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

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ABSTRACT

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

1. Introduction

Wine regions can be located in areas presenting climatic conditions favorable for the occurrence of leaf fungal diseases, mainly mildew (*Plasmopara viticola*) (Brunetto et al., 2017). Thus, it is worth regularly using fungicides made of copper (Cu), such as the Bordeaux fungicide, to protect grapevines from fungi. Since successive leaf applications of these fungicides are conducted in the same area for many years, Cu accumulates in the soil, mainly in the upper soil layers (Brunetto et al., 2014). Annual applications can reach 30 kg Cu ha^{-1} (Casali et al., 2008), so, the Cu content in the soil can often reach toxic levels to plants (Miotto et al., 2014; Girotto et al., 2016; Guimarães et al., 2016). Accordingly, some winemakers start using fungicides made of zinc (Zn) to the detriment of copper fungicides. Therefore, one can observe the

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combination of high Cu and Zn contents in vineyard soils in traditional wine regions worldwide, fact that has been gaining researchers' attention in the last decades (Fernández-Calviño et al., 2012; Brunetto et al., 2014, 2016; Cambrollé et al., 2015; Tiecher et al., 2016a, 2016c; Tiecher et al., 2017).

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

High Cu and Zn concentrations in plant tissues may negatively influence the gas exchanges performed by plants, since they often induce the occurrence of considerable effects on the net photosynthesis and stomatal conductance rates (Cambrollé et al., 2012, 2015). Yet, Cu excess may affect the membrane transport function and the ionic channels (Janicka-Russak et al., 2008). Such changes may lead to increase in the non-specific permeability of the membrane, which results in nutritional imbalance (Cambrollé et al., 2013). Moreover, it is possible observing oxidative stress caused by unbalance between antioxidant responses and the increased production of reactive oxygen species (ROS) through Fenton reaction (Girotto et al., 2013). The excess of Zn may also generate oxidative stress, because of its influence on the antioxidant defense system of plants (Gratão et al., 2005), as well as negatively affect the photosynthetic efficiency by inhibiting chlorophyll biosynthesis. Such inhibition leads to leaf chlorosis and carbon assimilation reduction, since it compromises the electron transport chain (De Magalhães et al., 2004; Castiglione et al., 2007; Chen et al., 2008; Dhir et al., 2008), fact that helps inhibiting plant growth (Girotto et al., 2013).

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

2. Materials and methods

2.1. Experiment description

The soil used in the experiment was classified as Typic Hapludalf (Soil Survey Staff, 2006); it was collected in natural non-anthropized field (30°47′23.5″S and 55°22′7.0″W), which presented naturally low Cu and Zn concentration. The area was located adjacent to a vineyard in Campanha Gaúcha region, Santana do Livramento County – RS (Southern Brazil). The soil was collected from the 0.00 to 0.15 m layer in August 2015. Subsequently, it was air dried, ground, sieved through 2 mm mesh and reserved. Soil physicochemical characterization is shown in Table 1.

Table 1
Physical and chemical characteristics of the 0.0–0.15 m layer in a Typic Hapludalf soil under natural field [data from Tiecher et al. (2016b)].

Physical and chemical characteristics	Natural field
Clay (g kg^{-1})	54
Sand (g kg^{-1})	894
Silt (g kg^{-1})	52
Organic matter (g kg^{-1})	9.0
pH_{H2O} (1:1)	5.2
Exchangeable Al ($cmol_c$ kg^{-1})	0.4
Available Cu by EDTA (mg kg^{-1})	0.7
Available Zn by EDTA (mg kg^{-1})	0.9
Available K by Mehlich − 1 (mg kg^{-1})	132
Available P by Mehlich − 1 (mg kg^{-1})	7.0
Available Fe by EDTA (mg kg^{-1})	21.9
Available Mn by EDTA (mg kg^{-1})	40.7
Exchangeable Ca ($cmol_c$ kg^{-1})	0.5
Exchangeable Mg ($cmol_c$ kg^{-1})	0.2
CEC_{ef}[a], $cmol_c$ kg^{-1}	1.4
$CEC_{pH\ 7.0}$[b], $cmol_c$ kg^{-1}	3.2

[a] CEC_{ef} = Effective cation exchange capacity.
[b] $CEC_{pH\ 7.0}$ = Potential cation exchange capacity at pH 7.0.

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

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

Young grapevine plants were obtained by micropropagation and acclimatized in sterile substrate. Explants were cultivated for 30 days in test tubes in a growth room with a temperature of 25 ± 1 °C, photoperiod of 16 h daylight and photosynthetically active radiation of 75 µmol photons m^{-2} s^{-1}. The plants were subsequently transferred into 200 mL plastic pots containing horticultural substrate and thin vermiculite (1:1 ratio) and cultivated for another 30 days in a growth room. Later, the plants were transferred for another 15 days into a greenhouse and kept with 50% interference of the radiation. Finally, in November 2015, one young grapevine plant (*Vitis vinifera*) was transplanted – after incubation – to each rizobox containing 2.4 kg of soil,

and cultivated for 60 days. The rizoboxes were 20 cm wide, 32 cm tall and 4 cm deep, they had lateral wood frames and the inner sides were coated with acrylic. Both outer rizobox faces were covered with aluminum paper to avoid light incidence inside the soil mass, thus simulating real soil condition. Rizoboxes were allocated on wood supports, at inclination 45°, throughout the grapevine cultivation. During the experimental period, the temperature in greenhouse was kept at 25 ± 5 °C, the relative air humidity was approximately 70%, and there was no interference in the photoperiod (natural conditions). Soil humidity was kept at 80% MWRC through daily irrigation to replace the evapotranspirated water. The total of 50 mL of a solution containing 50 mg N kg^{-1}, was added to the samples 15 days after transplant (DAT).

