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Growth, heavy metal uptake, and photosynthesis in 'Paulsen 1103' (*Vitis berlandieri* x *rupestris*) grapevine rootstocks inoculated with arbuscular mycorrhizal fungi from vineyard soils with high copper contents

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Summary

Soils in old vineyards in southern Brazil have high copper accumulation due to fungicide applications over the years, which can affect physiology and growth of young grapevine plants. Arbuscular mycorrhizal fungi (AMF) alleviate toxic effects of metals and increase photosynthesis and plant growth. We evaluated whether inoculation with *Rhizophagus clarus* (Rh) from a mining area or with a trap-culture-enriched AMF community (Tc) isolated from a high-copper vineyard soil, improved growth and photosynthesis in grapevine rootstocks planted in young (< 10 years) and old (> 60 years) vineyards soils of Vale da Uva Goethe, SC, Brazil. Mycorrhizal colonization was higher in grapevines installed in young vineyard soil than those planted in old vineyard soil.

Plants grew more in the old vineyard soil than in the soil from a young vineyard, and that was related to plant nutrient concentration in the soil. In both soils, Tc-inoculated grapevines had higher photosynthetic activity, while those inoculated with *R. clarus* had higher carbon assimilation. In conclusion, grapevines showed a positive response to AMF inoculation in different soil conditions, and the native AMF community from high copper soils are promising for inoculation of grapevines.

K e y w o r d s : copper; native mycorrhizal fungi; soils; *Vitis*.

Introduction

Management of fruit trees and grapevines often includes the use of cupric fungicides such as Bordeaux mixture [$CuSO_4$ + $Ca(OH)_2$]. Those products are applied several times in a growing season to control plant pathogens and prevent foliar fungal diseases. Part of the products falls into the soil, and continuous use over the years leads to a substantial increase in soil Cu content (Brunetto *et al.* 2016, Tiecher *et al.* 2017 and 2018). Soil copper accumulation may cause plant toxicity and environmental contamination (Miotto *et al.* 2014), and excess Cu in the soil may cause toxicity to young grapevines and inhibit their growth, especially in vineyard renewal. Copper is an essential plant micronutrient, involved in many metabolic processes such as photosynthesis, respiration, and protein metabolism (Marschner 1995). It is a constituent of plant enzymes such as superoxide dismutase, cytochrome oxidase, amine oxidase, plastocyanin, and polyphenol oxidase (Yruela 2005). However, high Cu concentrations in plant tissue lead to nutritional unbalance (Ferreira *et al.* 2015), decrease in plant photosynthesis (Cambrollé *et al.* 2015, Tiecher *et al.* 2018), and oxidative stress (Girotto *et al.* 2013). In addition to those harmful effects, soil copper contents above 60 $mg \cdot kg^{-1}$ indicate the need for preventive measures to preserve soil functionality, or restorative practices to recover soil quality (CONAMA 2009). Therefore, in soils with high levels of Cu, actions are necessary to reduce soil Cu availability and, consequently, its potential toxicity to grapevines.

A possible strategy to reduce Cu toxicity in grapevines is the inoculation of arbuscular mycorrhizal fungi (AMF), which can alleviate metal toxicity to plants (Leyval *et al.* 2002, Meier *et al.* 2011). AMF may also avoid metal toxicity by immobilizing metals in their external hyphae (Cornejo *et al.* 2013, Cabral *et al.* 2015) and through the production of glomalins, glycoproteins with high affinity for metals (González-Chávez *et al.* 2004, Folli-Pereira *et al.* 2012). Nevertheless, the positive effects of AMF on phytoprotection depend on fungal isolates, as well as on the metal and plant species involved (Meyer *et al.* 2017). A direct effect of the excess of trace elements, including copper, on AMF is inhibition of spore germination and hypha development, which can delay or inhibit mycorrhiza establishment, or alter the AMF community favoring fungi more adapted to that condition (Klauberg-Filho *et al.* 2002, Cardoso *et al.* 2002, Silva *et al.* 2005). Thus, a strategy to increase the intensity of the mycorrhizal association may be inoculation with AMF communities adapted to a high concentration of trace elements, in this case, copper.

This work aimed to verify if or an AMF community from a high-copper vineyard soil and *Rhizophagus clarus* improved growth and photosynthesis-related physiological parameters of grapevine rootstocks grown in soils with different cultivation times and copper concentrations.

Material and Methods

S o i l a n d p l a n t m a t e r i a l : Rootstock plantlets 'P1103' (*Vitis berlandieri x rupestris*) (Dry 2007), obtained by *in vitro* propagation (Lima Da Silva and Doazan 1995)

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were supplied by EMBRAPA-UVA and VINHO, Bento Gonçalves, Brazil. The plantlets were established, in a greenhouse, on soils coming from vineyards in the municipality of Pedras Grandes, Santa Catarina (28° 26 '09 "S, 49° 11' 06" W, 39 m altitude). Extension agents and researchers working on the region indicated the vineyards, which had known ages, similar management practices and soil types. The soils had different cultivation times, V10 vineyard is ten years old, and V60 vineyard is 60 years old (Tab. 1). The region's annual rainfall, temperature, and relative air humidity are 1540 mm, 19.2 °C, and 81.5 %, respectively, with a predominance of Humic Cambisol type soils (Santos *et al.* 2018).

The inoculation treatments were divided into two soils with natural diversity, V10 and V60, with ten and four spores of AMF per g, respectively, without external inoculation (Ni), and two treatments with external inoculum (Tab. 2). One source of external inoculum came from trap culture (Tc) of soil from the V60 vineyard, which contained six AMF spores per gram in a mixture of vermiculite, sand and soil from the V60 vineyard (35:35:20, v:v:v). The other external source was a positive control, with *Rhizophagus clarus* UFSC-14 (Rh), obtained from the AMF collection of the Soil Microbiology laboratory (UFSC, Florianópolis) and the one isolated from soil samples from coal mining areas, in the municipality of Criciúma (SC). *R. clarus* is an AMF with a ruderal ecological strategy (Chagnon *et al.* 2013), which has shown positive results in the growth of grapevines under high copper concentration in the soil (Ambrosini *et al.* 2015, Rosa *et al*. 2016). The inoculum contained 15 spores per gram in a mixture of vermiculite, sand, and soil (60:30:10, v:v:v).

Soil physical and chemical properties: Methods for soil analysis were those adopted by farmers in the region and described in Tedesco (1995). Soil pH was measured in water 1:1 (v:v), and O.M. was estimated with sulfochromic digestion (Walkley and Black 1934) and titration. Available P was extracted with anion-exchange resin or Mehlich-I solution and measured by colorimetry (Murphy and Riley 1962). Mehlich-I extracted K was determined by flame photometry. Cu and Zn, extracted with 0.01 mol·L^{-1} EDTA solution (Chaignon *et al.* 2009), were quantified by atomic absorption spectrophotometry. Al, Ca, and Mg were extracted with a 1-mol·L^{-1} KCl solution and measured by atomic absorption spectrophotometry. Soil apparent density was quantified with the cylinder method (Claessen 1997).

