



Article

Influence of Different Defoliation Timings on Quality and Phenolic Composition of the Wines Produced from the Serbian Autochthonous Variety Prokupac (*Vitis vinifera* L.)

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Abstract: The variety Prokupac is the dominant variety in the vineyards of Southern Serbia, which produces quality wines of characteristic and unique tastes. In the agroecological conditions of the Prokuplje vine district, the influence of manual defoliation on the phenolic profile of the wine produced from the variety Prokupac was examined. Four experimental treatments with different timings of manual defoliation were applied: early defoliation—treatment I, early defoliation—treatment II, late defoliation—treatment III and the control. The phenolic profile of the wine was determined for the three treatments of defoliation and the control treatment. Additionally, a multivariate analysis was applied on the obtained results, together with already published data (grape seeds and skins phenolic profiles). Identification and quantification of the phenolic compounds was performed using ultra-high-performance liquid chromatography (UHPLC) with an ultraviolet multi-diode detector (DAD) and mass detector with three analyzers—triple quadrupole (QQQ). Based on the obtained results, it was determined that there are significant differences between the experimental treatments in the content of individual polyphenols, total polyphenols and the antioxidant capacity. Twenty (20) phenolic compounds were identified in the wine samples of the experimental treatments. Defoliation significantly affected the variations of the contents of phenolic acids and flavonoids. In treatment III, the highest content of gallic acid was obtained, while the treatments with early defoliation did not differ in relation to the control sample. Early defoliation in treatments I and II had an effect on the phenolic composition of the wine by favoring the accumulation of flavonol, while the content of hydroxycinnamic acid and total anthocyanins (TAC) was higher in treatment III. The TAC increases with later defoliation. The wines obtained by the defoliation treatments did not show higher antioxidant activity compared to the control sample. A principal component analysis resulted in clustering of the samples based on the phenolic components characteristic for each group of samples.

Keywords: leaf removal; wine; anthocyanin; autochthonous grapevine variety; treatments; polyphenols

1. Introduction

The grape (*Vitis vinifera* L.) is one of the most important fruit species cultivated all over the world [1]. Autochthonous grapevine varieties represent an important historical heritage of each country [1–3]. Collecting, preserving and characterizing local grape cultivars is an important task, because the populations of some local cultivars are faced with genetic erosion [4]. For getting new cultivars with higher yields and better quality, it is essential to characterize and conserve grape genetic resources in each country [2]. In the vineyards of Southern Serbia, in addition to international varieties, more and more attention is being paid to old autochthonous grapevine varieties that have been adapted to the edaphic and climatic conditions of Serbia. Prokupac is an autochthonous grapevine variety in Serbia, and it is used to make quality and table red wines with recognizable and unique tastes [5,6]. It belongs to the ecological geographical group *Convarietas pontica*. It is a very lush and high-yielding variety that requires short pruning [7].

The growing demand for quality and premium wine has induced the application of defoliation as an ampelotechnical measure that affects the quality of the grapes and the wine. Leaf removal in the cluster zone is a widely used practice of managing the leaf surface at any time from the berry set, onset of the ripening of the grapes—veraison, also carried out and later than veraison [8–10]. By removing the part of the assimilation surface, changes occur over the course of the physiological processes, but, on the other hand, defoliation changes the microclimatic conditions of the vine, the intensity of light, temperature, humidity and ventilation in the cluster zone [7]. The defoliation of four to six young, photosynthetically active basal leaves in early defoliation causes a photosynthetic shock and results in a reduction in the amount of assimilatives that occur in very sensitive phases—flowering and berry set [11]. As a result, there is an imbalance in the supply of inflorescence and berry set with assimilatives, which causes a reduction of the percentage of the fertilized flowers, an increase of the rate of coulure, a reduction of the number and size of the berries and, also, a change in the proportion between the berry skin and mesocarp of the berries [7,12–14]. In contrast, Aćimović et al. (2016) [15] stated that the application of defoliation before flowering and in the phase of full flowering, where six leaves were removed, was insufficient to cause such stress of the reduction of organic matter that would cause a decrease in the number of clusters every year. Leaf removal before flowering decreased the grape yield of the Sangiovese variety but not the other phenolic compounds due to changed microclimatic conditions in the zone of grape clusters [16]. Removing all basal leaves up to the first bunch after flowering increases the contents of monoterpenes and higher alcohols and decreases the concentration of volatile esters in grapes, compared with defoliation at veraison [17]. Defoliation around bunches is usually applied in colder climates, and many reports have pointed to the significant effects of such practice on ripened grape quality parameters [9,18,19]. In a dense system, improved microclimate after defoliation is mainly attributed to the increased temperature of bunches and a modified light ratio [20]. Open systems, especially in warm climates, should be defoliated carefully to prevent a risk of berry burns and overheating, which can diminish grape and wine quality [21–23]. Changes in the structures of the clusters and berries are most pronounced when defoliation is performed during the initial phase of the berry development, when an intensive division of the pericarp cells occurs. In this case, the application of early defoliation yields smaller and more loose clusters, with a better ratio of the proportion of the berry skin to the mesocarp [8,24]. Depending on the ecological conditions and the canopy management practices, changing the microclimatic conditions of the vine and the structures of the grapes and berries should lead to an improvement in the quality of the grapes. The greatest impact of these changes is reflected in the increase in the sugar content; phenolic compounds and colored, fragrant and aromatic substances [9,18,19,25,26].