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

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

The samples were ground in Wiley mill after drying; the total Cu and Zn content in the shoot and roots was set after the digestion of 0.1 g of tissue in 3.0 mL of HNO_3 65% P.A. (Vetec, ≤ 0.2 ppm of heavy metals) and 1 mL of $HClO_4$ 70% P.A. (Vetec, ≤0.1 ppm of Cu and Zn) (Embrapa, 2009). Sample digestion was conducted in open system by using the digester block Velp Scientifica (Milan, Italy), which was heated at 130 °C for 4 h. The total Cu and Zn content was analyzed in atomic absorption spectrophotometer with detection limit of 1.5 μg L^{-1} of Cu and Zn (AAS, Perkin Elmer AAnalyst 200, USA). To calibrate the AAS, a calibration curve was made from a standard Merck Certipur 1000 mg L^{-1} of Cu and Zn.

2.3. Morphological analysis of the root system

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

2.4. Photosynthetic pigment extraction and quantification

Leaf discs were collected from the same leaf used to set the gas exchanges at 60 DAT in order to analyze the photosynthetic pigments in each treatment repetition. The discs were frozen in liquid N_2 and stored at − 80 °C. The chlorophylls *a* (Chl *a*) and *b* (Chl *b*), as well as carotenoid contents were quantified according to the methodology described by Hendry and Price (1993). Subsequently, the leaf discs were macerated with liquid N_2 and the tissue was homogenized in 5 mL of 80% acetone; samples were then transferred to 15-mL falcon tubes and centrifuged at 4000*g* for 4 min at 25 °C (3–18 K Centrifuge, Sigma, Germany). Finally, supernatant absorbance was set at 480, 645 and 663 nm in order to determine the Chl *a*, Chl *b* and carotenoid contents, respectively, using spectrophotometer model SF325NM (Bel Engineering, Italy). The pigment contents were calculated according to the methodology suggested by Lichtenthaler (1987).

2.5. Chlorophyll a fluorescence

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

2.6. Superoxide dismutase (SOD) activity

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

2.7. Gas exchanges

Gas exchanges were measured 60 DAT with the aid of an open circuit system incorporating an infrared gas analyzer (LI-6400XT LI-COR, Inc., Lincoln, NE, EUA). The readings were applied to each treatment repetition in the first completely expanded leaf. Net photosynthetic rate (A), and intercellular CO_2 concentration (Ci) were measured at ambient CO_2 concentration 400 μmol mol^{-1}, temperature 20/25 °C, relative humidity 50% ± 5% and photon flux density 1000 μmol m^{-2} s^{-1}.

2.8. Statistical analysis

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

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

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

3. Results

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

There was interaction between Cu and Zn in the root and shoot dry matter production of grapevine plants (Table 2). The higher the Cu and

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

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

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

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

There was interaction between the Cu and Zn contents in the roots (Table 2). Regarding treatments based on Cu addition, the higher the Zn dose, the higher the Cu contents in the roots (Fig. 1c, d). On the other hand, Cu contents in the shoot just responded to Cu addition to the soil (Fig. 1e), whereas Zn contents in the shoot responded to Zn addition to the soil (Fig. 1f); in both cases, the higher the dose applied to the soil, the higher the Cu and Zn contents in plant shoot.