Experimental treatments and design: The experiment was arranged in a 2 x 3 factorial scheme, with 10 replicates (N = 60). The factors were: soils from two vineyards with different cultivation histories: 10 years (V10) and 60 years (V60) of age, and inoculation, with three treatments: soil with non-inoculated natural diversity (Ni), trap inoculum (Tc), and positive control with *Rhizophagus clarus* (Rh). The treatments Ni (V10 and V60) had 486 and 222 AMF spores respectively, and each plant in the Tc and Rh treatments received approximately 100 spores of each AMF inoculum directly under the roots, at the time of transplantation to 1.7 L^{-1}. For the Tc and Rh inoculants, the spores were counted by the wet sieving method, followed by sucrose gradient centrifugation (Gerdemann and Nicolson 1963).

Collection of plant material: At 90 days, the plants were removed from the pots and cut close to the soil. Thin root segments corresponding to approximately 1.0 g were cut from each root and stored in capsules for staining. The remaining roots and shoots were oven-dried at 65 °C until constant mass and weighed.

Mycorrhizal colonization: The fine root samples were cleared in 10 % KOH at 80 °C for 180 min, and the procedure was repeated for three days. Subsequently, the roots were acidified with 1.0 % HCl and stained with trypan blue (Koske and Gemma 1989). Colonization was estimated by the slide intersection method (Mcgonigle *et al.* 1990): roots cut into 1.0 cm sections were placed in glycerin on microscope slides, covered with slipcovers, and observed at 200x magnification.

Plant growth measurements: Plant height (height), number of leaves (leaf n.), stem diameter (stem diam.), shoot dry mass (SDM), root dry mass (RDM), and total dry mass (TDM) were determined at the end of the experiment.

Chlorophyll fluorescence: Chlorophyll fluorescence emission was quantified with a modulated light fluorimeter MINI-PAM (Walz, Germany) at 90 days, recording estimated initial chlorophyll fluorescence (Fo); maximum chlorophyll fluorescence (Fm); photosynthetic efficiency of photosystem II (Fv/Fm); apparent electron transport rate (ETR); photochemical extinction coefficient (qP); non-photochemical extinction coefficient (qN), and photosystem II quantum efficiency of energy dissipation (NPQ). The measurements were performed with metal clamps (DLC-8) coupled to the MINI-PAM sensor, placed

Table 1

Physical and chemical attributes of soils used as substrates for grapevine rootstocks.
V10 = 10 year-old vineyard soil; V60 = 60 year-old vineyard soil.
P res = phosphorus extracted with resin; P Mel = phosphorus extracted with Mehlich-I solution;
CEC = cation exchange capacity; O.M. = organic matter; Ad = apparent density

	pH	P res	P Mel	K	Cu	Zn	Ca	Mg	Al	CEC	O.M.	Clay	Ad
			mg·dm^{-3}				mmolc·dm^{-3}				g·kg^{-1}		g·cm^{-3}
V10	5.5	23	23	182	15.9	15.1	62	17	2.6	145	26.0	284	1.25
V60	6.1	81	174	216	582	49.1	126	42	0.0	218	66.8	468	1.15

Table 2

Occurrence of AMF species in soils of vineyards planted ten (V10) or 60 years (V60) and in AMF trap culture with the soil V60 (Tc)

Families of AMF/Species / Number of spores in 50 cm^3	V10 486	V60 222	Tc 298
Family Acaulosporaceae			
Acaulospora cavernata Błaszk.	■		
Acaulospora colombiana (Spain & N.C. Schenck) Kaonongbua, J.B. Morton & Bever	■		
Acaulospora mellea Spain & Schenck	■		
Acaulospora morrowiae Spain & N.C. Schenck	■		
Acaulospora foveata Trappe & Janos	■		
Acaulospora scrobiculata Trappe	■		
Family Glomeraceae			
Funneliformis sp1		■	
Glomus ambisporum G.S. Sm. & N.C. Schenck	■	■	■
Glomus macrocarpum Tul. & C. Tul.	■		
Glomus glomerulatum Sieverd.	■		
Glomus microaggregatum Koske, Gemma & Oleixa		■	
Glomus sinuosum (Gerd. & B.K. Bakshi) R.T. Almeida & N.C. Schenck		■	
Glomus sp1	■		
Glomus sp2	■	■	■
Glomus sp3		■	
Rhizophagus sp1	■	■	
Family Claroideoglomeraceae			
Claroideoglomus claroideum (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler	■		
Family Gigasporaceae			
Gigaspora margarita W.N. Becker & I.R. Hall	■		
Family Paraglomeraceae			
Paraglomus sp1	■		
Family Archaeosporaceae			
Archaeospora trappei (Ames & Linderman) Morton & Redecker	■	■	
Family Ambisporaceae			
Ambispora callosa (Sieverd.) C. Walker, Vestberg & A. Schüßler		■	■

in the leaf median region, on one side of the leaf limb, avoiding the central nervure. Before each measurement, a leaf portion was kept in the dark for at least 30 min, until all reaction centers in that region acquired the "open" condition, as indicated by Maxwell and Johnson (2000).

Chlorophylls and carotenoids: Leaf chlorophyll and carotenoid contents were measured 90 days after extraction with dimethyl sulfoxide in a water bath at 65 °C for two h, without maceration by spectrophotometry (Hiscox and Israelstam 1979). Calculations for the determination of chlorophyll A, chlorophyll B, total chlorophyll, and carotenoid concentrations were performed using the Wellburn (1994) formulas.

Gas exchanges: Gas exchanges were quantified at 90 days with a portable infrared gas analyzer (IRGA), LI-6400XT (LICOR, USA), with a 2 cm^2 chamber and 400 ppm CO_2 concentration. CO_2 assimilation (As), stomatal conductance (gs), transpiration (Tr), and internal CO_2 concentration in the cell (Ci) were evaluated. Measurements were done between 9 and 11 a.m., with a luminosity of 1000 μmol photon $m^{-2} \cdot s^{-1}$, and a healthy, complete leaf of the middle third of the plant was used as standard.

Plant tissue chemical analysis: P, Cu, and Zn in grapevine shoots were quantified, according to Tedesco *et al.* (1995). The shoots were dried in a forced-air oven at 60-70 °C until constant mass, and ground in a Willey type mill. Plant material for P, Cu and Zn quantification was digested at 300 ºC in HNO_3 and $HClO_4$ (Tedesco *et al.* 1995). Cu and Zn were determined in an inductively coupled plasma optical emission spectrometer (ICP-OES; Perkin Elmer Optima 8000), and P by colorimetry according to Murphy and Riley (1962). P, Cu and Zn accumulations were calculated multiplying shoot dry mass by P, Cu, and Zn contents. Phosphorus and copper root/shoot accumulation

ratios were calculated to verify each element translocation from root to shoot.