The polyphenolic composition of grapes and wines and their antioxidant activity are of great importance, mostly due to the great influence of polyphenols on the organoleptic properties of the wine, especially on color, bitterness and astringency [27]. The contents of the phenolic compounds is influenced by the variety, the ecological conditions and the mi-

croclimate of the vine [28]. Having this in mind, the application of various ampelotechnical measures can influence their contents in grapes, and the increase of polyphenolic and aromatic substances through defoliation has been the subject of research in numerous papers.

Phenolic acids are present in all grapevine organs in different concentrations. They occur as hydroxy derivatives of benzoic and cinnamic acids. Of the hydroxybenzoic acid derivatives, gallic acid is most common, but vanillic, protocatechuic, gentisic and ellagic acids are also present [29–31]. The grapevine variety and other factors (geographical location, edaphic and climatic conditions) that affect the development of the berries have a significant impact on the polyphenolic composition of the wine, while the content of phenols in the wine varies depending on the grape variety and the harvest time [32]. Rustioni et al. (2011) [33] underlined the connection between grapevine eco-physiological conditions, grape ripening and enological practices in concentrations of anthocyanins in berry skin.

The grapevine is one of the plants that is very rich in various phenolic compounds. There are simpler phenolic compounds, such as phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), but also more complex flavonoids (anthocyanins, flavonols, flavan-3-ols, procyanidins, etc.). These compounds are widespread in all organs of the grapevine. The berry is rich in different classes of phenolic compounds that are differently distributed in the skin, seeds and pulp.

Flavan-3-ols found in the grape seed are: catechin (C), epicatechin (EC) and epicatechin-gallate (ECG). Procyanidins (tannins) are formed in the oligomerization process and comprised of a flavan-3-ol terminal subunit and extension subunits connected by interflavan linkages (C4–C8 or C4–C6). In comparison to seed tannins, skin tannins contain epigallocatechin units and have a lower proportion of galloylated units and higher average molecular weights (mDP) [34].

The quality of grapes significantly determines the polyphenolic composition of the wine. The concentration of phenolic compounds in the wine is related to their concentration in the berries [35], and since the biosynthetic pathways of anthocyanins [36] and flavonols are regulated by temperature-sensitive enzymes [37], all changes in the microclimatic conditions, such as those transmitted by defoliation, can significantly affect the synthesis and accumulation of these compounds in the berries and their concentrations in the wine [18]. Moreover, defoliation affects the different accumulations of aromatic compounds in the berry and the change of the aromatic complex of the wine. Early defoliation has an impact on higher concentrations of monoterpenes, higher alcohols and volatile esters [17].

The aim of the research was to determine the influence of different defoliation timings on the quality and the phenolic composition of the wine made from the grape variety Prokupac. The effects of leaf removal treatments, which differ in relation to the time of defoliation, were compared with each other, as well as with the control treatment in which defoliation was not performed, with special reference to their impact on the quality and the phenolic composition of the wine produced from the autochthonous variety Prokupac.

2. Materials and Methods

2.1. Site Description and Experiment Design

The Prokupac variety of vine (*Vitis vinifera* L.) has been investigated in a productive vineyard of the Toplicki Vinogradi Winery near Prokuplje, Serbia. Location of the trial (lat. 43°12'57" N; long. 21°25'31" E; alt. 359 m) belongs to the vine-growing region of Toplica, the wine district of Prokuplje. The vineyard was planted in 2009 with a planting space 2.5 × 0.8 m (5000 plants/ha), and the rootstock variety was Kober 5BB. The training system applied was spur-trained Cordon de Royat, with a trunk height of 60 cm. The investigated vines were loaded by six buds per plant.