3.2. Morphological analyses of the root system in grapevines

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

3.3. Photosynthetic pigments

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

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

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

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

4. Discussion

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

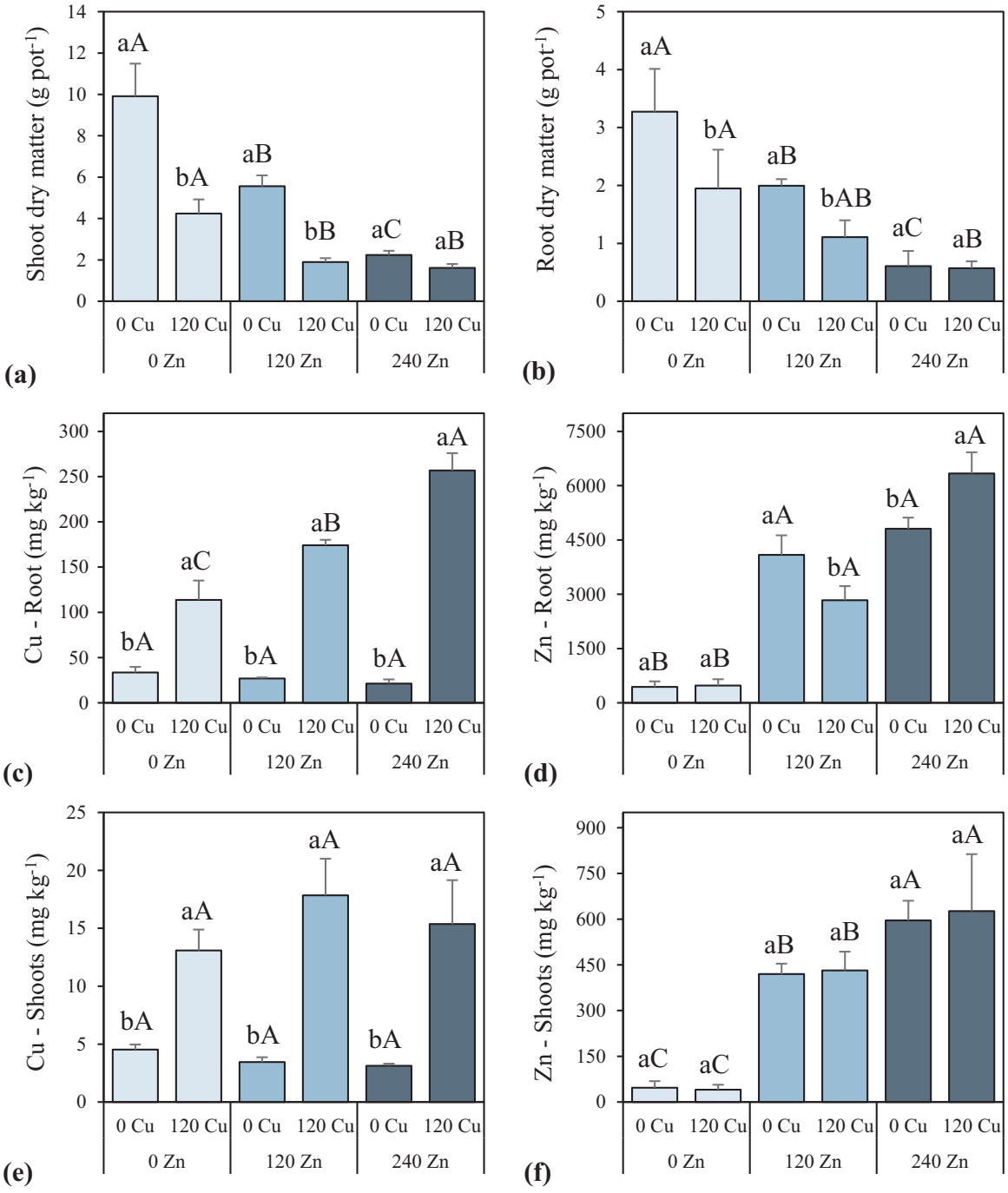

Fig. 1. Dry matter production of the shoots (a) and roots (b), total Cu content (c) and Zn content (d) in the roots of young grapevine in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn, and upper-case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

influences the absorption, transport and regulation of essential ions (Wang et al., 2009). Such process results in the disruption of metabolic processes such as transpiration and photosynthesis, as well as in reduced growth (Sagardoy et al., 2009).

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

On the other hand, the Zn content in the shoot of plants was higher than 200 mg kg^{-1} in all treatments added with Zn (Fig. 1f). Such result may be attributed to the high Zn amounts found in the soil solution, along with similarity between the ionic radii of bivalent cations such as

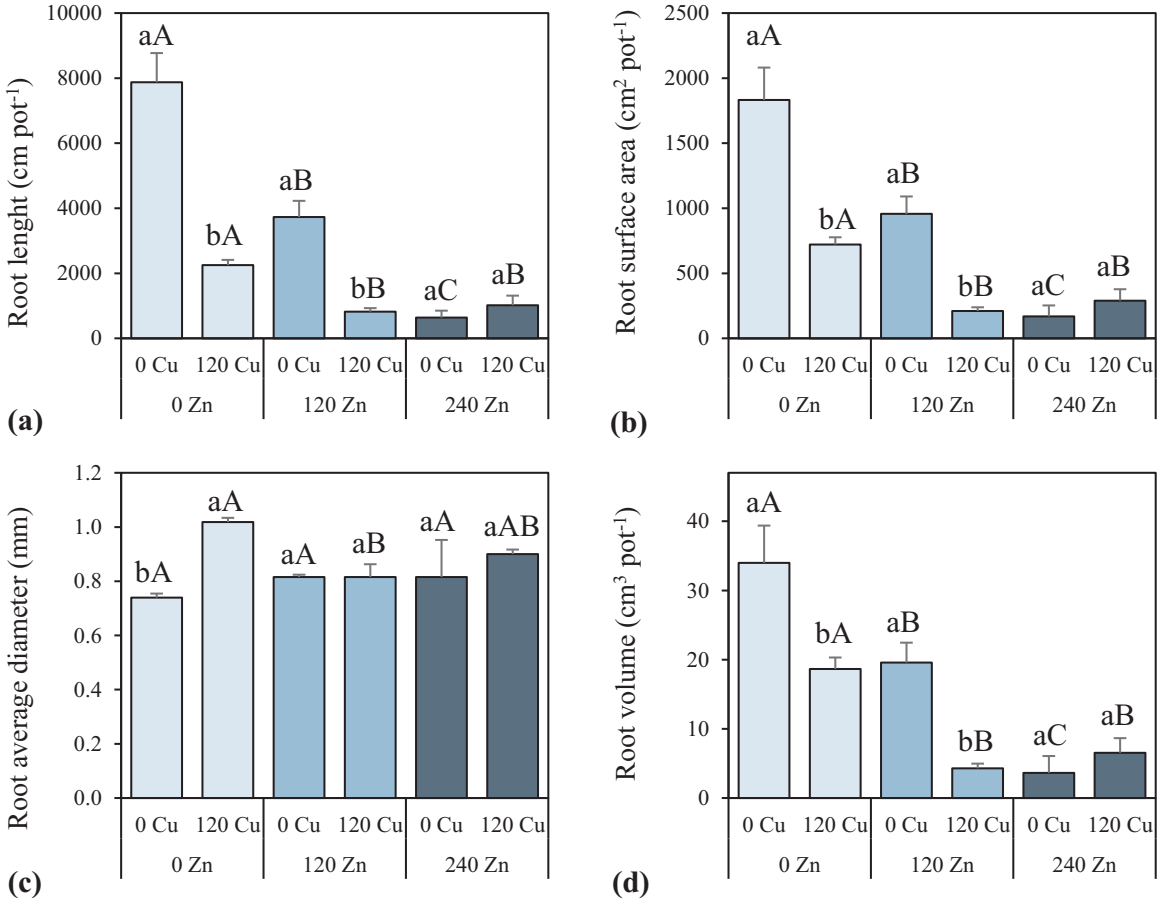

Fig. 2. Length (a), surface area (b), average diameter (c), and volume (d) in roots young grapevine in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn, and upper-case letters compare doses of Zn within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