Statistical analyses: All data were submitted to Bartlett's test to verify homoscedasticity. Root colonization percentages were transformed with the square root function. Two-way analyses of variance were performed, and when there was statistical significance, Tukey's mean separation test ($p < 0.05$) was applied. Pearson's linear correlation analysis between dependent variables was performed, and significance was assessed by the t-test ($p < 0.05$). The vegan package (Oksanen *et al.* 2013) in the Rstudio 3.4.3 program was used for statistical analysis.

Results

Mycorrhizal colonization: Mycorrhizal colonization ranged between 40 %, and 70 % in all inoculation treatments. Colonization in V10 soil was significantly higher than in V60 soil, ranging from 15 to 30 % (Fig. 1). In the V10 soil, root colonization in the inoculated treatment with the trap culture (Tc) was higher than in the other two treatments, which did not differ from each other.

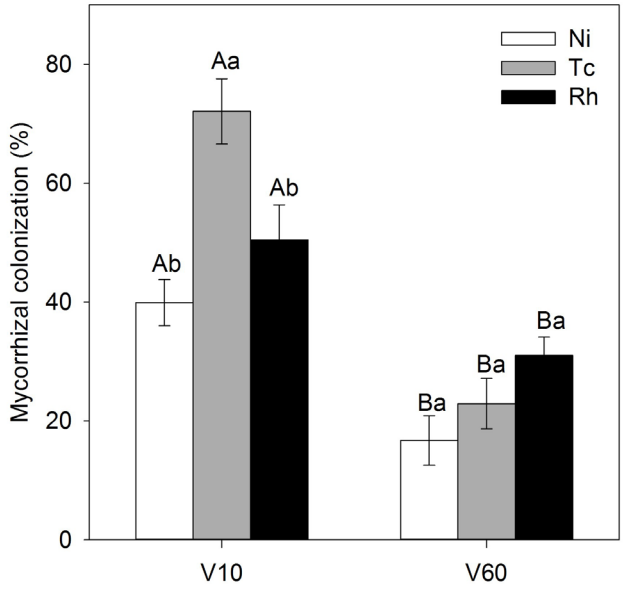

Fig. 1: Root mycorrhizal colonization of 'Paulsen' grapevine rootstock (1103), inoculated with AMF from V60 soil trap cultures (Tc), or with *Rhizophagus clarus* (Rh), or not inoculated (Ni), in soils from ten-year (V10) or 60-year (V60) old vineyards. Means followed by the same lowercase letter do not differ (Tukey test, $p < 0.05$, n = 10) in inoculation effect in each soil; means followed by the same capital letter do not differ between soils in each inoculation treatment. Bars represent standard error of the mean.

Plant growth: In general, plant growth was higher in V60 than V10 soil. Plants were taller in V60 soil than in V10 soil (Fig. 2A), for all inoculation treatments. In V10 soil, grapevines with Tc and Rh inoculation were taller than non-inoculated plants (Ni) (Fig. 2A). The number of leaves per plant had a different pattern; it was higher in V10 soil than in V60 soil. In V10 soil, Ni and Tc treatments had more leaves than Rh treatment (Fig. 2B). In V60 soil, the

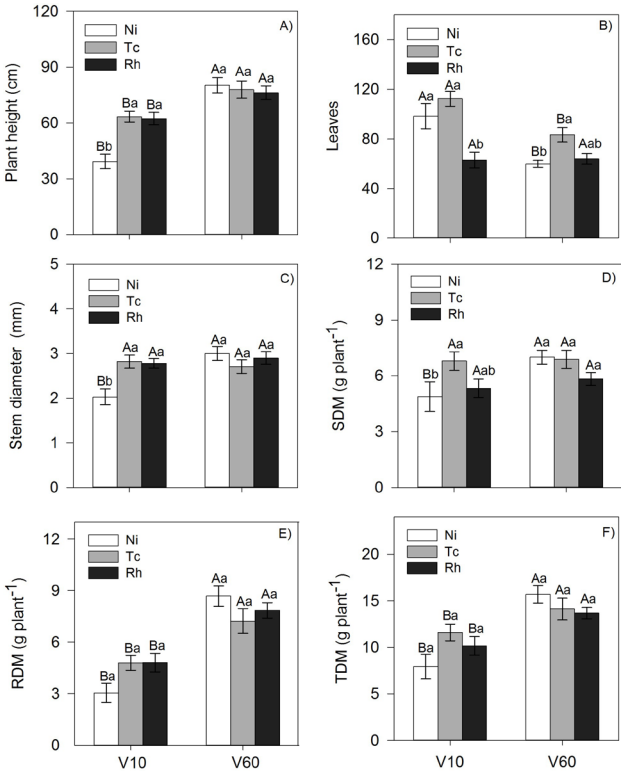

Fig. 2: Plant height (**A**), number of leaves (**B**), stem diameter (**C**), shoot dry mass - SDM (**D**), root dry mass - RDM (**E**), and total dry mass - TDM (**F**) of 'Paulsen' grapevine rootstocks (1103) in soils from ten-year (V10) or 60-year (V60) old vineyards, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc), or with *Rhizophagus clarus* (Rh). Means followed by the same lowercase letter do not differ (Tukey test, $p < 0.05$, n = 10) in inoculation effect in each soil; means followed by the same capital letter do not differ between soils in each inoculation treatment. Bars represent standard error of the mean.

number of leaves in the Tc treatment was higher than in the Ni treatment, while Rh-inoculated plants had intermediate values. Ni plants in V10 soil had thicker stems than in V60 (Fig. 2C), while in V10 soil, Ni plants had thinner stems than those inoculated with Tc or Rh.

Ni plants grown in V10 soil had higher shoot biomass than those in V60 (Figure 2D). In V10 soil, Shoot dry matter (SDM) was higher in Tc inoculated plants than in Ni plants, and Rh showed an intermediate pattern. Root dry matter (RDM) (Fig. 2E) and total dry matter (TDM) (Fig. 2F) were higher at V60 than at V10 in all inoculation treatments. No differences were found between the inoculation treatments at V10 and V60.

Chlorophyll fluorescence: Although initial chlorophyll fluorescence (Fo) showed no differences between soils or among inoculation treatments (Fig. 3A), there were differences in maximum chlorophyll fluorescence (Fm) and potential photosystem II efficiency (Fv/Fm) (Figs 3B and 3C). Fm was higher in V10 soils than in V60 in all inoculation treatments (Fig. 3B), and Ni and Tc-inoculated plants had higher values than Rh-inoculated plants in V60 soils. Fv/Fm (Fig. 3C) was higher in V10 plants than in V60 plants in the Rh inoculation treatment. Relative electron transport rate (ETR) (Fig. 4A) and photochemical quenching (qP) (Fig. 4B) in the V10 soil were lower for Tc plants than