The trials have been set in a random complete block design (RCBD) with three blocks and four treatments per block, with 15 plants per treatments, over three years (2014–2016). Defoliation was carried out in different vine developmental stages as follows: treatment I—early defoliation at the flowering stage when 50% flowers were open (BBCH scale 65),

treatment II—early defoliation at the stage when grape sizes were 3–5 mm (BBCH scale 73), treatment III—late defoliation at onset of grape ripening veraison (BBCH scale 81) and control—no defoliation. The first six leaves of each primary and secondary shoots were removed from the all the defoliated vines.

2.2. Chemicals and Materials

Standards of the phenolic compounds (gallic, protocatechuic, *p*-hydroxyphenylacetic, gentisic, ellagic, vanilic, caffeic, *p*-coumaric, ferulic and sinapic acids; catechin; gallic acid gallate; epigallocatechin gallate; rutin; kaempferol-3-*O*-glucoside; quercetin-3-*O*-galactoside; naringin; hesperetin; luteolin-7-*O*-glucoside and phloridzin) used for UH-PLC MS/MS analysis and Trolox were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, acetonitrile (both HPLC grade), formic acid, ethyl acetate and Folin–Ciocalteu reagent were purchased from Merck (Darmstadt, Germany), while 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Fluka AG (Buch, Switzerland). Standard solutions and dilutions were prepared using ultrapure water (TKA Germany MicroPure water purification system, 0.055 $\mu\text{S}/\text{cm}$; Niederelbert, Germany). All other reagents were of analytical grade. Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA).

2.3. Microvinification

The grapes were crushed by hand. The obtained must was enzymed with 2 g/hL of pectolytic enzyme, then sulphitized with 10 g/100 kg of potassium metabisulphite; after which, 25 g/100 kg of activated yeast D254 (Lallemand, Montreal, QC, Canada) were sown. Maceration lasted 24 h. Three replications of microvinifications were performed for each treatment. Ten liters of must were fermented for each replication. Alcoholic fermentation with maceration was carried out at the temperature of 22–25 °C for a period of 15 days, and during all this time, the must was soaked twice a day. After the alcoholic fermentation was completed, the young wine (free-run wine—without applying pressure) was separated from the pomace, stood for 14 days and further nurtured (racking, removal from sediment and sulphitation), stabilized and then prepared for analysis. Microvinification was done in buckets. The obtained results represented the mean values of the three replications.

2.4. Evaluation of Total Phenolic Content (TPC), Radical-Scavenging Activity (RSA) and Total Anthocyanin Content (TAC)

The measurements were performed in triplicate on a Cintra 6 UV–Vis spectrophotometer (GBC Scientific Equipment Ltd., Hampshire, IL, USA) by partially modified methods described by Pavlović et al. (2013) [38] and Pantelić et al. (2016) [39]. Prepared extracts were filtered using 0.45- μm membrane filters prior to the analyses.

The concentration of total phenolic compounds in the wine was determined by the Folin–Ciocalteu spectrophotometric method. The absorbance was measured at 765 nm, and a standard curve was constructed with 20, 40, 60, 80 and 100 mg of gallic acid per liter. Based on that, TPC was expressed as milligrams of gallic acid equivalents per liter of wine (mg GAE/L). Radical-scavenging activity was assessed using DPPH radical solution, and the reduction of DPPH \cdot was monitored by recording the absorbance at 515 nm. Trolox was used as the standard solution in the concentration range 100–600 $\mu\text{mol}/\text{L}$. The calibration curve was shown as the dependence of Trolox concentrations and the percentage inhibition by DPPH \cdot . The results were presented as millimoles of Trolox equivalents per liter of sample (mmol TE/L). The pH differential method was used to determine the total anthocyanins. Test solutions were prepared by diluting the extract in buffer solutions of pH 1.0 (HCl/KCl, 0.025 mol/L) and pH 4.5 (CH₃COOH/CH₃COONa, 0.4 mol/L). The absorbances were measured at two wavelengths: at 520 nm and 700 nm. The content of total anthocyanins was presented as mg of the equivalents of malvidin 3-glucoside per liter of wine (mg mal 3-glu/L).

