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The Effect of Early and Late Defoliation on Phenolic Composition and Antioxidant Properties of Prokupac Variety Grape Berries (*Vitis vinifera* L.)

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Abstract: The influence of leaf area and various variants of manual defoliation on the phenolic profile of the Prokupac variety grape berry were investigated in the agroecological conditions of southern Serbia. The following four trial variants of manual defoliation were assessed: Early defoliation—variant I (flowering stage, 50% open flowers); early defoliation—variant II (grape size 3–5 mm); late defoliation—variant III (onset of grape ripening, veraison); and control (no defoliation). The first six leaves of each primary shoot were removed from all defoliated vines. The greatest assimilation area of primary and lateral shoots during the study was observed in the control, i.e., the trial variant with no defoliation. Defoliation significantly decreased the grape yield of the all three defoliated variants in regard to the control. The phenolic profile of the three variants and control was established by analyzing the grape seeds and skin. Based on the collected results for the Prokupac variety, significant differences between the trial variants were established regarding the content of phenols and total polyphenols, as well as radical scavenging activity. Defoliation variants showed a significant effect on the total phenols content of grape skin. In all defoliation variants, as well as in the control, high amounts of ellagic acid were measured. Resveratrol was identified only in grape skin samples of the control variant. The removal of leaves increased the concentration of phenolic compounds in variants where early defoliation was applied. The highest total anthocyanins content was found in 2015 in variant I, where leaves were removed during the full flowering stage.

Keywords: skin anthocyanin; leaf removal; treatments; grape seeds; assimilation area; polyphenols

1. Introduction

Prokupac is the predominant vine variety in southern Serbia, and quality wines with distinctive and unique taste are made of it. It is an autochthonous vine variety that produces table and quality red wines, and belongs to the ecologically geographic group *Convar pontica*, *Convarietas balcanica*. It is a very vigorous and high-yielding variety, which demands short pruning. Besides international vine varieties, vineyards of southern Serbia are often made of old, native vine varieties, adapted to the soil

and climatic conditions of Serbia. Native vine varieties represent an important historical heritage of every country. During the last decade, the Prokupac variety has again become the predominant vine variety in the wine districts of southern Serbia. Quality wines with distinctive and unique tastes are characteristic of this part of Serbia [1,2].

Since the beginning of the 20th century, numerous studies on the chemical composition of vine organs have demonstrated that the chemical composition of different varieties and genotypes is highly variable [3–5]. The vine an uncommon plant that is very rich in various phenolic compounds. It involves simple phenolic compounds, such as phenolic acids (derivatives of hydroxybenzoic and hydroxycinnamic acid), but also more complex ones, such as flavonoids (anthocyanins, flavonols, flavan-3-ols, procyanidins, etc.). These compounds are present in all vine organs. Grape berry is rich in various classes of phenolic compounds, and their content is different in skin, seeds and pulp. The distribution of polyphenols in the berry is not uniform, and about 60% to 70% of total soluble phenols are found in the seeds, 25% to 35% in the skin, and only 10% in the pulp [6]. Seeds are rich in monomers and oligomers of flavan-3-ols and derivatives of gallic acid [7].

The assimilation area strongly affects grape quality, not only its sugar and total acid content but also other traits of the bunch and berry, colored and aromatic compounds, and secondary metabolites [6,7]. Many reports point to the importance of reaching and retaining the optimal ratio between the assimilation area and grape yield for a high-quality grape and wine [8–12]. Defoliation is a viticultural practice carried out during the vegetation period in order to regulate canopy density and shoot exposition, with the aim of improving the grape quality [13,14]. Phenolic compounds represent an important grape quality indicator [15], and those compounds are not synthesized in the same grape development stage, so that the defoliation term can affect grape composition [16]. The effect of leaf removal depends on the timing [16], number of removed leaves [17], vine variety [18], and climate [12]. Early defoliation decreases berry weight and thus grape yield itself [10,13], while defoliation during or after veraison affects primary and secondary synthesis of metabolites [19,20]. On the other hand, some reports have shown that early leaf removal is capable of improving the grape and wine quality [21,22]. Leaf removal before flowering decreased the grape yield of the Sangiovese variety, but on the other hand, it increased concentrations of anthocyanins and other phenolic compounds, due to changed microclimatic conditions in the zone of grape clusters [16]. The removal of all basal leaves up to the first bunch after flowering increases the content of monoterpenes (linalool, geraniol, nerol, etc.) and higher alcohols, and decreases the concentration of volatile esters in grapes, compared with defoliation at veraison [23]. Defoliation around bunches is usually applied in colder climates, and many reports point to the significant effects of such a practice on ripened grapes' quality parameters [8,11,17,24,25]. In dense systems, an improved microclimate after defoliation is mainly attributed to the increased temperature of bunches and modified light ratio [26–28]. Open systems, especially in warm climates, should be defoliated carefully to prevent the risk of berry burning and overheating, which can diminish grape and wine quality [29–31].

