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Mycorrhizal Fungi Enhance Yield and Berry Chemical Composition of in Field Grown "Cabernet Sauvignon" Grapevines (*V. vinifera* L.)

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Abstract: The aim of this study was to assess the influence of the application of mycorrhizal fungal inoculum on "Cabernet Sauvignon" (*Vitis vinifera* L.) leaf gas exchange, yield parameters, as well as grape berry composition, especially regarding phenolic compounds. The experiment was conducted over two years under natural vineyard conditions of the Zagreb wine-growing area, the continental region of Croatia. "Cabernet Sauvignon" grapevines were grafted on SO_4 rootstock, both being commonly used in all wine production areas in Croatia. Results obtained demonstrated that symbiotic grapevines, in general, expressed improved leaf gas exchange parameters and higher yield parameters, especially regarding the number of clusters per vine. It should be emphasized that mycorrhizal fungi affected higher total flavan-3-ols, total anthocyanins, and total polyphenols in berry skin in both experimental years. Despite variation in some yield parameters, generally, it is possible to obtain higher yields together with the improved phenolic composition of grapes.

Keywords: mycorrhizal fungi; *Vitis vinifera*; yield; grape berry composition; polyphenols

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1. Introduction

Mycorrhizal fungi live in symbiosis with plant roots. During this mutualism, mycorrhizal fungus helps the plant to acquire water and mineral nutrients from the soil, gaining photosynthetic products from a plant in return [1]. Mycorrhizal symbiosis plays a key role in nutrient cycling in the ecosystem and also protects plants against environmental stress and enhances plant resistance to pests and diseases [2–4]. Mycorrhizal fungi also increase tolerance to adverse soil conditions [1]. Most tree species are in symbiosis with endomycorrhizal or arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (ECM) fungi, or both [5]. AM fungi form tree-like hyphal structures (arbuscules) within root cells. The other association formed by ECM fungi is characterized by hyphal growth and the "Hartig net" formation and a sheath (mantle) around the root tips [1].

AM fungi can improve the nutritional quality of edible parts of crops and play essential roles in the maintenance of host plant fitness under stressed environments [6]. Previous studies have shown that inoculation of grapevines with AM fungi increased shoot and root growth [7–10]; Fe and chlorophyll concentrations in the leaves [11]; photosynthesis rate [12]; N, P, Mn, and Cu concentrations in leaf fresh matter [7,9,13]; as well as increased phenol content in leaf tissues as compared to control grapevines [14]. Furthermore, the AM fungi improved the plants' water status; induced an improvement in the photosynthetic performance that increased the water use efficiency; promoted the uptake of phosphorus

(P), potassium (K), and calcium (Ca); and led to a mobilization of starch reserves in the apex in winter, which was possibly responsible for enhancing root development. Moreover, inoculated plants had significantly increased yield and improved quality of grapes, which led to early grape maturation [15,16]. Although the variations in photosynthesis and transpiration rates coupled with chlorophyll fluorescence-derived parameters provide a quick way to characterize the plant response to water stress, comparison of photosynthetic characteristics of 20 cultivars of grapevine (*Vitis vinifera* L.) showed high variability for gas-exchange parameters (net CO_2 assimilation, stomatal conductance, and intrinsic water use efficiency) but not for chlorophyll fluorescence parameters [17]. Phenolic compounds, which are present in grape berries and wines in quite low concentrations, play a very important role in wine quality and sensory attributes. They strongly contribute to the color, bitterness, and astringency of red wines [18,19]. They are also important because of great health benefits due to antioxidant action in the human body [20–22].

The vast majority of experiments with AM fungi effect on grapevines were conducted under controlled soil and/or climatic conditions, where grapevines were inoculated and planted in pots. There are only a few research works with AM fungi conducted under field (vineyard) conditions [23–25], and even fewer of them [8,26–29] are concerned with the chemical composition of *Vitis vinifera* L. grapes and wines. To our knowledge, there are little known researches dealing with mycorrhizal fungi impact on grapevine physiology, vegetative growth, and grape quality in field conditions.