Cu, Manganese (Mn) and Iron (Fe). Thus, Zn ions can replace any of these divalent cations, be absorbed by the roots (Tewaru et al., 2008) and, subsequently, translocate to the shoot. Once these ions get inside the plant, the excess of Zn can change the physiological balance due to the competition with other cations found in different locations (Tewaru et al., 2008), such as the primary absorption sites or nutrient transport zones in the roots (Yang et al., 2011).

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

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

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

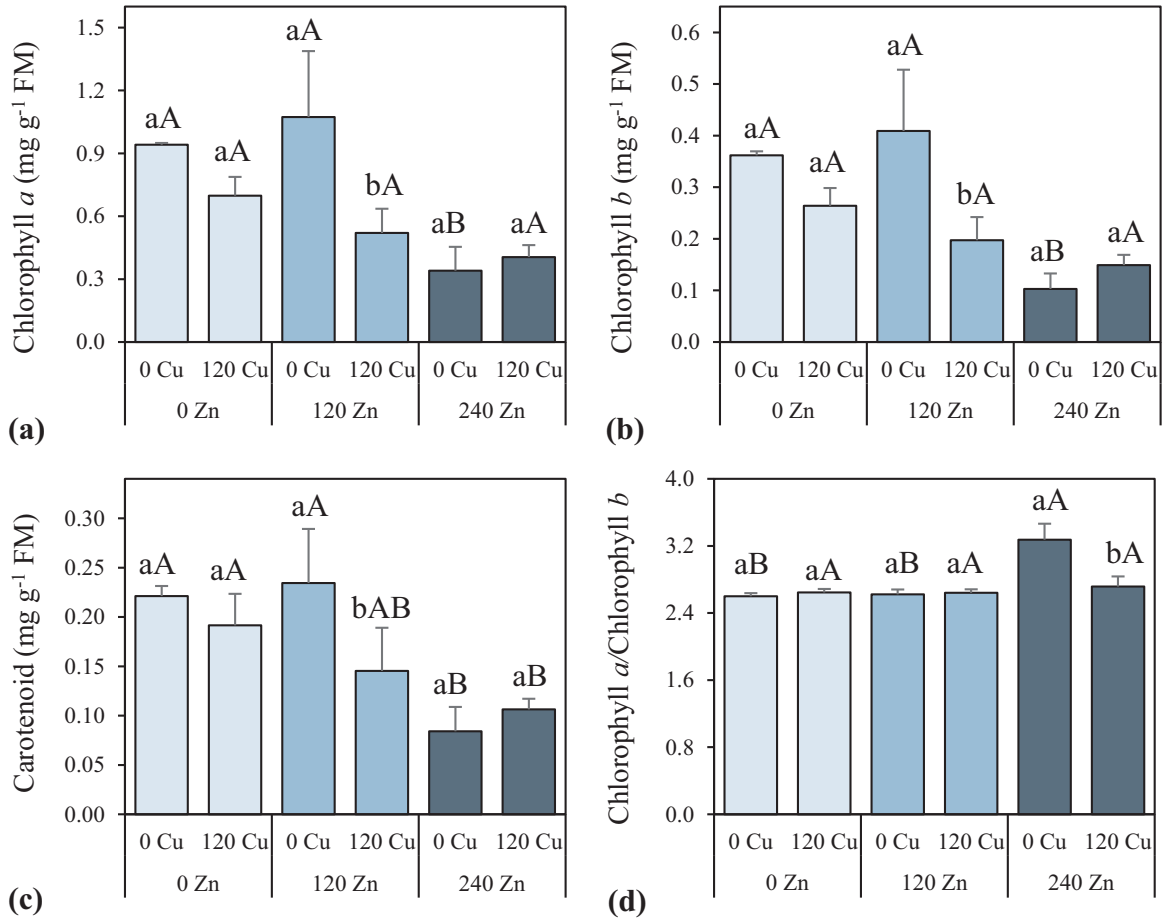

Fig. 3. Chlorophyll *a* (a), chlorophyll *b* (b), and carotenoid (c) content, and chlorophyll *a*/chlorophyll *b* ratio (d) in young grapevine leaves in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters compare Cu doses within each dose of Zn,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

(Sofo et al., 2013; Bochicchio et al., 2015).

The photosynthetic pigment decrease (Fig. 3a, b, c), induced by the high Zn contents in the plant root-growth environment, can be attributed to the adverse effect from Zn excess on the electron transport during photosynthesis, which causes chlorophyll synthesis decrease or increase in its degradation (Cambrollé et al., 2012). Moreover, Cu excess can lead to degradation in the structure and in the inner content of the chloroplast (Ciscato et al., 1997; Ouzounidou, 1996). The magnesium central ion (Mg) can also be replaced by Cu in the chlorophyll molecule, and it can impair chlorophyll synthesis and energy capture (Küpper et al., 2002). The increased Chl *a*/Chl *b* ratio in some treatments (Fig. 3d), in its turn, can be associated with PSII protection, since Chl *b* is found at high concentrations in the antenna complex (Pearcy and Yang, 1998). An efficient way to reduce the energy reaching PSII lies on the partial destruction of these pigments (Eckhardt et al., 2004). The Chl *b* absorbs the luminous energy at wavelength in the red range, consequently, it is more energetic than Chl *a*. Accordingly, the Chl *b* concentration reduction acts in such way that a smaller amount of energy is captured by the antenna complexes. Such process reduces the chlorophyll excitation state and prevents the formation of reactive oxygen species (ROS) capable of irreversibly damaging proteins, lipids and photosynthetic membrane pigments (Horton and Ruban, 2004). Carotenoids are also important protectors of the photosynthetic system. They can absorb the excess of energy from the excited chlorophylls and dissipate it in the form of heat, preventing the production of ROS and, consequently, the degradation of the chloroplast membrane (Mittler, 2002; Havaux, 2014).