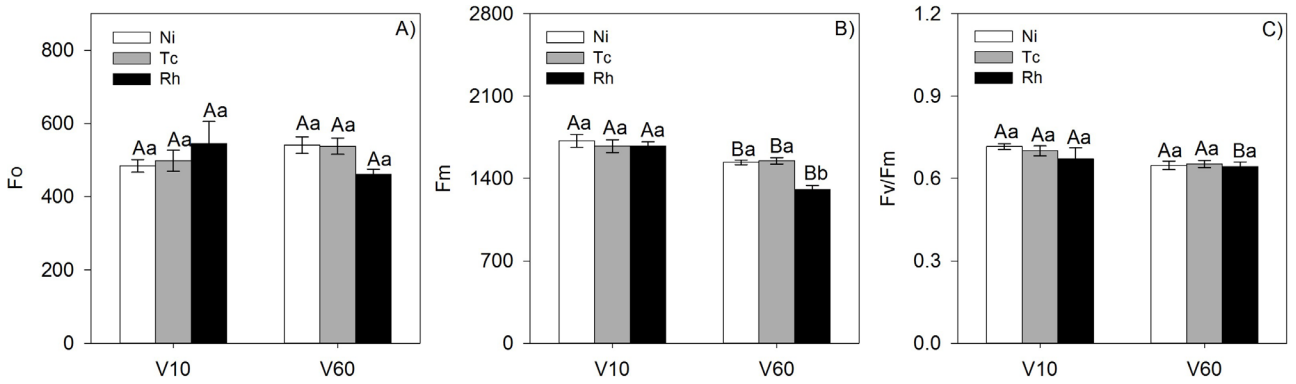

Fig. 3: Initial (Fo) (**A**) and maximum (Fm) (**B**) chlorophyll fluorescence, and potential photosystem II quantum efficiency (Fv/Fm) (**C**) in 'Paulsen' (1103) rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from ten-year (V10) or 60-year (V60) old vineyards. Means followed by the same lowercase letter do not differ (Tukey test, $p < 0.05$, n = 10) in inoculation effect in each soil; means followed by the same capital letter do not differ between soils in each inoculation treatment. Bars represent standard error of the mean.

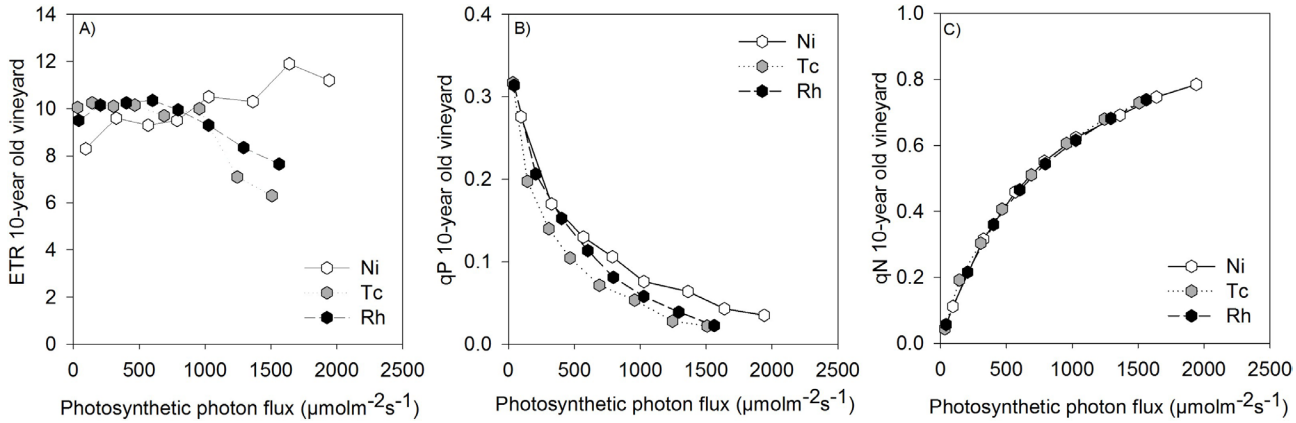

Fig. 4: Effect of photosynthetic photon flux on the relative electron transport rate curves (ETR) (**A**), photochemical quenching (qP) (**B**), and non-photochemical quenching (qN) (**C**) in 'Paulsen' (1103) rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from ten-year (V10) old vineyards.

Ni plants, while Rh plants showed intermediate values. In plants grown in the V60 soil, relative electron transport rate (ETR) (Fig. 5A) and photochemical quenching (qP) (Fig. 5B) were higher in the Tc treatment than in the Rh, while Ni did not differ from either of them. Non-photochemical quenching (qN) showed no differences among inoculation treatments (Fig. 5C).

Chlorophylls and carotenoids: Chlorophyll A, chlorophyll B, total chlorophyll, and carotenoid concentrations (Tab. 3) were higher in plants growing in V10 soil than in V60 soil, in all inoculation treatments. In plants growing in V60 soil, chlorophyll concentration was higher in the Tc treatment than in the Rh treatment, and Ni plants had intermediate values.

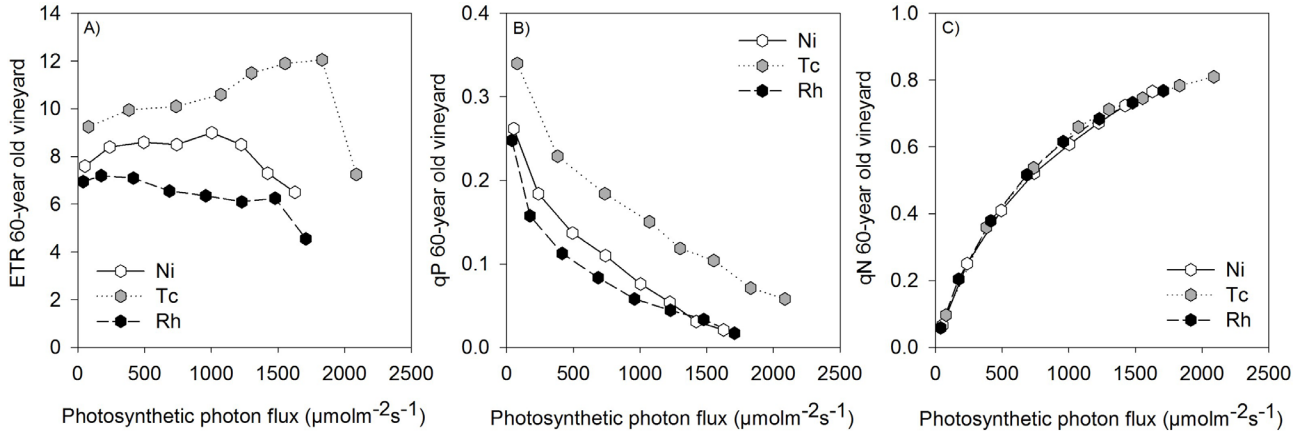

Fig. 5: Effect of photosynthetic photon flux on the relative electron transport rate curves (ETR) (**A**), photochemical quenching (qP) (**B**), and non-photochemical quenching (qN) (**C**) in 'Paulsen' (1103) rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from 60-year (V60) old vineyards.