The aim of this study was to assess the influence of the application of mycorrhizal fungal inoculum on "Cabernet Sauvignon" (*Vitis vinifera* L.) photosynthetic rate, yield parameters, as well as grape composition, especially regarding phenolic compounds. The experiment was conducted over two years under natural vineyard conditions of the Zagreb wine-growing area, the continental region of Croatia. "Cabernet Sauvignon" grapevines were grafted on SO_4 rootstock, both being commonly used in all wine production areas in Croatia.

2. Materials and Methods

2.1. Vineyard Site and Plant Material

A two-year experiment (2016–2017) was conducted on "Cabernet Sauvignon" cultivar (*Vitis vinifera* L.) at Jazbina experimental field (University of Zagreb, Faculty of Agriculture, lat. 45°51′ N, long. 16°0′ E), which is characterized by a moderately warm and rainy continental climate. Experimental vines were planted in 2005 on the spacing of 1.2 × 2.0 m (4167 vines/ha). Rows were east–west oriented. Vines were grafted on SO_4 rootstock and double Guyot trained, leaving 24 buds per vine. The fruit-bearing wire was set up to 80 cm above ground, with the addition of two sets of catch wires at 40 cm intervals from the bearing wire. The maximum canopy height was 180 cm.

Meteorological data during two consecutive growing seasons (1 April–31 October) are presented in Table 1. Soil type was anthropogenic pseudogley with a clay texture. The vines received no fertilization in 2015. Soil analysis was done in the first experimental year (March 2016) and showed that it was very acid with a surface pH (in KCl) of 4.26 (0 to 30 cm depth). The soil was poor in organic matter, ranging from 1.14% (0 to 30 cm depth) to 1.28% (30 to 60 cm depth). The deep horizon of soil was richer in organic matter due to the trenching of soil up to 60 cm depth, performed prior to the planting of vines. Available P and K were very low ranging from 8.6 mg of P_2O_5/100 g soil and 20.0 mg of K_2O/100 g soil, respectively. The soil was moderately supplied with nitrogen, ranging from 0.10% (0 to 30 cm depth) to 0.13% (30 to 60 cm depth). Three soil samples were pooled together to get the average data analysis.

Viticultural practices were performed as usual for this viticultural area. This includes the application of glyphosate beneath the vine rows (0.6 m wide strip) to keep the soil weed-free. Shoots exceeding the height of the trellis were hedged to 20 cm above the last wire, 4 weeks before veraison. Vines were not irrigated, and no fertilization was applied during the experiment.

Table 1. Average temperature (°C) and precipitation sum (mm) during the growing season, Zagreb, 2016–2017.

Year	Average Temperature (°C)	Precipitation Sum (mm)
2016	18.0	518.9
2017	18.2	596.0

2.2. Mycorrhiza Inoculation and Experimental Design

There were two treatments: (1) non-inoculated control (C) and (2) inoculation with commercial inoculum Mykoflor (Mykoflor, Polland). In June 2015, 40 vines of "Cabernet Sauvignon" were inoculated with fungal inoculum. Inoculation was performed by injecting 20 mL of suspension under the vine roots, containing about 2000 propagules of live fungal mycelium. Control vines were located at a distance of ~50 m to avoid potential contamination with inoculated fungi. There were three replications within treatment, with 10 consecutive vines in each replication, so there were 30 vines in each treatment. The experiment had a total of 6 units (plots), distributed in two rows.

2.3. Root Sampling and Mycorrhizal Assessment

Young roots were sampled only in 2016 at ten control and ten inoculated vines, from different parts of the vineyard. After washing under tap water, they were stained with Trypan blue and analyzed under light microscopy (magnification, 200×) [30]. One-hundred-and-fifty root intersections per plant were analyzed to determine colonization rate, according to the magnified intersections method [31], with the percentage of arbuscules, vesicles, only hyphal and total colonization quantified.

2.4. Yield Components and Grape Juice Analysis

Grapes were harvested at their full maturity when the total soluble solids (°Brix) of 100 randomized collected berries remained constant for a few days, which was on 30 September 2016 [274 days of the year (DOY)] and 3 October 2017 (277 DOY). Both treatments were harvested on the same day and processed separately. The number of clusters and yield (g) per vine were counted and weighed in the vineyard, so cluster weight (g) was calculated based on collected data. Immediately after harvest, 100 randomized chosen berries per treatment and repetition were collected and weighed to obtain the average berry weight. Clusters were separately destemmed and crushed for each experimental plot and submitted to juice analysis. Sugar content in musts was determined by refractometer (expressed in °Brix) and titratable acidity of the must (g L^{-1}) was by titration with 0.1 M NaOH according to the O.I.V. method (2013). pH was measured with an electronic pH meter (850 LAB, Schott, Germany).