2.5. UHPLC-DAD MS/MS Analysis of Phenols

Ultra-high-performance liquid chromatography (UHPLC Dionex Ultimate 3000, Thermo Fisher Scientific, Bremen, Germany) with an ultraviolet multi-diode detector (DAD) and mass detector with three analyzers—triple quadrupole (QQQ, TSQ Quantum Access Max, Thermo Fisher Scientific, Bremen, Germany) was used to quantify the polyphenols. The TSQ Quantum Access Max mass spectrometer was equipped with an ion source in the form of electrospray ionization at a temperature of 200 °C, the spray voltage was 5 kV and the capillary temperature 300 °C.

The mass spectrometer records masses in negative ionization mode in the range of 100–1000 m/z . In order to quantify the polyphenols for each standard, a molecular ion and the two most intense fragments from the MS² spectrum were generated by Gašić et al. (2015) [40]. Xcalibur software (version 2.2) was used to control the instruments. Polyphenols were identified by direct comparison with the commercial standards. The total content of each compound was calculated by integrating the peak areas and will be calculated as mg/kg [40].

2.6. The Method of Data Processing and Presentation

Standard statistical methods and the statistical program Statistical Analysis System—SAS (9.031) were used for data processing. The results for the total phenols (TPC) and the antioxidant activity (RSA) were expressed as the mean values of three measurements \pm standard deviations (SD). One-way analysis of variance (ANOVA) was used to examine the experimental data (MS Excel (Microsoft Office 2007 Professional; Redmond, WA, USA)), followed by Duncan's test for detecting differences ($p < 0.05$) between means (NCSS software package) (www.ncss.com (accessed on 1 March 2022)). A principal component analysis (PCA) was performed using PLS_Tool Box software package for MATLAB (Version 7.12.0). Previous to the PCA, all data were group-scaled, and the singular value decomposition algorithm (SVD) and a 0.95 confidence level for Q and Hotelling T² limits for outliers were chosen.

3. Results and Discussion

3.1. Wine Phenolic Composition

Defoliation has significantly influenced the fluctuation in the content of phenolic acids and flavonoids in the tested wines (Table 1). Twenty (20) phenolic compounds were identified in the wine samples of the experimental treatments.

Of the quantified polyphenolic compounds, gallic acid is the most prevalent in the wines, ranging from 8.87 mg/L (the treatment with early defoliation in the flowering phase) to 11.13 mg/L (the treatment with late defoliation), which is less than the values stated by Pantelić et al. (2018) [41] for the variety Prokupac (34.23 mg/L). The dominant content of gallic acid in wines has also been indicated in the data available in the literature [42]. In a review published by Arribas et al. (2012) [43], it was stated that the amounts of gallic acid in red wines range from 2 to 500 mg/L, which was confirmed in our research. In the wine obtained from the treatment with late defoliation (11.13 mg/L), a statistically and significantly higher content of gallic acid was procured in comparison to the wines obtained from the treatments with early defoliation. There was no statistically significant difference in the content of gallic acid ($p > 0.05$) found in the control wine samples and the treatment with late defoliation. The wine from the control treatment contains a statistically and significantly higher content of gallic acid in comparison to the treatment with early defoliation in the flowering phase. Pantelić et al. (2018) [41] stated that the highest content of ellagic acid (2.61 mg/L) was found in the wine of the autochthonous variety Prokupac in comparison to other experimental wines from their research. In our research, a higher content of ellagic acid was obtained in the wine samples of all tested treatments with different defoliation timing and the control sample. In the examined wine samples, the highest content of protocatechuic acid was quantified in treatment II with early defoliation in the phase of berry growth of 3–5 mm (1.08 mg/L). Vanillic acid was found in treatment I with early defoliation in the flowering stage (0.11 mg/L) and treatment III (0.18 mg/L),

where late defoliation was performed, while it was not identified in the control sample and treatment II with early defoliation in the phase of berry growth of 3–5 mm. In the samples of the treatment with early defoliation in the phase of berry growth of 3–5 mm and the treatment with late defoliation, a significantly higher content of protocatechuic acid was obtained in comparison to the control treatment (0.60) ($p < 0.05$). The content of vanillic acid was lower than the values stated by Pantelić et al. (2018) [41].

Table 1. Phenolic compound contents of wine mg/L (average for three years).