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

Although Zn does not present redox reaction, its excess can induce ROS formation, which damages organic molecules. The ROS naturally form inside the cells, mainly in the chloroplasts and mitochondria, where the electron transport takes place (Ferreira et al., 2015). However, ROS can have their production significantly increased under high heavy metal contents in the soil. When ROS formation increases, the strategy used by plants lies on activating the enzymatic antioxidant system (Gill and Tuteja, 2010); SOD, and its isoforms, belong to the

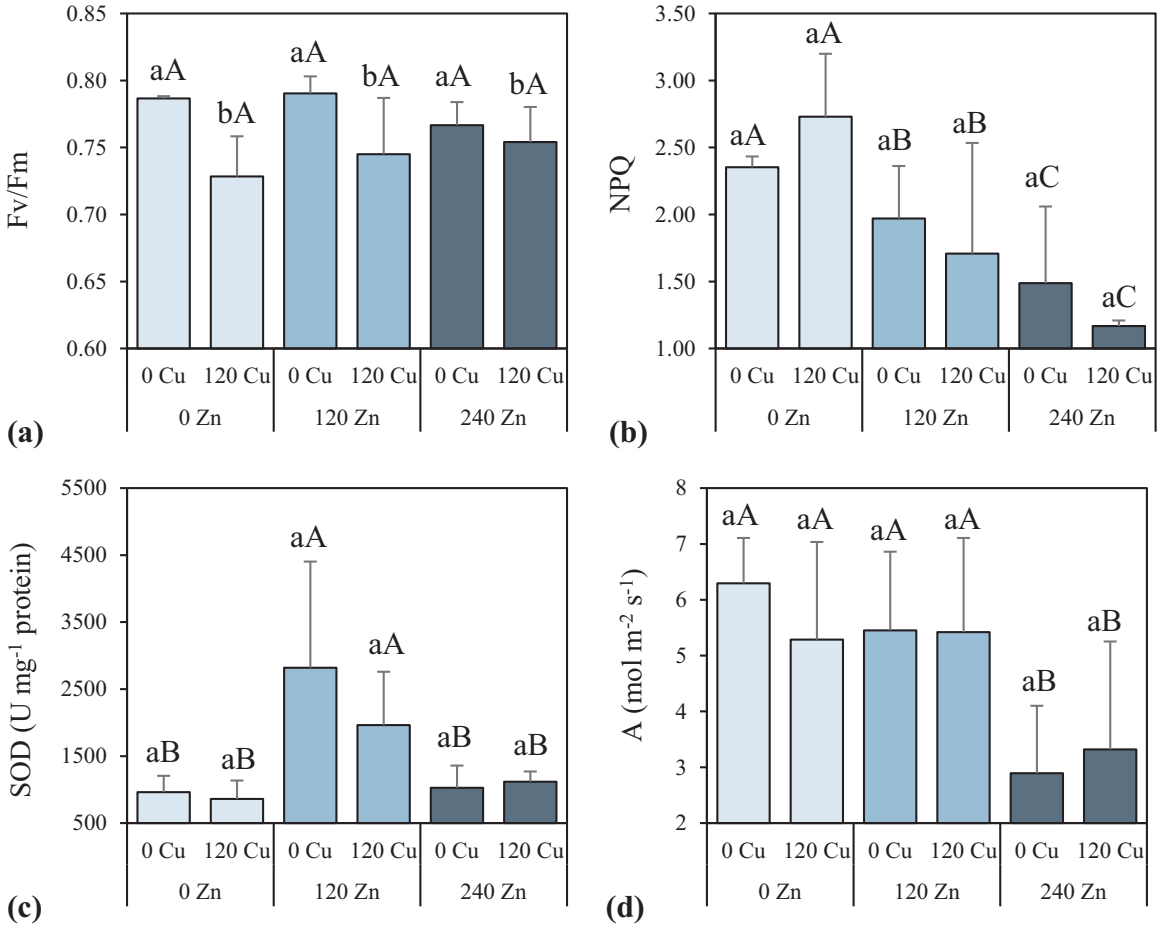

Fig. 4. Maximum quantum yield of PSII (Fv/Fm) (a), non-photochemical quenching (NPQ) (b), superoxide dismutase (SOD) (c) activity, and net photosynthetic rate (A) (d) in young grapevine leaves in response to the addition of Cu and Zn to a sandy acidic soil and cultivated for 60 days. Lower-case letters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

primary defense system in plants and work to eliminate O_2^- radicals. With regard to the present study, the increased SOD activity in plants subjected to treatments added with intermediate Zn dose (Fig. 4c) indicates the activation of an enzymatic response to prevent oxidative damages caused by Zn toxicity (Jain et al., 2010). It suggests that the produced O_2^- is predominantly degraded in the cell as an attempt to avoid oxidative stress in plant cells. However, reduction in the activity of this enzyme, in treatments added with higher Zn doses, indicates that higher Zn concentrations do not protect SOD in grapevine plants. Besides, it can lead to ROS increase in the tissues, as well as potentiate oxidative damages, fact that leads to plant growth reduction and to lack of protection to structures such as chloroplast, as well as to photosynthetic rate reduction.