2.5. HPLC Analysis

Acetonitrile HPLC grade was purchased from J. T. Baker (Deventer, The Netherlands). Formic acid and 85% orthophosphoric acid were obtained from Fluka (Buchs, Switzerland).

The concentration of tartaric, malic, and citric acids (g L^{-1}) was determined by HPLC in a sample of fresh juice obtained from 100 g of berries as described above. Analyses were performed isocratically at a 0.6 mL min^{-1} flow and 65 °C column temperature with a 300 × 7.8 mm i.d. Aminex HPX-87H cation exchange column and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.065% H_3PO_4 as the mobile phase and Agilent Diode Array Detector (Series 1100; Agilent, Palo Alto, CA) set to 210 nm. Data analysis was carried out using the ChemStation chromatography data system (Agilent, Palo Alto, CA, USA).

The berry skins were manually removed from the pulp and seeds and freeze-dried in an Alpha 1–2 Ldplus freeze-dryer (Martin Christ Gefriertrocknungaslangen GmbH, Osterode am Harz, Germany). Dry skins were ground (Coffee Grinder SMK150, Gorenje, Slovenia), and the powder obtained was immediately extracted and analyzed. The extrac-

tion was performed according to the method described by Tomaz et al. [32]. In brief, grape skin powder (160 mg) was extracted by a 10 mL of 20% aqueous acetonitrile containing 1% formic acid for 1 h at 50 °C on the magnetic stirrer. The extract was centrifuged in an LC-321 centrifuge (Tehtnica, Železnik, Slovenia) for 20 min at 5000 rpm at room temperature. The supernatant was removed and brought to a final volume of 10 mL with eluent A (water: phosphoric acid, 99.5:0.5, *v*/*v*). The extract was filtered with Phenex-PTFE 0.20 µm syringe filter (Phenomenex, Torrance, CA, USA) and analyzed by HPLC. The separation, identification, and quantification of flavonoids from grape skin extracts were performed according to the method described by Tomaz and Maslov [33] on an Agilent 1100 Series system (Agilent, Germany). The separation was performed with a reversed-phase column Luna Phenyl-Hexyl (4.6 × 250 mm; 5 µm particle (Phenomenex, Torrance, CA, USA)). The solvents were water:phosphoric acid (99.5:0.5, *v*/*v*, eluent A) and acetonitrile:water:phosphoric acid; 50:49.5:0.5, *v*/*v*/*v*, eluent B). Using DAD, flavonol glycosides were detected at 360 nm, and anthocyanins at 518 nm. Using FLD, flavan-3-ols were detected at λ_{ex} = 225 nm and λ_{em} = 320 nm. Quantification was obtained from calibrating curves of external standards All analyses were performed in triplicate. The results are expressed in mg kg^{-1} of dry weight (d.w.) of grape skin.

2.6. Leaf Gas-Exchange Parameters

Leaf gas-exchange parameters (net photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i), were measured with an LCpro portable photosynthesis system (ADC, Bio Scientific Ltd., Hoddesdon, UK) equipped with a 6.25 cm^2 clamp-on leaf cuvette. The chlorophyll content index (CCI) was determined using a Chlorophyll content meter (CCM-200 by Opti-Sciences, Inc., Hudson, OH, USA). Measurements were taken three times during the growing period: berries setting (end of flowering), berries pea size (berry growth), and berries with intermediate sugar values (berry ripening), which represent stages 27, 31, and 36 according to modified Eichorn and Lorenz system [34]. Measurements were performed on three fully developed and undamaged leaves opposite to clusters from three vine plants per treatment. Average values were calculated from nine recorded measurements per treatment. Measurements were taken on cloudless days between 9:30 and 11:30 h a.m. at 1200 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and 380 ± µmol^{-1} CO_2 concentration. WUE (water use efficiency) was calculated as net photosynthetic rate (A)/transpiration rate (E).