Table 3

Chlorophyll and carotenoid contents in leaves of 'Paulsen 1103' rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from ten-year (V10) or 60-year (V60) old vineyards

Treatment	Chlorophyll A		Chlorophyll B		Total Chlorophyll		Carotenoids	
	$mg \cdot g^{-1}$							
	V10	V60	V10	V60	V10	V60	V10	V60
Ni	0.97 Aa	0.74 Bab	0.35 Aa	0.25 Bab	1.31 Aa	0.99 Bab	0.22 Aa	0.16 Bab
Tc	1.01 Aa	0.81 Ba	0.35 Aa	0.27 Ba	1.36 Aa	1.07 Ba	0.24 Aa	0.19 Ba
Rh	0.93 Aa	0.53 Bb	0.33 Aa	0.18 Bb	1.25 Aa	0.71 Bb	0.22 Aa	0.13 Bb

For each variable, means followed by the same capital letters in each line, and lower case letters in each column do not differ according to Tukey's test ($p < 0.05$, n = 10).

Table 4

CO_2 assimilation rates (As), stomatal conductance (gs), internal CO_2 concentration in the cell (Ci) and transpiration (Tr) of 'Paulsen' grapevine rootstock (1103) leaves, in soil of vineyard of ten years old (V10), not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh)

Treatment	As		gs		Ci		Tr	
	$\mu mol \cdot m^{-2} \cdot s^{-1}$		$mmol \cdot m^{-2} \cdot s^{-1}$		$\mu mol \cdot mol^{-1}$		$mmol \cdot m^{-2} \cdot s^{-1}$	
	V10	V60	V10	V60	V10	V60	V10	V60
Ni	4.81Aa	4.71Aa	0.27Ab	0.23Aa	453Aa	436Aa	0.08Ab	0.06Aa
Tc	6.65Aa	3.26Ba	0.22Ab	0.23Aa	451Aa	426Ba	0.07Ab	0.07Aa
Rh	7.31Aa	3.30Ba	0.41Aa	0.14Ba	436Aa	443Aa	0.13Aa	0.05Ba

For each variable, means followed by the same capital letters in each line, and lower case letters in each column do not differ according to Tukey's test ($p < 0.05$, n = 10).

Gas exchanges: CO_2 assimilation rate (As) of Rh- and Tc-inoculated plants was higher in V10 soil than in V60 soil (Tab. 4), while no differences between soils were found in Ni plants. Stomatal conductance (gs) was higher in Rh-inoculated plants in V10 soil than in V60 soil. In V10 soil, stomatal conductance was higher in Rh than in Tc and Ni plants. Cell internal CO_2 concentration (Ci) in Tc-inoculated plants was higher in V10 soil than in V60 soil. Transpiration rate (Tr) of Tc plants was also higher in V10 soil than in V60 soil. In V10 soil, transpiration rate was higher in Rh plants than in the Ni and Tc treatments.

P, Cu, and Zn concentrations in leaves and roots: P concentration and accumulation in grapevine shoots (Fig. 6A and C) were higher in V60 soil than in V10 soil in all inoculation treatments. In the V10 soil, shoot P concentration of Ni plants was around 4 $mg \cdot kg^{-1}$, significantly higher than the other inoculation treatments, while shoot P accumulation was higher in Ni and Tc plants than in Rh-inoculated plants. P concentration and accumulation in roots (Fig. 6B and D) were higher in V60 soil than in V10 soil. In V10 soil, root P concentration in Ni plants was higher than in Rh, with Tc in an intermediate position. The phosphorus translocation ratio from roots and shoots (Fig. 6E) in V10 soil was higher in Rh plants than in the other treatments, while in the old vineyard soil (V60), the treatment inoculated with *R. clarus* (Rh) had the smallest value and therefore the highest phosphorus translocation.

Shoot Cu concentration and accumulation (Fig. 7A and C) in the Ni and Rh treatments were higher in V60 soil than in V10 soil, while there were no differences between soils in Tc plants. In V10 soil, shoot P concentration and accumulation were higher in Tc plants than in Rh plants, and Ni had intermediate values. Cu concentration and accumulation in roots (Fig. 7B and D) were higher in V60 soil than in V10 soil. In V10 soil, copper translocation ratios between roots and shoots (Fig. 7E) in the non-inoculated treatment (Ni) were higher, resulting in less copper translocation to leaves, than in the other treatments. In V60 soil, the plants inoculated with *R. clarus* (Rh) had the highest translocation values, and therefore the least copper translocation.

Shoot Zn concentration (Fig. 8A) was higher in grapevines grown in V10 soil than in V60 soil. Zn accumulation in shoots (Fig. 8C) of Tc-inoculated plants was higher in V10 soil than in V60 soil. On the other hand, the opposite occurred with Rh-inoculated plants, and there was no difference between soils for Ni plants. In V10 soil, Tc treatment plants had higher Zn concentration than Ni and Rh plants, while no differences were found between inoculation treat-

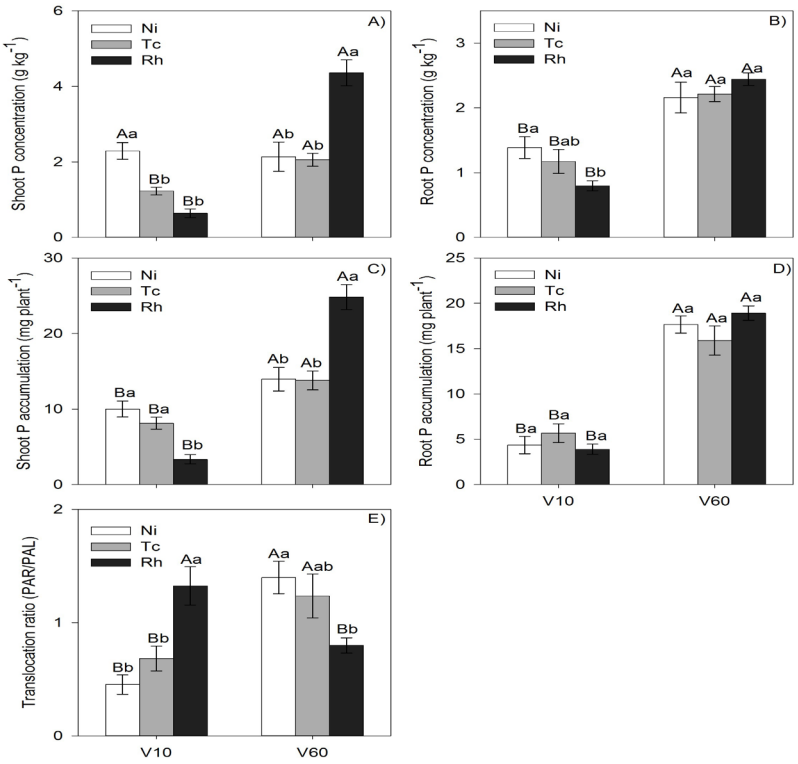

Fig. 6: Phosphorus concentration and accumulation in shoots (**A** and **C**), roots (**B** and **D**) and translocation ratio (**E**) of 'Paulsen' (1103) rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from ten-year (V10) or 60-year (V60) old vineyards. PAR = phosphorus accumulated on the root, PAL = phosphorus accumulated on the leaf. Means followed by the same lowercase letter do not differ (Tukey test, $p < 0.05$, n = 10) in inoculation effect in each soil; means followed by the same capital letter do not differ between soils in each inoculation treatment. Bars represent standard error of the mean.