Phenolic Compounds	Experiment Treatments				F ¹
	Control	Treatment I	Treatment II	Treatment III	
Hydroxybenzoic acids					
gallic acid	10.63 ^{bc}	8.87 ^a	9.46 ^{ab}	11.13 ^c	*
protocatechuic acid	0.60 ^a	0.90 ^{ab}	1.08 ^b	0.99 ^b	ns
<i>p</i> -hydroxyphenylacetic acid	– ²	–	–	0.38	–
gentisic acid	0.15 ^a	0.16 ^a	0.21 ^a	0.22 ^a	ns
ellagic acid	3.90 ^a	2.33 ^a	2.95 ^a	3.59 ^a	ns
vanillic acid	–	0.11 ^a	–	0.18 ^a	ns
Hydroxycinnamic acids					
caffeic acid	0.94 ^{ab}	0.79 ^a	0.83 ^{ab}	0.97 ^b	ns
<i>p</i> -coumaric acid	0.33 ^a	0.21 ^a	0.34 ^a	0.39 ^a	ns
ferulic acid	–	0.42	–	–	–
sinapic acid	0.11 ^a	–	–	0.72 ^a	ns
Flavan-3-ols					
catechin	8.17 ^a	8.53 ^a	7.87 ^a	7.87 ^a	ns
gallo catechin gallate	0.18 ^a	0.27 ^a	0.37 ^a	0.13 ^a	ns
epigallocatechin gallate	0.15 ^a	0.34 ^b	0.17 ^a	0.34 ^b	*
Flavonols					
kaempferol-3- <i>O</i> -glucoside	–	0.01	–	–	–
quercetin-3- <i>O</i> -galactoside	0.32 ^a	1.27 ^a	1.29 ^a	1.06 ^a	ns
rutin	0.01 ^a	0.01 ^a	0.02 ^a	0.01 ^a	ns
Flavanones					
naringenin	0.004 ^a	0.010 ^{ab}	0.018 ^b	0.017 ^b	*
hesperetin	–	–	–	0.005	–
Flavones					
luteolin-7- <i>O</i> -glucoside	0.03 ^a	0.18 ^a	0.09 ^a	0.12 ^a	ns
Dihydrochalcone derivatives					
phlorizin	0.36 ^a	0.33 ^a	0.38 ^a	0.37 ^a	ns

^{a,b,c} Values are grouped based on Duncan's multiple range test ($\alpha = 0.05$), where different letters within the same row denote significant differences between treatments. ¹ Significance based on the F test: ns = $p > 0.05$, * = $p < 0.05$. ² Stands for "measurement has not been carried out".

Hydroxycinnamic acids (caffeic and *p*-coumaric) were found in all wines of the experimental treatments, while ferulic acid was identified in the wine of treatment I and sinapic acid in the wine of treatment III and the control sample. The highest total content of all hydroxycinnamic acids was determined in the wine of treatment III with defoliation in the veraison stage. Hydroxycinnamic acids are a characteristic of most red wines, except for chlorogenic acid, which was not identified in the samples of the experimental treatments. The defoliation performed in the veraison stage (treatment III) significantly affected the higher content of caffeic acid (0.97) in comparison to treatment I with early defoliation in the full flowering stage (0.79) ($p < 0.05$). In our research, early defoliation did not affect the concentration of hydroxybenzoic and hydroxycinnamic acids, in contrast to the results reported for the Tempranillo variety by Moreno et al. (2015) [44], who pointed out that the concentration of these compounds increases in drier years due to the application of early defoliation before flowering, which exposes the clusters to sunlight. Contrary to the above-mentioned authors, in the years of research, we had a large amount of precipitation [45], above the multi-year average, which affected negatively their concentration. In the three-year research, a uniform concentration of hydroxycinnamic acids was obtained in the wine samples from the treatments with early and late defoliation, as well as the control sample. In accordance with our results, Tardaguila et al. (2010) [46] stated that

early leaf removal significantly improved the color of the wine and the concentration of the phenolic compounds in both cultivars tested. Diago et al. (2012) [47] stated that early defoliation has affected the phenolic composition of wine by favoring the accumulation of hydroxycinnamic acid, flavonols and anthocyanins, which was partly confirmed in our research on flavonols, while the contents of hydroxycinnamic acid and anthocyanins were higher in the treatment with late defoliation in the veraison stage. One study showed that the anthocyanin synthesis decreased and flavonol concentration increased when grapes were exposed to sunlight due to defoliation [48], which was proven in our studies in the treatment with defoliation in the flowering stage and the treatment where defoliation was performed in the phase of berry growth of 3–5 mm.