2.7. Statistical Analysis

All analyses were performed using a two-way analysis of variance (ANOVA), with year, treatment, and year*treatment interaction as independent variables in a model. Multiple tests of differences between means of the significant factor levels ($p < 0.05$) were performed using Bonferroni correction. When the interaction year*treatment was found significant in the model, multiple comparisons were made between means of different treatments within the same year with appropriate Bonferroni correction. Data were analyzed using SAS statistical software, version 9.4 (SAS Institute, Cary, NC, USA).

3. Results

This mycorrhizal colonization was relatively high in inoculated vines (79–82%), similar to the average values in the region [35]. Although control plants had a significantly lower percentage of the total (t = −4.671, $p = 0.001$) and arbuscular (t = −4.843, $p < 0.001$) root colonization (t = −4.361, $p = 0.003$), they also developed a high level of colonization (48–74%), being spontaneously mycorrhized by the native AMF present in the vineyard soil (Figure 1).

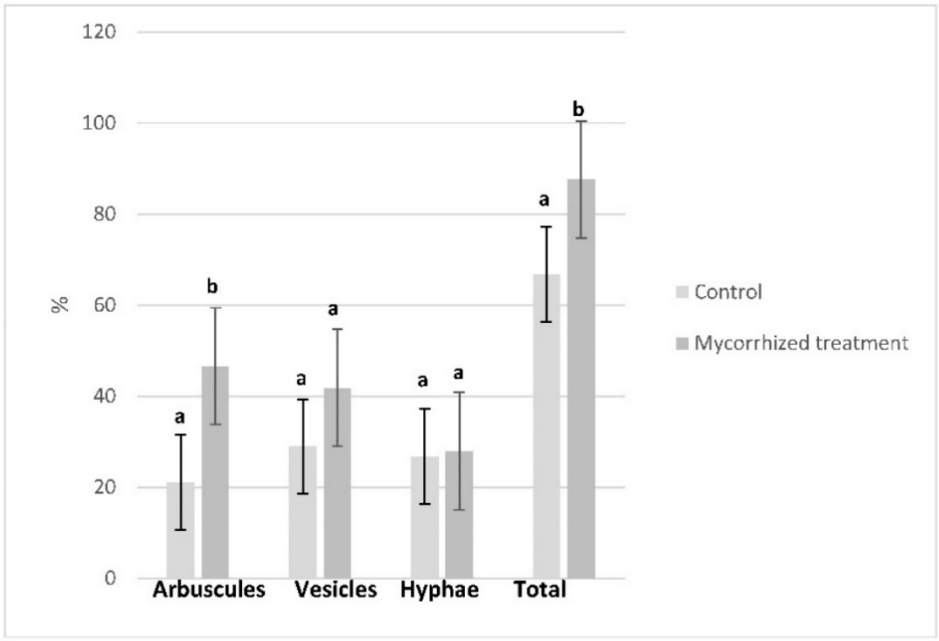

Figure 1. Percentage of arbuscules, vesicles, hyphae only, and total colonization of AMF in control and mycorrhized treatments. Means with different letters are significantly different within treatments (mean separation by Bonferroni correction at $p \leq 0.05$). Error bars represent standard deviation.

Mean values for gas-exchange parameters and CCI are shown in Table 2. In the first year of research, significant differences were determined during the second measurement for Ci, E, and gs, and in the third measurement for Ci. At the verasion stage, Myc treatment had significantly higher values for Ci (145.67 μmol mol^{-1}), E (4.21 mmol H_2O m^{-2} s^{-1}), and gs (0.16 mol H_2O m^{-2} s^{-1}). However, at the beginning of ripening, a significantly higher value for Ci was determined in the control treatment (161.78 μmol mol^{-1}). In the second year, significant differences were found in all measurements: in the first measurement for all parameters, in the second for Ci E, gs, and in the third for A and WUE. At the berry setting stage, Myc had significantly higher values for Ci (191.44 μmol mol^{-1}), E (4.80), and gs (0.22 mol H_2O m^{-2} s^{-1}). However, at the berry growth stage, those values were significantly higher on the control treatment (Ci = 128.56 μmol mol^{-1}; E = 2.92 mmol H_2O m^{-2} s^{-1}; gs = 0.06 mol H_2O m^{-2} s^{-1}). A significant effect for A; WUE and LUE were determined in the second year at the berry setting stage where Myc had significantly lower values (A = 20.00 μmol CO_2 m^{-2} s^{-1}; WUE = 4.17; LUE = 0.0167). However, at berry ripening stage had significantly higher values for these parameters (A = 11.8 μmol CO_2 m^{-2} s^{-1}, WUE = 10.71; LUE = 0.0098). Although CCI at Myc treatment was higher in all measurements, significant differences were not determined.