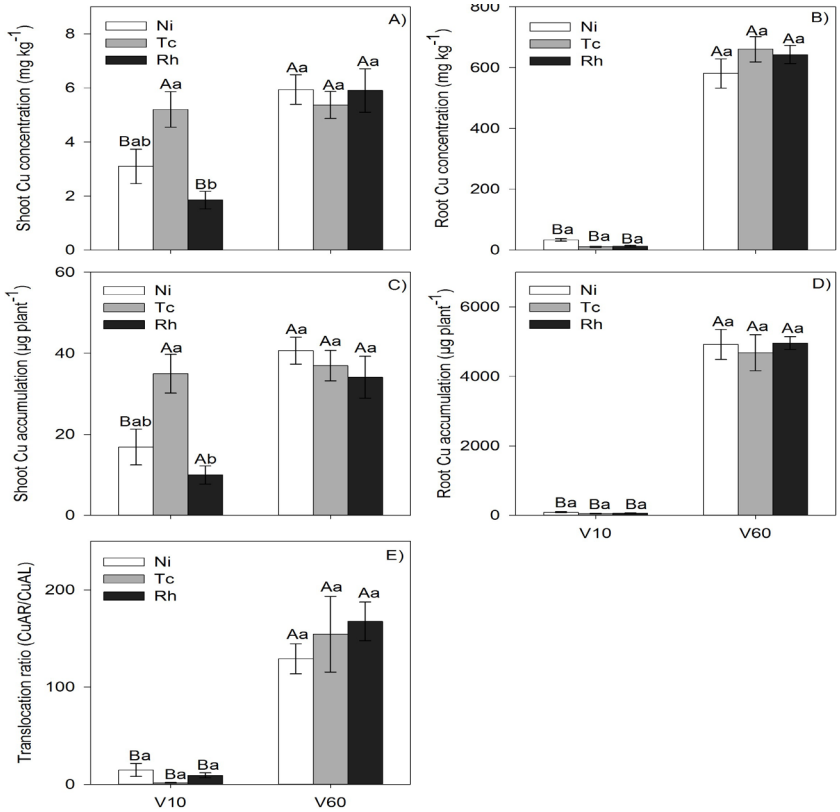

Fig. 7: Copper concentration and accumulation in shoots (**A** and **C**), roots (**B** and **D**) and translocation ratio (**E**) of 'Paulsen' (1103) rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from ten-year (V10) or 60-year (V60) old vineyards. CuAR = copper accumulated on the root, CuAL = copper accumulated on the leaf. Means followed by the same lowercase letter do not differ (Tukey test, $p < 0.05$, n=10) in inoculation effect in each soil; means followed by the same capital letter do not differ between soils in each inoculation treatment. Bars represent standard error of the mean.

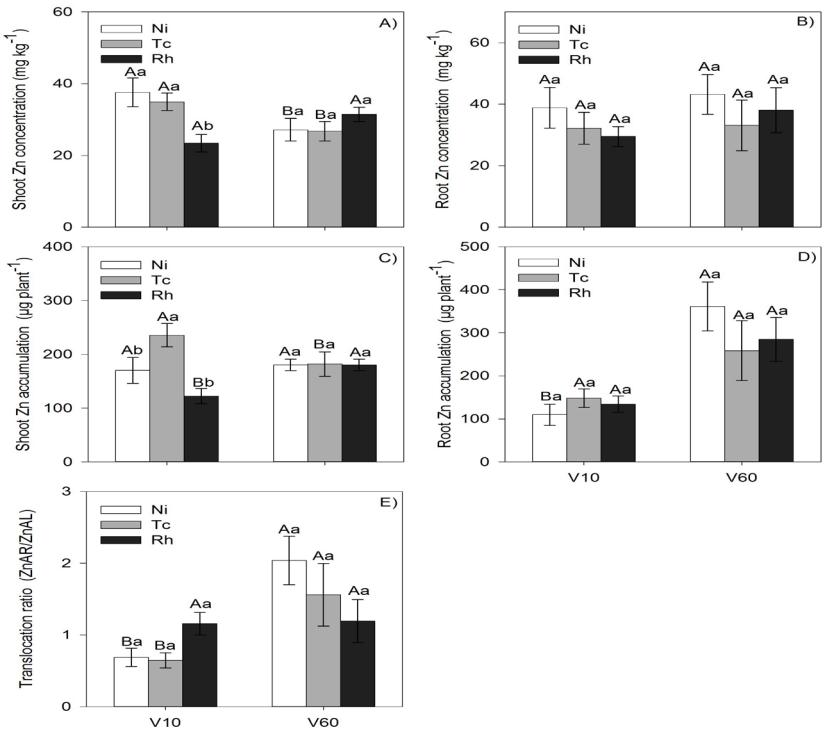

Fig. 8: Zinc concentration and accumulation in shoots (**A** and **C**) and roots (**B** and **D**) and translocation ratio (**E**) of 'Paulsen' (1103) rootstock grapevines, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh), in soils from ten-year (V10) or 60-year (V60) old vineyards. ZnAR = zinc accumulated on the root, ZnAL = zinc accumulated on the leaf. Means followed by the same lowercase letter do not differ (Tukey test, $p < 0.05$, $n = 10$) in inoculation effect in each soil; means followed by the same capital letter do not differ between soils in each inoculation treatment. Bars represent standard error of the mean.

ments in soil V60. Although root Zn concentrations (Fig. 8B) did not differ between soils, Zn accumulation in roots (Fig. 8D) was higher in V60 soil than in V10 soil for Ni and Tc-inoculates plants, while there was no difference between soils for Rh-inoculated plants. Root/shoot zinc translocation ratios b (Fig. 8E) in V10 soil had the treatment inoculated with *R. clarus* (Rh) with a higher value, therefore with less copper translocation to the leaves than the other treatments. In the V60 soils, the non-inoculated treatment (Ni) had the highest value, and therefore the least copper translocation.

Correlations of mycorrhizal colonization, plant P, Cu, and Zn with other plant variables: Mycorrhizal colonization had positive correlations (Tab. 5) with maximum chlorophyll fluorescence (Fm), CO_2 assimilation rate (As), cell internal CO_2 concentration (Ci), number of leaves (leaf n.), chlorophyll A, chlorophyll B, total chlorophyll, and carotenoid concentrations, and negative correlations with plant height and dry root mass (RDM). Shoot and root P concentration correlated negatively with mycorrhizal colonization (Coln), maximum chlorophyll fluorescence (Fm), potential quantum efficiency of the photosystem II (Fv/Fm), CO_2 assimilation rate (As), stomatal conductance (gs), transpiration rate (Tr), shoot dry mass (SDM), chlorophyll A, chlorophyll B, total chlorophyll, and carotenoid concentrations. In addition, root P concentration had a positive correlation with plant height and root dry mass (RDM). Shoot Cu concentration correlated positively with height and dry root mass (RDM), and negatively with maximum chlorophyll fluorescence (Fm), CO_2 assimilation rate (As), stomatal conductance (gs), and transpiration rate (Tr). Root Cu concentration correlated negatively with mycorrhizal colonization (Coln), maximum chlorophyll fluorescence (Fm), potential quantum efficiency of the photosystem II (Fv/Fm), CO_2 assimilation rate (As), stomatal conductance (gs), transpiration rate (Tr), number of leaves (leaf n.), chlorophyll A, chlorophyll B, total chlorophyll, and carotenoid concentration. Shoot Cu concentration correlated positively with height and dry root mass (RDM).