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

5. Conclusions

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

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References

Alves, P.L., Magalhães, A.C.N., Barja, P.R., 2002. The phenomenon of photoinhibition of photosynthesis and its importance in reforestation. Bot. Rev. 68, 193–208.

Ambrosini, V.G., Rosa, D.J., Prado, J.P.C., Borghezan, M., Melo, G.W.B., Soares, C.R.F.S., Comin, J.J., Simão, D.G., Brunetto, G., 2015. Reduction of copper phytotoxicity by liming: a study of the root anatomy of young vines (*Vitis labrusca* L.). Plant Physiol. Biochem. 96, 270–280.

Arduini, I., Godbold, D.L., Onnis, A., 1994. Cadmium and copper change root growth and morphology of *Pinus pinea* and *Pinus pineaster* seedlings. Physiol. Plant 92, 675–680.

Arduini, I., Godbold, D.L., Onnis, A., 1995. Influence of copper on root growth and morphology of *Pinus pinea* L. and *Pinus pinaster* Ait. seedlings. Tree Physiol. 15, 411–415.

Beauchamp, C.O., Fridovich, I., 1971. Superoxide dismutase. Improved assays and an assay applicable to acrylamide gel. Anal. Biochem. 44, 276–287.

Bochicchio, R., Sofo, A., Terzano, R., Gattullo, C.E., Amato, M., Scopa, A., 2015. Root architecture and morphometric analysis of *Arabidopsis thaliana* grown in Cd/Cu/Zn-gradient agar dishes: a new screening technique for studying plant response to metals. Plant Physiol. Biochem. 91, 20–27.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Broadley, M., Brown, P., Cakmak, I., 2012. Function of nutrients: micronutrients. In: Marcher, P. (Ed.), Mineral Nutrition of Higher Plants, third edition. Academic Press, New York, pp. 649.

Brunetto, G., Miotto, A., Ceretta, C.A., Schmitt, D.E., Heinzen, J., Moraes, M.P., Canton, L., Tiecher, T.L., Comin, J.J., Girotto, E., 2014. Mobility of copper and zinc fractions in fungicide-amended vineyard sandy soils. Arch. Agron. Soil Sci. 60, 609–624.

Brunetto, G., Melo, G.W.B., Terzano, R., Del Buono, D., Astolfi, S., Tomasi, N., Pii, Y., Mimmo, T., Cesco, S., 2016. Copper accumulation in vineyard soils: rhizosphere processes and agronomic practices to limit its toxicity. Chemosphere 162, 293–307.

Brunetto, G., Ferreira, P.A.A., Melo, G.W., Ceretta, C.A., Toselli, M., 2017. Heavy metals in vineyards and orchard soils. Rev. Bras. Frutic. 39, 263–274.

Cambrollé, J., Mancilla-Leytón, J.M., Muñoz-Vallés, S., Luque, T., Figueroa, M.E., 2012. Zinc tolerance and accumulation in the salt-marsh shrub *Halimione portulacoides*. Chemosphere 86, 867–874.

Cambrollé, J., García, J.L., Ocete, R., Figueroa, M.E., Cantos, M., 2013. Growth and photosynthetic responses to copper in wild grapevine. Chemosphere 93, 294–301.

Cambrollé, J., García, J.L., Figueroa, M.E., Cantos, M., 2015. Evaluating wild grapevine tolerance to copper toxicity. Chemosphere 120, 171–178.

Casali, C.A., Moterle, D.F., Rheinheimer, D.S., Brunetto, G., Corcini, A.L.M., Kaminski, J., 2008. Copper forms and desorption in soils under grapevine in the Serra Gaúcha of Rio Grande do sul. R. Bras. Ci. Solo 32, 1479–1487.

Castiglione, S., Franchin, C., Fossati, T., Lingua, G., Torrigiani, P., Biondi, S., 2007. High zinc concentrations reduce rooting capacity and alter metallothionein gene expression in white poplar (*Populus alba* cv. Villafranca). Chemosphere 67, 1117–1126.

Chen, W., Yang, X., He, Z., Feng, Y., Hu, F., 2008. Differential changes in photosynthetic capacity, chlorophyll fluorescence and chloroplast ultrastructure between Zn-efficient and Zn-inefficient rice genotypes (*Oryza sativa*) under low zinc stress. Physiol. Plant 132, 89–101.

Cherif, J., Mediouni, C., Ammar, W.B., Jemal, F., 2011. Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solanum lycopersicum*). J. Environ. Sci. 23 (5), 837–844.

Ciscato, M., Valcke, R., van Loven, K., Clijsters, H., Navari-Izzo, F., 1997. Effects of *in vivo* copper treatment on the photosynthetic apparatus of two *Triticum durum* cultivars with different stress sensitivity. Physiol. Plant 100, 901–908.

De Magalhães, C.C.P., Cardoso, D., Dos Santos, C.P., Chaloub, R.M., 2004. Physiological and photosynthetic responses of *Synechocystis aquatilis* f. *aquatilis* (Cyanophyceae) to elevated levels of zinc. J. Phycol. 40, 496–504.

Dhir, B., Sharmila, P., Pardha Sarad, P., 2008. Photosynthetic performance of *Salvinia natans* exposed to chromium and zinc rich wastewater. Braz. J. Plant Physiol. 20, 61–70.