Table 3 presents the yield parameters of "Cabernet Sauvignon" for both experimental years. The effect of Myc fungi on grapevine yield components became apparent already in the first experimental year, but only regarding the number of clusters per vine. Yield generally decreased in the second year, but all yield components were affected by Myc fungi. Myc fungi inoculation increased yield per vine, number of clusters per vine, and cluster weight. On the other hand, berry weight significantly decreased in Myc treatment. There was an effect of the experimental year on all measured yield components. The interaction between year and treatment was observed for cluster and berry weight.

Table 2. Leaf gas-exchange measurements in mycorrhized and control treatments.

Parameters [1]		CCI	C_i (µmol mol^{-1})	E (mmol H_2O m^{-2} s^{-1})	g_s (mol H_2O m^{-2} s^{-1})	A (µmol CO_2 m^{-2} s^{-1})	WUE
Treatments [2]/Year				2016			
21 June	C	32.54	83.11	3.64	0.14	19.03	5.23
	Myc	33.63	157.22	4.18	0.14	20.81	4.98
	Signif. [3]	ns	ns	ns	ns	ns	ns
19 July	C	26.97	105.67	3.69	0.14	18.09	5.23
	Myc	30.29	145.67	4.21	0.16	16.87	4.98
	Signif.	ns	*	*	*	ns	ns
23 August	C	19.90	161.78	2.81	0.09	9.78	3.48
	Myc	22.28	146.93	2.79	0.09	9.82	3.52
	Signif.	ns	*	ns	ns	ns	ns
Treatments/Year				2017			
16 June	C	25.80	163.89	4.28	0.20	21.49	5.02
	Myc	29.94	191.44	4.80	0.22	20.00	4.17
	Signif.	ns	*	*	*	*	*
17 July	C	27.00	128.56	2.92	0.08	10.77	3.68
	Myc	28.94	108.89	2.41	0.06	9.21	3.82
	Signif.	ns	*	*	*	ns	ns
5 September	C	29.50	34.78	1.13	0.04	9.54	8.44b
	Myc	29.74	42.11	1.10	0.06	11.80	10.71
	Signif.	ns	ns	ns	ns	*	*
Year							
	2016	27.60	133.40	3.55	0.13	15.73	4.44
	2017	28.71	111.61	2.80	0.11	13.80	5.98
	Signif.	ns	ns	*	ns	ns	*
Treatment * Year		ns	ns	ns	ns	ns	ns

[1] CCI indicates chlorophyll content index; C_i indicates intracellular CO_2 concentration; E indicates transpiration rate; g_s indicates stomatal conductance; A indicates net photosynthetic rate; WUE indicates water use efficiency. [2] C indicates control treatment without mycorrhizal inoculation. Myc indicates treatment with mycorrhizal fungi inoculation. [3] Results of analysis of variance: * and ns indicate significance at $p = 0.05$ and not significant respectively, within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

Table 3. Yield components in mycorrhized and control treatments.