Zn concentration in grapevine shoots correlated positively with initial fluorescence (Fo) and the number of leaves (leaf n.), and it correlated negatively with stem diameter (Stem diam.), shoot dry mass (SDM), and root dry mass (RDM). Root Zn concentration did not correlate with the plant variables.

Discussion

'Paulsen 1103' grapevine rootstocks planted in soils from young and old vineyards had contrasting responses to single-species and trap-culture AMF inoculants. In soil from a 10-year old orchard (V10), inoculation resulted in higher rates of root colonization, especially when the trap-culture AMF inoculum was used. Inoculation had positive effects on grapevine growth, photosynthesis, and gas exchange. That indicates the potential of the native AMF, and associated microbiota, to improve plant establishment and growth. The low rate of root colonization by AMF in soil from a 60-year old vineyard (V60) may be associated with some soil attributes, but also to the inoculum potential of local AMF. AMF colonization occurs primarily when there is low availability of plant nutrients, especially P (Smith and Read

Table 5

Pearson's correlation between mycorrhizal colonization, P, Cu and Zn values of leaves and roots and variables of physiological parameters and growth in 'Paulsen' grapevine rootstocks (1103) at 10 (V10) or 60 (V60) year old vineyard soils, not inoculated (Ni), inoculated with AMF from soil trap cultures V60 (Tc) or with *Rhizophagus clarus* (Rh)

			Leaves						Roots					
			P		Cu		Zn		P		Cu		Zn	
	Coln		-0.34	**	-0.21	ns	0.08	ns	-0.50	***	-0.64	***	-0.08	ns
Fo	-0.05	ns	-0.06	ns	-0.05	ns	0.26	*	0.08	ns	0.01	ns	-0.09	ns
Fm	0.27	*	-0.61	***	-0.27	*	0.03	ns	-0.53	***	-0.57	***	-0.01	ns
Fv/Fm	0.19	ns	-0.28	*	-0.09	ns	-0.21	ns	-0.36	**	-0.31	*	0.09	ns
As	0.39	**	-0.29	*	-0.27	*	-0.02	ns	-0.36	**	-0.38	**	0.06	ns
gs	0.04	ns	-0.38	**	-0.41	**	-0.08	ns	-0.32	*	-0.38	**	0.05	ns
ci	0.32	**	0.04	ns	0.05	ns	0.09	ns	-0.20	ns	-0.18	ns	0.03	ns
Tr	0.05	ns	-0.37	**	-0.42	**	-0.07	ns	-0.33	**	-0.40	**	0.05	ns
Height	-0.28	*	0.24	ns	0.33	**	-0.13	ns	0.46	**	0.60	***	0.10	ns
Leaf n.	0.46	***	-0.11	ns	0.05	ns	0.46	**	-0.22	ns	-0.36	**	-0.06	ns
Stem diam.	0.11	ns	-0.05	ns	0.12	ns	-0.33	**	0.09	ns	0.19	ns	0.10	ns
ADM	0.10	ns	-0.25	*	0.24	ns	-0.36	**	0.16	ns	0.18	ns	-0.15	ns
RDM	-0.28	*	0.10	ns	0.29	*	-0.47	**	0.38	**	0.60	***	-0.07	ns
Chlorophyll A	0.24	*	-0.46	**	-0.24	ns	0.24	ns	-0.57	***	-0.63	***	0.07	ns
Chlorophyll B	0.23	*	-0.46	**	-0.25	ns	0.25	ns	-0.55	***	-0.63	***	0.06	ns
Total Chlorophyll	0.24	*	-0.46	**	-0.24	ns	0.24	ns	-0.56	***	-0.64	***	0.07	ns
Carotenoids	0.30	**	-0.48	***	-0.24	ns	0.22	ns	-0.60	***	-0.65	***	0.06	ns

*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$, ns = not significant. Mycorrhizal colonization (Coln), initial fluorescence (Fo), maximum chlorophyll fluorescence a (Fm), potential quantum efficiency of PSII (Fv/Fm), CO_2 assimilation rates (As), stomatal conductance (gs), internal CO_2 concentration in the cell (Ci) transpiration (Tr), Plant height (Height), number of leaves (Leaf n.), stem diameter (Stem diam.), aerial dry mass (ADM), root dry mass (RDM).

2008), and high concentrations of plant nutrients in the soil have been shown to reduce AMF colonization in grapevines (Schreiner 2005).

The old vineyard soil (V60) had higher K, O.M., Ca, Mg, Zn than V10 soil. V60 had soil P above 80 $mg \cdot kg^{-1}$, four times higher than in V10 soil, and Cu concentration above 500 $mg \cdot kg^{-1}$, 36 times higher than in V10 soil. High available P in the soil may decrease or even suppress root colonization by AMF (Sorensen *et al.* 2005, Kahiluoto *et al.* 2001), and Schreiner (2007) found 67 % and 43 % of mycorrhizal colonization in grapevine roots cultivated in soil with low and high P concentrations, respectively, which is similar to our results. High soil Cu can also inhibit root colonization by AMF (Ambrosini *et al.* 2015, Rosa *et al.* 2016). In soil from the older vineyard, we were not able to establish whether native AMF have bioremediation effects, as previously shown under controlled conditions (Ferrol *et al.* 2009, Cornejo *et al*. 2013). It would also be necessary to verify whether root copper analysis included soil residues that resulted in overestimation of the values, both in the V10 and V60 vineyards.

In V10 soil, root colonization in plants inoculated with the trap culture (Tc) was higher than in plants inoculated with the single-fungus inoculum (Rh) or with the non-enriched native AMF community (Ni). That suggests that the community of native AMF enriched by the trap culture, and its associated microbiota, have higher affinity for grapevines than the inoculant with a single species or the non-enriched native AMF community. The AMF in Tc originate from a soil with high Cu concentration, a condition that affects AMF selection (Joner *et al.* 2000, Gonzáles-Chávez *et al.* 2002, Gonzáles-Guerrero *et al.* 2008). Ambrosini *et al.* (2015) found higher root colonization by *Rhizophagus clarus* (Rh) in soils with higher Cu concentrations and suggested that this fungus has potential for areas with high Cu availability. In the soil with low Cu concentration, inoculation with Tc AMF resulted in higher colonization than inoculation with the single-species Rh inoculant, but in the soil with high Cu concentrations, no differences occurred. That lack of difference may be due to the low level of root colonization in V60 soil, probably caused by high nutrient availability.