Eckhardt, U., Grimm, B., Hörtensteiner, S., 2004. Recent advances in chlorophyll biosynthesis and breakdown in higher plants. Plant Mol. Biol. 56, 1–14.

Embrapa, 2009. (Empresa Brasileira de Pesquisa Agropecuária – Brazilian Agricultural Research Corporation). Manual de análises químicas de solos plantas e fertilizantes (Manual of chemical analysis of soils, plants and fertilizers), 2nd ed, Brasília, p. 627.

Fernández-Calviño, D., Pateiro-Moure, M., Nóvoa-Muñoz, J.C., Garrido-Rodrigues, B., Arias-Estévez, M., 2012. Zinc distribution and acid–base mobilization in vineyard soils and sediments. Sci. Total Environ. 414, 470–479.

Ferreira, P.A.A., Ceretta, C.A., Soriani, H.H., Tiecher, T.L., Soares, C.R.F.S., Rossato, L.V., Nicoloso, F.T., Brunetto, G., Paranhos, J.T., Cornejo, P., 2015. *Rhizophagus clarus* and phosphate alter the physiological responses of *Crotalaria juncea* cultivated in soil with a high Cu level. Appl. Soil Ecol. 91, 37–47.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutase I. Occurrence in higher plants. Plant Physiol. 59, 309–314.

Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930.

Girotto, E., Ceretta, C.A., Rossato, L.V., Farias, J.G., Tiecher, T.L., De Conti, L., Schmatz, R., Brunetto, G., Schetinger, M.R.C., Nicoloso, F.T., 2013. Triggered antioxidant defense mechanism in maize grown in soil with accumulation of Cu and Zn due to intensive application of pig slurry. Ecotoxicol. Environ. Saf. 93, 145–155.

Girotto, E., Ceretta, C.A., Rossatto, L.V., Farias, J.G., Brunetto, G., Miotto, A., Tiecher, T.L., De Conti, L., Lourenzi, C.R., Schmatz, R., Giachini, A., Nicoloso, F.T., 2016. Biochemical changes in black oat (*Avena strigosa* Schreb) cultivated in vineyard soils contaminated with copper. Plant Physiol. Biochem. 103, 199–207.

Gratão, P.L., Polle, A., Lea, P.J., Azevedo, R.A., 2005. Making the life of heavy metal-stressed plants a little easier. Funct. Plant Biol. 32, 481–494.

Guimarães, P.R., Ambrosini, V.G., Miotto, A., Ceretta, C.A., Simão, D.G., Brunetto, G., 2016. Black Oat (*Avena strigosa* Schreb.) Growth and root anatomical changes in sandy soil with different copper and phosphorus concentrations. Water Air Soil Pollut. 227, 192–201.

Havaux, M., 2014. Carotenoid oxidation products as stress signals in plants. Plant J. 79, 597–606.

Hendry, G.A.F., Price, A.H., 1993. Stress indications: chlorophylls and carotenoids. In: Hendry, G.A.F., Grime, J.P. (Eds.), Methods in Comparative Plant Ecology. A Laboratory Manual. Chapman & Hall, London, pp. 148–152.

Henriques, A.R., Chalfun-Junior, A., Aarts, M., 2012. Strategies to increase zinc deficiency tolerance and homeostasis in plants. Braz. J. Plant Physiol. 24 (1), 3–8.

Hewitt, E.J., 1983. Essential and functional methods in plants. In: Robb, D.A., Pierpoint, W.S. (Eds.), Metals and Micronutrients: Uptake and Utilization by Plants. Academic Press, New York, pp. 313–315.

Horton, P., Ruban, A., 2004. Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. J. Exp. Bot. 56, 365–373.

Jain, R., Srivastava, S., Solomon, S., Shrivastava, A.K., Chandra, A., 2010. Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (*Saccharum* spp.). Acta Physiol. Plant 32, 979–986.

Janicka-Russak, M., Kabała, K., Burzynski, M., Kłobus, G., 2008. Response of plasma membrane H^+-ATPase to heavy metal stress in *Cucumis sativus* roots. J. Exp. Bot. 59, 3721–3728.

Kabała, K., Janicka-Russak, M., Burzynski, M., Kłobus, G., 2008. Comparison of heavy metal effect on the proton pumps of plasma membrane and tonoplast in cucumber root cells. J. Plant Physiol. 165, 278–288.

Kabata-Pendias, A., 2011. Trace Elements in Soils and Plants. CRC Press, Boca Ratón, Florida.

Kloke, A., Sauerbeck, D.R., Vetter, H., 1984. The contamination of plants and soils with heavy metals and the transport of metals in terrestrial food chains. In: Nriagu, J. (Ed.), Changing Metal Cycles and Human Health. Springer-Verlag, Berlin, pp. 113.

Küpper, H., Setlik, I., Spiller, M., Küpper, F.C., Prásil, O., 2002. Heavy metal-induced inhibition of photosynthesis: targets of in vivo heavy metal chlorophyll formation. J. Phycol. 38, 429–441.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Method Enzymol. 148, 350–382.

Mateos-Naranjo, E., Andrades-Moreno, L., Cambrollé, J., Perez-Martin, A., 2013. Assessing the effect of copper on growth, copper accumulation and physiological responses of grazing species *Atriplex halimus*: ecotoxicological implications. Ecotoxicol. Environ. Saf. 90, 136–142.

Maxwell, K., Johnson, G.N., 2000. Chorophyll fluorescence – a practical guide. J. Exp. Bot. 51, 659–668.