		Yield (g/Vine)	Clusters/Vine	Cluster Weight (g)	Berry Weight (g)
Treatments [1]					
2016	C	3744.4	31.1	122.6	1.13
	Myc	3762.2	43.2	91.0	1.09
	Signif. [2]	ns	*	*	ns
2017	C	2676.7	29.4	91.0	1.22
	Myc	3443.3	36.5	94.3	1.09
	Signif.	*	*	*	*
Year					
	2016	3753.3	37.2	106.8	1.11
	2017	3060.0	33.0	92.7	1.15
	Signif.	*	*	*	*
Treatment * Year		ns	ns	*	*

[1] C indicates control treatment without mycorrhizal inoculation. Myc indicates treatment with mycorrhizal fungi inoculation. [2] Results of analysis of variance: * and ns indicate significance at $p = 0.05$ and not significant respectively, within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

The effect of Myc fungi on grape juice quality was not consistent among experimental years (Table 4). In 2016, Myc affected higher soluble solids, lower titratable acidity, and

higher pH value thus implying enhanced grape ripening. In 2017, Myc affected lower soluble solids, titratable acidity, and pH value, which was expected regarding significantly higher yield parameters in Myc grapevines. The concentration of tartaric acid was higher in grapes of Myc treatment in both years, while the concentration of malic acid varied between experimental years.

Table 4. Grape juice quality in mycorrhized and control treatments.

		Soluble Solids (°Brix)	Titratable Acidity (g L^{-1})	pH	Tartaric Acid (g L^{-1})	Malic Acid (g L^{-1})	Citric Acid (g L^{-1})
Treatments [1]							
2016	C	21.2	9.2	2.92	5.8	3.9	0.1
	Myc	21.6	8.9	2.96	6.3	2.5	0.2
	Signif. [2]	*	*	*	*	*	*
2017	C	24.8	6.6	3.21	4.3	0.7	0.1
	Myc	23.0	6.4	3.19	5.1	0.8	0.1
	Signif.	*	*	*	*	*	ns
Year							
	2016	21.4	9.0	2.94	6.1	3.2	0.2
	2017	23.9	6.5	3.20	4.7	0.8	0.1
	Signif.	*	*	*	*	*	*
Treatment * Year		*	ns	*	*	*	*

[1] C indicates control treatment without mycorrhizal inoculation. Myc indicates treatment with mycorrhizal fungi inoculation. [2] Results of analysis of variance: * and ns indicate significance at $p = 0.05$ and not significant, respectively, within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

The effect of ECM fungi inoculation on berry skin flavonols, flavan-3-ols, anthocyanins, as well as total polyphenols is presented in Tables 5–7. Regarding flavonols, Myc treatment affected the higher concentration of quercetin-glucuronide, quercetin-glucoside, and finally total flavonols, but only in 2016. In the second year of the experiment, the concentration of all flavonols was higher in Myc treatment but without statistical significance. Only isorhamnetin concentration was significantly higher in the 2016 year when compared to 2017. Flavanols responded more strongly to Myc treatment, which affected a higher concentration of all measured flavanols in 2016. A similar effect was observed in 2017, but differences in concentration of epigallocatechin and epicatechin were not statistically significant. There was a strong effect of the experimental year, causing higher concentrations of flavanols in the 2016 year. The interaction between year and treatment was observed for catechin, procyanidin b2, and total flavanols. Myc treatment also affected a higher concentration of all berry skin anthocyanins and total polyphenols in 2016. In 2017, Myc treatment affected a higher concentration of delphinidin-3-glucoside, as well as total anthocyanins and polyphenols concentration. The effect of the experimental year was not observed for cyanidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside, while the effect of interaction between year and treatment was the opposite.

Table 5. Berry skin flavonols (mg kg^{-1} of the dry weight of grape skin) in mycorrhized and control treatments.

		Myricetin	Quercetin-Glucuronide	Quercetin-Glucoside	Kaempferol	Isorhamnetin	Total Flavonols
Treatments [1]							
2016	C	369.1	77.9	656.5	115.6	75.5	1294.6
	Myc	426.6	96.8	785.1	150.0	80.1	1538.5
	Signif.[2]	ns	*	*	ns	ns	*
2017	C	420.6	100.3	783.6	128.8	44.0	1477.5
	Myc	519.8	119.2	1005.3	132.8	57.1	1834.2
	Signif.	ns	ns	ns	ns	ns	ns

Table 5. *Cont.*

		Myricetin	Quercetin-Glucuronide	Quercetin-Glucoside	Kaempferol	Isorhamnetin	Total Flavonols
Year							
	2016	397.8	87.4	720.8	132.8	77.8	1416.6
	2017	470.2	109.8	894.5	130.8	50.68	1655.8
	Signif.	ns	ns	ns	ns	*	ns
Treatment * Year		ns	ns	ns	ns	ns	ns

[1] C indicates control treatment without mycorrhizal inoculation. Myc indicates treatment with mycorrhizal fungi inoculation. [2] Results of analysis of variance: * and ns indicate significance at $p = 0.05$ and not significant, respectively, within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

Table 6. Berry skin flavanols (mg kg^{-1} of the dry weight of grape skin) in mycorrhized and control treatments.