The higher root, shoot, and total dry matter in plants growing in the V60 soil, as compared with those in V10 soil, are probably due to the higher concentration of available nutrients in V60 soil. Mycorrhizal inoculation did not affect grapevine growth in V60 soil because high concentrations of nutrients in the soil inhibit mycorrhizal colonization (Kahiluoto *et al.* 2001, Sorensen *et al.* 2005). Previous works (Ambrosini *et al.* 2015, Rosa *et al.* 2016) have shown that high soil Cu concentrations also inhibit AMF colonization, and even impair grapevine growth, but fertility of the soils used in those works was lower than in V60 soil. High soil

Cu concentration decreases root elongation and damages the root system, negatively affecting water and nutrient uptake (Chen *et al.* 2013). Those factors explain the reduction in growth parameters of grapevines cultivated on soil with high Cu contents (Lequeux *et al.* 2010, Kopittke *et al.* 2011). However, organic matter in V60 soils is very high, and under such conditions, formation of organic chelates and complexation of Cu into insoluble organic forms may decrease or prevent the toxicity of this metal to grapevines (Karlsson *et al.* 2006). On the other hand, in V10 soil, with lower fertility than V60 soil, Tc- and Rh-inoculated grapevines showed increased growth, in comparison with non-inoculated plants. The plants that grew more had a higher percentage of mycorrhizal colonization, similar to results of other works using 'Paulsen 1103' grapevine rootstocks (Ambrosini *et al.* 2015, Rosa *et al.* 2016).

Chlorophyll A, chlorophyll B, total chlorophyll, and carotenoid contents differed between V10 and V60 soils, and there was an interaction with inoculation treatments. That might be related to higher Cu availability, and consequent translocation to the plant shoot, as this metal may replace Mg in the chlorophyll molecule, negatively affecting photosynthesis (Yruela 2009). The higher relative proportion of chlorophyll in soil V10 indicates better capture of energy with different wavelengths, improving the whole photosynthesis process (Engel and Poggiani 1991). In general, plants grown in substrates with a high concentration of heavy metals have lower contents of chlorophyll A, chlorophyll B, and carotenoids (Cambrollé *et al.* 2015, Tiecher *et al.* 2017 and 2018). In V60 soil, with higher fertility and copper content, photosynthetic activity was more marked in plants inoculated with the trap culture (Tc), intermediate in the non-inoculated ones (Ni), and lower in the plants inoculated with a single AMF species (Rh). That suggests that the native AMF community, especially when enriched by a trap culture, promotes physiological changes that increase photosynthesis.

AMF may affect gas exchanges related to chlorophyll activity. In all inoculation treatments (Ni, Tc, and Rh), both in V10 and V60 soils, potential photosystem II quantum efficiency (Fig. 3C) was higher than 0.6. When the plant photosynthetic apparatus is intact, in non-stress conditions, the relation between Fv and Fm, which expresses the maximum quantum efficiency, will vary between 0.75 and 0.85 (Bolhàr-Nordenkampf *et al.* 1989). The results obtained indicate low or null inhibition of photochemical processes due to stress that high Cu contents in V60 soil might have caused. The good nutritional conditions provided by the V60 soil may have prevented metal damage to photosynthetic processes.

The electron flux data show that in V10 soil, the inoculated treatments (Tc and Rh) had lower photochemical quenching (qP) than the non-inoculated treatment (Ni), indicating better photosynthetic assimilation capacity in the treatments associated with increases in AMF. Additionally, qN showed no differences among treatments in V10 soils, indicating a similar efficiency in heat dissipation, as qN measures heat dissipation due to increases in proton gradient between the lumen and the chloroplast stroma (Genty *et al.* 1989, Maxwell and Johnson 2000). The values of electron flux in V60 soils showed similar patterns to V10, but Tc-inoculated plants in V60 soils had high qP, which indicates high photosynthetic assimilation efficiency in plants inoculated with the enriched native AMF community. An explanation for the improved photosynthetic performance of the inoculated plants in V10 soils would be higher P uptake promoted by the AMF (Smith and Read 2008). P stimulates photophosphorylation and ATPase activity in leaves, causing an increment in photosynthetic rate in plants, acting like as regulator of photosynthetic processes (Paul and Pellny 2003).

Gas exchange variables showed that carbon assimilation (As) and stomatal conductance (gs) were increased by AMF inoculation in V10 soil, in contrast to the behavior in V60 soil (Tab. 4). In this old vineyard soil, with high levels of copper and phosphorus, inoculated plants decreased their carbon assimilation, in comparison to the young soil, with lower copper and phosphorus. That may be linked to the drain AMF represent for plants, in a condition where there is no advantage in terms of increased nutrient uptake, as amply demonstrated for mycorrhizal symbioses (Smith and Read 2008). AMF change plant metabolism by increasing enzymatic activity and stomatal opening, thus enhancing CO_2 absorption, which results in higher photosynthetic rates (Smith and Read 2008). Increases in photosynthetic rates in AMF-inoculated grapevines indicate that mycorrhizal plants assimilate higher amounts of CO_2, improving growth under unfavorable environmental conditions (Krishna *et al.* 2005).

The correlations showed that P and Cu in grapevine shoots negatively affected root colonization, growth, and chlorophyll concentrations in plants grown in V60 soil, corroborating results obtained in the same region (Ambrosini *et al.* 2015, Rosa *et al.* 2016). Besides, in V10 soil, shoot P and Cu concentrations were higher in Tc-inoculated grapevines than in plants inoculated with the single-species inoculant (Rh). Native AMF populations have mechanisms to regulate metal uptake that are more efficient than fungi from soils with low metal concentrations (Meier *et al.* 2011). Although Cu concentrations in V10 soil are not toxic, AMF from environments with high Cu concentrations may work better, as they have probably developed a variety of strategies to mitigate stresses and survive in high-Cu environments (Ferrol *et al.* 2009, Cornejo *et al.* 2013).

In short, the high levels of phosphorus and copper in the soil were linked to a low frequency of AMF root colonization in soil from old vineyards, even when native AMF were used. However, in soils with low phosphorus and copper accumulation, native AMF led to positive responses in grapevine photosynthetic activity and, therefore, greater growth. In soils with high copper concentration, photosynthesis showed a marked response to native AMF, and carbon absorption was higher with inoculation of *R. clarus*. This resulted in higher photosynthetic rates, and therefore the response to AMF inoculation was, in general, positive for grapevines in different soil conditions. The high copper concentration in old vineyards can cause growth problems in young grapevines and alternatives like AMF inoculation may help overcome such problems. However, other variables, such as high phosphorus fertilization also need to be managed to obtain better results in grapevine growth and development.

Conclusions

We confirmed that high levels of soil nutrients lead to lower colonization of grapevine roots by AMF, even when an enriched inoculum of native AMF is used. In contrast, in a soil with lower fertility, AMF had positive effects on the grapevine photosynthetic capacity, and thus enhanced plant growth. In soils with high nutrients levels, plant photosynthetic performance responded to inoculation with native AMF. Carbon uptake was higher with inoculation of *Rhizophagus clarus*, resulting in higher photosynthetic rates, and the response to AMF inoculation was generally positive for grapevine plants in different soil conditions.

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