Miotto, A., Ceretta, C.A., Brunetto, G., Nicoloso, F.T., Girotto, E., Farias, J.G., Tiecher, T.L., De Conti, L., Trentin, G., 2014. Copper uptake, accumulation and physiological changes in adult grapevines in response to excess copper in soil. Plant Soil 374, 593–610.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Mysliwa-Kurdziel, B., Prasad, M.N.V., Strzalka, K., 2004. Photosynthesis in metal plants. In: Prasad, M.N.V. (Ed.), Heavy Metal Stress in Plants. From Molecule to Ecosystems. Springer, Berlin, pp. 146–181.

Ouzounidou, G., 1996. The use of photoacoustic spectroscopy in assessing leaf photosynthesis under copper stress: correlation of energy storage to photosystem II fluorescence parameters and redox change of P–700. Plant Sci. 113 (2), 229–237.

Overvoorde, P., Fukaki, H., Beeckman, T., 2010. Auxin control of root development. Cold Spring Harb. Perspect. Biol. 2, 1–16.

Pearcy, R.W., Yang, W., 1998. The functional morphology of light capture and carbon gain in Redwood forest understorey plant *Adenocaulon bicolor* (Hook.). Funct. Ecol. 12, 543–552.

Potters, G., Pasternak, T.P., Guisez, Y., Palme, K.J., Jansen, M.A.K., 2007. Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci. 12, 98–105.

Quint, M., Barkawi, L.S., Fan, K.T., Cohen, J.D., Gray, W.M., 2009. *Arabidopsis* IAR4 modulates auxin response by regulating auxin homeostasis. Plant Physiol. 150, 748–758.

R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. Available at: ⟨https://www.r-project.org/⟩.

Sagardoy, R., Morales, F., Lopez-Millan, A.F., Abadia, A., Abadia, J., 2009. Effects of zinc toxicity on sugar beet (*Beta vulgaris* L.) plants grown in hydroponics. Plant Biol. 11, 339–350.

Sheetal, K.R., Singh, S.D., Anand, A., Prasad, S., 2016. Heavy metal accumulation and effects on growth, biomass and physiological processes in mustard. Ind. J. Plant Physiol. 21, 219–223.

Sofo, A., Vitti, A., Nuzzaci, M., Tataranni, G., Scopa, A., Vangronsveld, J., Remans, T., Falasca, G., Altamura, M.M., Degola, F., Sanità di Toppi, L., 2013. Correlation between hormonal homeostasis and morphogenic responses in Arabidopsis thaliana seedlings growing in a Cd/Cu/Zn multi-pollution context. Physiol. Plant 149, 287–298.

Soil Survey Staff, 2006. Soil Taxonomy: A Basic System of Soil Classification for Making and interpreting Soil Surveys, 2nd ed. US Government Printing Office, Washington DC.

Souza, E.P., Silva, I.F., Ferreira, L.E., 2011. Mecanismos de tolerância a estresses por metais pesados em plantas. Rev. Bras. De. Agrociência 17, 167–173.
Tewaru, R.K., Kumar, P., Sharma, P.N., 2008. Morphology and physiology of zinc-stressed mulberry plants. J. Plant Nutr. Soil Sci. 171, 286–294.
Tiecher, T.L., Ceretta, C.A., Ferreira, P.A.A., Lourenzi, C.R., Tiecher, T., Girotto, E., Nicoloso, F.T., Soriani, H.H., De Conti, L., Mimmo, T., Cesco, S., Brunetto, G., 2016a. The potential of *Zea mays* L. in remediating copper and zinc contaminated soils for grapevine production. Geoderma 262, 52–61.
Tiecher, T.L., Ceretta, C.A., Tiecher, T., Ferreira, P.A.A., Nicoloso, F.T., Soriani, H.H., Rossato, L.V., Mimmo, T., Cesco, S., Lourenzi, C.R., Giachini, J.A., Brunetto, G., 2016b. Effects of zinc addition to a copper-contaminated vineyard soil on sorption of Zn by soil and plant physiological responses. Ecotoxicol. Environ. Saf. 129, 109–119.
Tiecher, T.L., Tiecher, T., Ceretta, C.A., Ferreira, P.A.A., Nicoloso, F.T., Soriani, H.H., Tassinari, A., Paranhos, J.T., De Conti, L., Brunetto, G., 2016c. Physiological and nutritional status of black oat (*Avena strigosa* Schreb.) grown in soil with interaction of high doses of copper and zinc. Plant Physiol. Biochem. 106, 253–263.
Tiecher, T.L., Tiecher, T., Ceretta, C.A., Ferreira, P.A.A., Nicoloso, F.T., Soriani, H.H., De Conti, L., Kulmann, M.S.S., Schneider, R.O., Brunetto, G., 2017. Tolerance and translocation of heavy metals in young grapevine (*Vitis vinifera*) grown in sandy acidic soil with interaction of high doses of copper and zinc. Sci. Hortic. 222, 203–212.
Wang, C., Zhang, S.H., Wang, P.F., Hou, J., Zhang, W.J., Li, W., Lin, Z.P., 2009. The effect of excess Zn on mineral nutrition and antioxidative response in rapeseed seedlings. Chemosphere 75, 1468–1476.
Yang, Y., Sun, C., Yao, Y., Zhang, Y., Achal, V., 2011. Growth and physiological responses of grape (*Vitis vinifera* "Combier") to excess zinc. Acta Physiol. Plant 33, 1483–1491.
Yruela, I., 2005. Copper in plants. Braz. J. Plant Physiol. 171, 145–156.
Zhu, Z., Wei, G., Li, J., Qian, Q., Yu, J., 2004. Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt stressed cucumber (*Cucumis sativus* L.). Plant Sci. 167, 527–533.