		Procyanidin b1	Epigallocatechin	Catechin	Procyanidin b2	Epicatechin	Total Flavan-3-ols
Treatments [1]							
2016	C	18.5	85.0	68.3	38.1	35.0	243.9
	Myc	27.2	129.0	144.9	78.4	46.1	425.7
	Signif. [2]	*	*	*	*	*	*
2017	C	8.5	52.9	36.5	31.1	18.3	147.4
	Myc	15.6	77.5	63.3	33.8	25.9	216.1
	Signif.	*	ns	*	*	ns	*
Year							
	2016	22.4	107.0	106.6	58.2	40.6	334.8
	2017	12.1	65.2	49.9	32.4	22.1	181.7
	Signif.	*	*	*	*	*	*
Treatment * Year		ns	ns	*	*	ns	*

[1] C indicates control treatment without mycorrhizal inoculation. Myc indicates treatment with mycorrhizal fungi inoculation. [2] Results of analysis of variance: * and ns indicate significance at $p = 0.05$ and not significant respectively, within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

Table 7. Berry skin anthocyanins and total polyphenols (mg kg^{-1} of the dry weight of grape skin) in mycorrhized and control treatments.

		Dp-3-g [3]	Cy-3-g	Pt-3-g	Pn-3-g	Mv-3-g	Total Anthocyanins	Total Polyphenols
Treatments [1]								
2016	C	4383.2	201.2	998.5	376.0	12,586.0	18,545.0	20,083.0
	Myc	1,0651.1	438.6	2226.7	618.4	21,649.0	35,584.0	37,548.0
	Signif. [2]	*	*	*	*	*	*	*
2017	C	3144.0	284.2	1089.6	408.1	10,053.0	14,979.0	16,604.0
	Myc	7323.0	258.9	1605.3	399.5	15,635.0	25,222.0	27,272.0
	Signif.	*	ns	ns	ns	ns	*	*
Year								
	2016	7517.1	319.9	1612.6	497.2	17,117.0	27,064.0	28,815.0
	2017	5233.4	271.6	1347.5	703.8	12,844.0	20,100.0	21,938.0
	Signif.	*	ns	ns	ns	*	*	*
Treatment * Year		ns	*	*	*	ns	ns	ns

[1] C indicates control treatment without mycorrhizal inoculation. Myc indicates treatment with mycorrhizal fungi inoculation. [2] Results of analysis of variance: * and ns indicate significance at $p = 0.05$ and not significant, respectively, within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$). [3] Abbreviations: Dp-3-g: delphinidin-3-glucoside; Cy-3-g: cyaniding-3-glucoside; Pt-3-g: petunidin-3-glucoside; Pn-3-g: peonidin-3-glucoside; Mv-3-g: malvidin-3-glucoside.

4. Discussion

The Myc treatment had higher CCI in all measurements, although without statistical significance. This is in accordance with Ambrosini et al. [36] and Mikiciuk et al. [37] who