From the group of flavan-3-ols, catechin, galocatechin gallate and epigallocatechin gallate were identified in all wine samples that were tested. In the wine obtained from the treatment with early defoliation in the flowering stage, the content of catechins (8.53 mg/L) was higher in relation to other wine samples tested. The content of catechin obtained in this study was higher than the data provided for the variety Merlot-Cabernet Franc by Auger et al. (2005) [49]. Defoliation significantly affected the content of epigallocatechin gallate. In the wine of the treatment with early defoliation in the phase of berry growth of 3–5 mm and the treatment with late defoliation, a significantly higher content of epigallocatechin gallate was obtained in comparison to the control sample and the treatment with defoliation in the flowering stage, between which no significant differences were found.

Out of the tested flavonols in the wines of all treatments, quercetin-3-*O*-galactoside and rutin were identified, while kaempferol-3-*O*-glucoside was identified in the wine of the treatment with early defoliation in the flowering stage. Myricetin was not quantified in the tested wine samples, which is not in accordance with the data given by Pantelić et al. (2018) [41], who stated that Prokupac wine contains 1.13 mg/L of myricetin. Contrary to the data from our research, Ristić et al. (2013) [50] did not find any differences in the concentration of phenolic compounds in the wine after applying defoliation. Tessarin et al. (2014) [51] stated that flavonols give bitterness to the wine and that late defoliation at the end of the veraison determined a decrease in epigallocatechin gallate in comparison to the control and the treatment with defoliation at the beginning of the veraison, which is contrary to our data, where a significantly higher concentration of epigallocatechin gallate was obtained in the treatment with late defoliation in the veraison stage and the full flowering stage with regard to the control. The same authors stated that the content of rutin was increased by defoliation in the veraison stage, which is contrary to our research, where the concentration of rutin was increased by early defoliation in the stage of berry growth.

In our research, defoliation significantly affected the concentration of naringenin in the wine of the experimental research treatments. A significantly higher content was obtained in the treatment with defoliation in the stage of berry growth and late defoliation in the veraison stage in relation to the control sample. Hesperetin was identified in the wine of the treatment with late defoliation.

The highest content of phlorizin was found in the wine of the treatment with early defoliation in the phase of berry growth of 3–5 mm (0.38 mg/L), which was more than the data reported by Pantelić et al. (2018) [41], while the content of luteolin-7-*O*-glucoside was higher in the wine of treatment I.

In our research, regarding the contents of phenolic compounds, the highest concentration was obtained in the treatments with early defoliation in the full flowering stage. Sabbatini et al. (2010) [24] stated that the application of defoliation in all three timings in the pre-flowering stage, flowering stage and veraison stage had a significant impact on the increase of content of phenolic substances as a result of better ventilation within the grape zone, which was confirmed in these tests in the treatment with early defoliation in the stage of full flowering. According to our research, Bešlić et al. (2013) [7] stated that the highest content of total phenols was obtained in the treatment with early defoliation in the stage of full flowering for the variety Prokupac. Song et al. (2018) [52] stated that, regardless of the

leaf removal treatment, the total phenol content was higher in the treatments with lower yields [45], which was confirmed in our research.

3.2. Total Polyphenolic Content, Radical Scavenging Activity and Total Anthocyanins Content

Table 2 presents the contents of the total polyphenolic compounds and the contents of the total anthocyanins, as well as the total antioxidant activity of the wine, according to the treatments and the years of research. The achieved TPC values varied significantly between the experimental treatments and the research years.

Defoliation significantly affected the content of total polyphenols in the wine. After the application of early defoliation in the full flowering stage, the highest concentration of total polyphenolic compounds was obtained in the berry skin and mesocarp [45]; on the other hand, the application of late defoliation in the veraison stage and early defoliation in the stage of berry growth of 3–5 mm had a positive effect on the contents of the total polyphenolic compounds in the wine. Bešlić et al. (2013) [7] stated that, by applying defoliation in the stage of berry growth, a significantly higher content of total polyphenols was obtained in comparison to the defoliation applied in the veraison stage, which was confirmed in our research. The lowest content of total polyphenolic compounds in the wine was obtained in the control sample. Contrary to our results, Tardaguila et al. (2008) [53] stated that the defoliation in the phase of berry growth and the veraison stage did not affect the content of the polyphenolic compounds in the wine of the experimental varieties.

