such as Cu, into their matrix, thus reducing the activity and toxic effects of this metal in the plant (Franceschi and Nakata, 2005). Moreover, Mg is involved in the synthesis of chlorophyll and is a component of

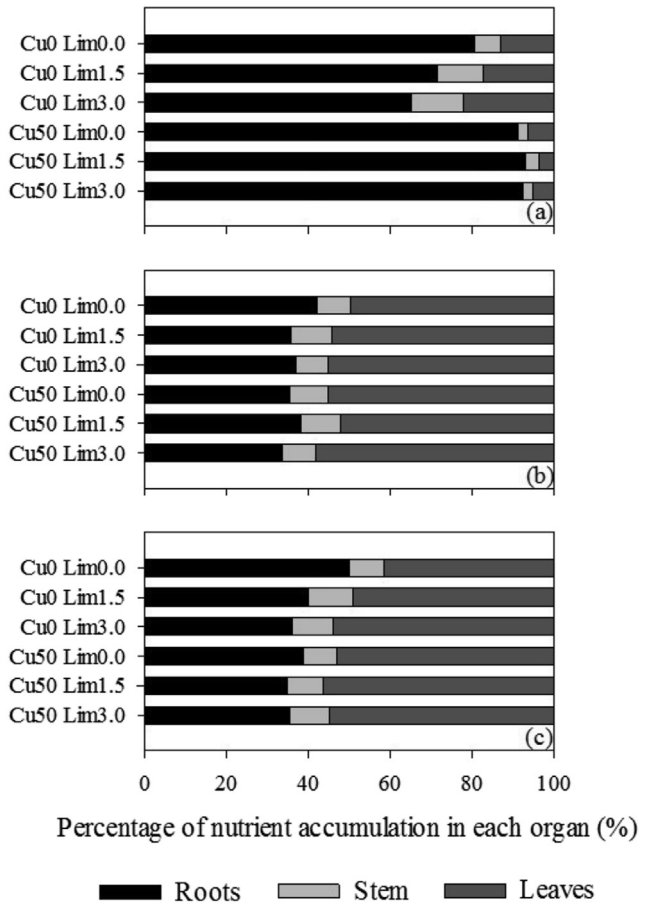

Fig. 5. Relative percentage of Cu (a), Ca (b) and Mg (c) accumulation in roots, stems and leaves of young vines 'Niágara Branca' (*Vitis labrusca* L.) cultivated in soil with and without addition of Cu and doses of lime. Cu0 Lim0.0 – without addition of Cu and without lime; Cu0 Lim1.5 – without addition of Cu and with 1.5 Mg ha^{-1} lime; Cu0 Lim3.0 – without addition of Cu and with 3.0 Mg ha^{-1} lime; Cu50 Lim0.0 – with addition of 50 mg kg^{-1} Cu and without lime; Cu50 Lim1.5 – with addition of 50 mg kg^{-1} Cu and with 1.5 Mg ha^{-1} lime; Cu50 Lim3.0 – with addition of 50 mg kg^{-1} Cu and with 3.0 Mg ha^{-1} lime.

chlorophyll molecules (Hawkesford et al., 2012). Increased Mg contents within the plant may compete with Cu and prevent it from replacing Mg as the central ion of the chlorophyll molecule and producing toxicity symptoms (Yruela, 2009).

Therefore, the practice of liming presented various mechanisms for reducing Cu toxicity. First, the increased soil pH and concentration of OH^- in the soil can precipitate Cu in hydroxide form and reduce its availability to plants (Agbenin and Olojo, 2004). Second, the addition of lime increased the Ca and Mg content in the soil, and these molecules can compete with Cu during uptake (Luo et al., 2008; Kopittke et al., 2011) and within the plant by preventing excessive Cu transport to the shoots (Chen et al., 2013; Juang et al., 2014).

In addition to the reduction of Cu availability and the increase of Ca and Mg in the soil, liming also increased the soil pH from 4.4 to 5.4 or 6.1, and it may have provided additional benefits to the young vines, such as by reducing the absorption of Al, which is toxic to plants, and by increasing the availability of phosphorus (P) to and the uptake of P by the roots, favoring growth (Bates et al., 2002). Increases of P within the plant increase the plant's nutritional status and also have an effect on Cu toxicity because within the roots, P binds to Cu and forms an insoluble complex, thus preventing interactions between Cu and the root cells, especially vascular tissue cells, and alterations to the root structure (Ferreira et al., 2014).