found no effect of AM fungi on chlorophyll content. Eftekhari et al. [38] claimed that AM fungi inoculation improved or at least maintain chlorophyll content. Generally, our results are similar to Zufferey et al. [39] who investigated water stress on grapevines. Major reductions in gas exchange (A, E) and g_s were measured from the end of July, and very low levels of g_s, A, and E were recorded during the grape ripening phase (August–September). The different transpiration rate by each year is probably caused by differences in the air to leaf vapor pressure deficit (VPD) in these two years, which caused a higher transpiration rate whereas did not significantly affect the rate of photosynthesis. Namely, due to the different concentration gradients and the size of the molecules, water vapor and CO_2 molecules have different diffusion rates, and there is a much higher concentration gradient between leaf and air for water vapor than for CO_2 molecules [40]. Thus, small changes in stomatal conductance caused by different VPD would have a more considerable effect on transpiration rate than on CO_2 diffusion and consequently on Ci and A. Martín et al. reported that inoculated plants during the first and second year had higher values of A and gs, while significant higher WUE values were found only in the second year of investigation [16]. Furthermore, Nikolaou et al. [9] reported that mycorrhizal inoculation had a beneficial effect on CO_2 assimilation in drought-stressed plants, but not for most of the irrigated plants. Nicolás et al. claimed that AM fungi improved photosynthetic performance and water use efficiency of grapevines [15]. Mycorrhizal fungi can increase the photosynthetic rate as a large proportion of assimilates are translocated toward the roots of plants infected with mycorrhizae [41]. Finally, Myc treatment had a positive effect on A and WUE values but only in the second experimental year and at the ripening stage. On the other hand, a positive effect of Myc treatment on g_s values was observed in the first experimental year at the berry growth stages and the second year at the berry setting stage. This is in accordance with Mikiciuk et al. who found that mycorrhizal fungi increased the intensity of CO_2 assimilation, transpiration, and stomatal conductance [37].

The ability of mycorrhizal fungi to enhance grapevine growth has been previously confirmed [23,42], but mostly in greenhouse conditions [43]. It is well known that mycorrhizal fungi can make a significant contribution to phosphorus (P) uptake from the soil [26,36,44]. Fertilization with P affects the increase in grapevine yield and cluster number [45]. The number of berries per cluster was higher in Myc treatment in the second year and this could be the reason for significantly lower berry weight. The described effect was not observed in the first experimental year. Nicolás et al. reported that grapevines inoculated with AM fungi had significantly increased yield [15].

Karagiannidis et al. [26] reported that non-mycorrhizal berries tended to have higher titratable acidity, which is in accordance with the present work. The same authors found no significant differences in soluble solids between mycorrhizal and non-mycorrhizal vines. Nicolás et al. [15] found improved quality of Crimson seedless grapes inoculated with AM fungi, which led to early grape maturation. Improved quality referred to increased soluble solids and reduced acid concentration in grapes.

According to Torres et al. [29], several studies reported enhanced polyphenols synthesis of mycorrhizal grapevines. This is very important regarding grapes and wine quality as well as vine tolerance to the environmental conditions. Previous works reported that inoculation of grapevines with AM fungi improves grapes' phenolic composition, especially anthocyanins content [28,29]. Gabriele et al. [27] found that symbiotic Sangiovese wines had a significantly higher level of total polyphenols and flavonols when compared to conventional wines. However, the same authors found significantly lower levels of monomeric anthocyanins in symbiotic wines than in conventional ones. On the contrary, they found a higher level of malvidin in symbiotic wines compared to conventional ones.

In the present study, the differences between experimental years are also obvious. This could be explained by the extremely hot summer of 2017 (June–August), which led to enhanced berry ripening. The summer of 2016 was slightly colder than the summer of 2017 (by 1.9 °C on average), which was not enough for a late variety like "Cabernet Sauvignon" to reach optimal maturity. On the other hand, extremely hot weather conditions, probably

accompanied by high UV radiation, resulted in a decrease in anthocyanin concentration in 2017, which is in accordance with previous findings of [46].

5. Conclusions

In the present study, the influence of mycorrhizal fungi on the in-field "Cabernet Sauvignon" (*Vitis vinifera* L.) vines was investigated. Although both inoculated and non-inoculated vines did develop symbiosis, mycorrhizal inoculation induced higher root colonization. Briefly, our result demonstrated that inoculated grapevines, in general, expressed improved leaf gas exchange parameters and higher yield parameters. Note that mycorrhizal inoculum resulted in an increase of total flavanols, total anthocyanins, and total polyphenols in berry skin in both experimental years. Therefore, it seems possible to obtain improved phenolic composition of grapes. This has yet to be confirmed by further investigations, in which the impact of yield indicators should be minimized. Besides, further investigation should focus on the nutritional and sanitary status of mycorrhized grapevines. Positive findings could confirm the use of mycorrhizal fungi in vineyards as an efficient tool for organic grape and wine production.

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