The wines in the treatment with late defoliation in the veraison stage (973.20 ± 17.67 mg GAE/L) and treatment II with early defoliation in the stage of berry growth of 3–5 mm (957.23 ± 4.38 mg GAE/L) had significantly higher contents of TPC in comparison to the control wines and the treatment with early defoliation in the flowering stage ($p < 0.05$). The highest values of TPC were obtained in 2014 in the treatment with late defoliation (1001.40 ± 14.43 mg GAE/L), while the lowest values were measured in the wine in 2015 in the treatment with early defoliation in the flowering stage (814.66 ± 18.76 mg GAE/L). Defoliation performed at the beginning of grape ripening affects the synthesis of the primary and secondary metabolism products, i.e., the increase in the content of total phenols [18,54], which was confirmed in this study.

Numerous papers can be found in the literature in which different values for the content of polyphenol in red wines were noted [55–58]. Comparing our results with the data from the literature, a similar content of total polyphenols in the wine from the Czech Republic [56] was noticed in the case of red wines (TPC range for Czech red wines was 0.85–1.97-g EGK/L). In the red wines from the Greek region, which were examined by Kallithraka et al. (2006) [57], the range of TPC polyphenols (0.62–3.20-g EGC/L) was slightly higher in comparison to our results. In our research, the total content of anthocyanins, on average for the research period, did not vary in a statistically significant way between the treatments with applied defoliation and the control sample. Many studies have shown that removing leaves can improve the content of anthocyanins in the berry skin and, thus, in the wine [59–63], which is mainly attributed to the increased exposure to sunlight and the temperature [21,64]. By analyzing the F test, the contents of total anthocyanins in the wine did not vary significantly between the years of research, as well as between the experimental treatments (Table 2) ($p > 0.05$). In comparison to the other two years of research, a higher content of total anthocyanins in the wine was obtained in 2014, but no statistically significant differences were found here either ($p > 0.05$). On average, a higher content of total anthocyanins was obtained in the wine from the control sample (128.46 ± 1.35 -mg mal 3-glu/L) in comparison to the treatment with early defoliation in the flowering stage (121.42 ± 2.42 -mg mal 3-glu/L), which confirms the assertions that anthocyanin synthesis decreases when the grapes are exposed to sunlight due to defoliation [48] ($p < 0.05$). On average, the highest content of anthocyanins was measured in the treatment with late defoliation in the veraison stage (150.07 ± 1.37 -mg mal 3-glu/L), while the lowest content was identified in the treatment with early defoliation in the flowering stage. Hunter et al. (1991) [37] stated that the concentration of anthocyanins increases with

later defoliation at the time of veraison, which was confirmed in this study. Intrierrri et al. (2008) [65] stated in their research that all defoliation treatments had a higher content of total anthocyanins in comparison to the control, which was not confirmed in our research. In our research, on average, the application of early and late defoliation did not affect the content of total anthocyanins in the wine.

Table 2. Total polyphenolic content, radical scavenging activity and total anthocyanins content of wine.

Years of the Investigation	Trial Treatments				F ¹
	Control	Treatment I	Treatment II	Treatment III	
Total polyphenolic content (mg GAE/L)					
2014	859.56 ± 7.22 ^a	828.95 ± 1.44 ^a	932.01 ± 8.66 ^b	1001.40 ± 14.43 ^b	*
2015	814.66 ± 18.76 ^a	867.72 ± 33.19 ^a	986.09 ± 4.33 ^b	958.54 ± 28.86 ^b	*
2016	916.10 ± 9.73 ^a	910.37 ± 1.62 ^a	953.58 ± 0.16 ^a	959.66 ± 9.73 ^a	*
AVG 2014-16	863.44 ± 11.90 ^a	869.01 ± 12.08 ^a	957.23 ± 4.38 ^b	973.20 ± 17.67 ^b	*
Total anthocyanins content (mg mal 3-glu/L)					
2014	163.81 ± 0.84 ^a	131.75 ± 3.36 ^a	138.45 ± 2.44 ^a	164.52 ± 1.07 ^a	ns
2015	134.57 ± 1.82 ^a	128.94 ± 1.37 ^a	118.72 ± 0.90 ^a	149.37 ± 1.15 ^a	ns
2016	87.01 ± 1.40 ^a	103.57 ± 2.53 ^{ab}	162.40 ± 2.41 ^c	136.33 ± 1.88 ^{bc}	ns
AVG 2014-16	128.46 ± 1.35 ^a	121.42 ± 2.42 ^a	139.86 ± 1.92 ^a	150.07 ± 1.37 ^a	ns
Radical scavenging activity (mmol TE/L)					
2014	5.50 ± 0.12 ^a	5.68 ± 0.20 ^{ab}	6.40 ± 0.29 ^{ab}	6.80 ± 0.12 ^b	ns
2015	5.89 ± 0.26 ^a	6.01 ± 0.09 ^a	6.95 ± 0.03 ^a	6.91 ± 0.09 ^a	ns
2016	4.58 ± 0.00 ^a	4.61 ± 0.42 ^a	4.82 ± 0.20 ^a	4.30 ± 0.19 ^a	ns
AVG 2014-16	5.32 ± 0.09 ^a	5.43 ± 0.24 ^a	6.06 ± 0.17 ^a	6.00 ± 0.13 ^a	ns