4.2. Concentration and accumulation of nutrients in plants

The addition of 50 mg kg^{-1} Cu in the soil increased the Cu concentration in the roots but not in the shoots of the young vines of the present study. The increased Cu concentration in the roots was accompanied by a higher relative percentage of heavy metal accumulation in the roots and lower percentage in the shoots. However, when Cu was not added to the soil, increased lime doses reduced Cu accumulation in the roots and increased Cu accumulation in the stems and leaves. This pattern indicates that under lower Cu concentrations in the soil, liming provided higher metal transport to the shoots, whereas with higher Cu concentrations, increased Cu transport from the roots to shoot did not occur, which indicates that the plant may regulate Cu transport to the shoot to prevent excess accumulations of Cu (Yruela, 2009; Lequeux et al., 2010).

These results are consistent with those of Lequeux et al. (2010) who worked with *A. thaliana* and also found greater Cu accumulation in roots exposed to high concentrations of the metal; these authors attributed this pattern to Cu retention in the cell wall in the apoplast, which is considered an important mechanism of stress tolerance. This effect was important for the young vines in the present experiment because it indicated that the Cu concentration in the leaves was maintained within the normal range for the vine, which is 10–20 mg kg^{-1} (Jones Jr. et al., 1991), and for most species, which is 5–30 mg kg^{-1} (Kabata-Pendias, 2011), thus confirming the results of Toselli et al. (2009).

The concentration of Ca in the roots of young vines in the present study was reduced by the addition of Cu to the soil, whereas the Ca concentration in the stems and leaves was not affected by the metal. The concentration of Mg in all the plant parts was not affected by the addition of Cu to the soil. A reduction in root Ca concentrations caused by exposure to high Cu concentrations was also observed by Toselli et al. (2009) and Chen et al. (2013). A reduction in plant Ca concentrations may have been caused by competition for adsorption on the root surface by ions with the same valence, such as Cu and Ca, which would cause a decline in Ca uptake (Luo et al., 2008; Kopittke et al., 2011).

As expected, an increase in lime doses caused an increase in Ca and Mg concentrations in the roots, stems and leaves, regardless of the Cu dose. As previously reported, an increase in these two nutrients within the plant can alleviate Cu toxicity symptoms in young vines (Chen et al., 2013; Juang et al., 2014).

5. Conclusions

Excessive concentrations of Cu in the soil changes the organization of cells in the root apex and increases the areas of the cortex and vascular cylinder, thus increasing root diameter; however, excess Cu also reduces the growth of young vines. In addition, exposure to Cu causes phenolic compounds to accumulate in the root apex cortical cells, Ca concentrations to decrease in the roots and Cu concentrations and percentages to increase in the plant roots.

Liming decreases Cu concentrations in the soil (extracted by $CaCl_2$) and increases Ca and Mg uptake by plants, and these effects prevent alterations to anatomical structures in the vine roots exposed to this contaminant.

6. Contributions

Vítor Gabriel Ambrosini: study design, accomplishment of the

experiment, all laboratory analysis, interpretation of results and writing of the article. Daniel José Rosa: study design, accomplishment of the experiment, nutrient analysis, interpretation of results and writing of the article. Jenny Paola Corredor Prado: study design, analysis of plant anatomy and writing of the article. Marcelo Borghezan: study design, accomplishment of the experiment and writing of the article. George Wellington Bastos de Melo, Cláudio Roberto Fonsêca de Sousa Soares and Jucinei José Comin: study design, interpretation of results and writing of the article. Daniela Guimarães Simão: study design, analysis of plant anatomy, interpretation of results and writing of the article. Gustavo Brunetto: coordinated the work, participated in the study design, interpretation of results and writing of the article.

Frequent applications of fungicides and solutions based on copper (Cu) to control foliar disease in vines causes accumulation of this heavy metal in the soil over the years. When in excess in soil, Cu can lead to anatomical and morphological changes in roots, reflecting in inhibition of nutrient absorption and plant growth, delaying the establishment of vine seedlings in the field. However, application of lime in the soil before vineyard planting can therefore be a strategy for minimizing the toxic effects of Cu in young vines. The present study therefore aimed to evaluate the effects of Cu toxicity on the root anatomy of young vines and the ameliorating effect of lime on contaminated sandy soil. Excess soil Cu altered the organization of cells in the root apex, reduced Ca content and increased the content and percentage of Cu uptake and reduced growth of the young vines. Liming reduced Cu phytotoxicity of the young vines by increasing absorption of Ca and Mg and by avoiding excess Cu transport to the shoots, besides to avoid the anatomical changes to the vine roots exposed to this contaminant. The results of this manuscript can be applied in the field production and are relevant to the advancement of research with copper toxicity in vines.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.08.012.

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