^{a,b,c} Values are grouped based on Duncan's multiple range test ($\alpha = 0.05$), where different letters within the same row denote significant differences between treatments. ¹ Significance based on the F test: ns = $p > 0.05$, * = $p < 0.05$.

The microclimate in the cluster zone, which changed under the influence of defoliation, has a significant role in raising the level of total anthocyanins [66]. It is known that a change in the microclimate can lead to a change in the chemical composition of grapes, and thus, certain amounts of esterified anthocyanins can occur. [33]. Other authors stated that, in warmer regions, the excessive exposure of clusters to the sun leads to their overheating and reduction of the amount of coloring matter [28,67]. The results of the research vary, depending on the grapevine variety and the climatic conditions; the average critical threshold for the accumulation of anthocyanins in the berry is at temperatures above 30 °C [68]. High exposure of clusters to sunlight in warm climates can increase the concentration of anthocyanins, although, if the temperatures are too high, their quality can be negatively affected [69]. During the research period, high temperatures were not a limiting factor [45] for the synthesis of total anthocyanins in the epidermis of the experimental treatments [45] and, thus, in the wine samples analyzed in this study. The obtained results are in accordance with the data on the higher concentration of total anthocyanins in warm and sunny years compared to in cold and rainy years with a higher amount of precipitation [47]. Namely, in the third (2016) year of the research, we had a large amount of precipitation during the summer months, which was reflected in a lower concentration of total anthocyanins in the experimental treatments, except in the treatment with defoliation in the phase of berry growth of 3–5 mm, where a significantly higher concentration of total anthocyanins in relation to the treatment with early defoliation in the flowering stage and the control sample was registered.

The antioxidant activity of phenolic compounds is conditioned by various factors [70,71]. One study showed that anthocyanins were responsible for a strong antioxidant activity [72] and another that flavanols were responsible for it [73]. Additionally photooxidative sunburn strongly affected the contents of antioxidants in wine [74]. In the research, on average, the antioxidant activity did not vary in a statistically significant way between the experimental treatments in the wine samples. The discovered values of RSA did not vary significantly between the treatments and the years of research. The highest content of

treatment I and treatment II with early defoliation, anthocyanin synthesis decreases, and the concentration of flavonols increases when the grapes are exposed to sunlight due to defoliation. Defoliation significantly affected the content of total polyphenols in the wine. Wines in treatment III with late defoliation and treatment II with early defoliation had a significantly higher content of total polyphenols in comparison to the wines from the control and the treatment with early defoliation in the flowering stage. Defoliation did not significantly affect the content of total anthocyanins in the wine samples of the experimental research treatments. The highest content of total anthocyanins was measured in treatment III with late defoliation, while the lowest content was identified in treatment I with early defoliation in the flowering stage. The concentration of anthocyanins increases with later defoliation in the veraison stage. The wines obtained from the treatments with early and late defoliation showed a higher antioxidant activity in comparison to the control sample but without any statistically significant differences. When the wine phenolic composition and total anthocyanins content is considered, the best results for the whole three-year period were found in treatments II and III with early and late defoliation applied. Finally, the PCA revealed clustering of the grape seeds, skins (analyzed in a previous publication) and corresponding wines according to the phenolic profiles, indicating the compounds characteristic for each group of samples. Wine samples stood out by the presence of *p*-coumaric, ferulic and sinapic acids.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae8040296/s1>: Table S1: Content of individual polyphenols (mg/kg of grape seeds and skins, mg/L of wines), total phenolic contents (TPC; g GAE/kg of grape seeds and skins, g GAE/L of wines) and radical scavenging activity (RSA; mmol TE/kg of grape seeds and skins, mmol TE/L of wines).

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