This study aimed to establish the influence of the leaf area and timing of defoliation (treatments of early defoliation at the flowering stage when 50% of the flowers were open, early defoliation at the stage when the grape size was 3 to 5 mm, and late defoliation at the onset of grape ripening) on grape skin and seed phenolic composition in cv. Prokupac, grown in the agroecological conditions of southern Serbia.

2. Materials and Methods

2.1. Site Description and Experiment Design

Prokupac variety of vine (*Vitis vinifera* L.) was investigated in a productive vineyard of the Toplicki Vinogradi Winery near Prokuplje, Serbia. The 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, wine district of Prokuplje. The vineyard was planted in 2009 with a planting space of 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 were set in a random complete block design (RCBD) with three blocks and four treatments per block, for three years (2014–2016). Defoliation was carried out in different vine developmental stages as follows: Variant I—early defoliation at the flowering stage when 50% of the flowers were open (BBCH scale 65), variant II—early defoliation at the stage when the grape size was 3 to 5 mm (BBCH scale 73), variant III—late defoliation at the onset of grape ripening veraison (BBCH scale 81), and control—no defoliation. The first six leaves of each primary shoot were removed from all defoliated vines. Defoliation of variant I in 2014 was carried out on 16 June, in 2015 on 13 June, while in 2016 it was carried out on 9 June. Removal of the first six leaves of each primary shoot of variant II in 2014 was carried out on 1 July, in 2015 on 29 June, and in 2016 on 25 June. Late defoliation at the veraison stage of variant III in 2014 was done on 8 August, in 2015 on 6 August, while in 2016 it was done on 2 August. The leaf area of primary and secondary shoots was measured in all three years of investigation immediately after defoliation, but the first measurement included the control and variant I, the second and third measurement included the control and variants I and II, and the fourth measurement included the control and the all three variants.

The leaf area of primary and secondary shoots and the total assimilation area per vine was established by the non-destructive method, based on the correlation between the lower side veins' length and leaf area [32]. Primary and secondary shoots' leaf area was calculated by multiple regression analysis based on the following variables: Shoot length, leaf number, and area of the largest and smallest leaf, while the total leaf area was established using the number of shoots per plant [33].

Chemical analyses were carried out in the laboratories of the Faculty of Chemistry, University of Belgrade.

2.2. Chemicals

Standards of phenolic compounds (gallic, protocatechuic, *p*-hydroxyphenylacetic, gentisic, ellagic, vanilic, chlorogenic, caffeic, *p*-coumaric, and ferulic acids; aesculin, gallocatechin, epigallocatechin, catechin, epicatechin, gallocatechin gallate, catechin-3-gallate, epigallocatechin gallate, morin, kaempferol, quercetin, rutin, myricetin, naringin, hesperetin, apigenin, and resveratrol) used for UHPLC 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 μS/cm). All other reagents were of analytical grade.

2.3. Materials

Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). The SPE cartridges used for fractionation of the extracts were Strata C18–E (500 mg/3 mL), obtained from Phenomenex (Torrance, CA, USA).

2.4. Grape Sampling

Grapes of the investigated variants were sampled in 2014, 2015, and 2016 during the technological maturity stage. Sampling was done by random berry picking from bunches of various shoots and vines within a variant. Every sample was taken in three repetitions (from three blocks); each comprised 100 berries from various parts of bunches. Collected samples were immediately frozen to −18 °C and stored until the moment of analysis.

2.5. Preparation of Grape Seeds and Skin Sample Extracts

Polyphenols from the skin and seeds were extracted by the modified method according to Pavlović et al. [34]. They were manually extracted from frozen berries. Seeds were dried in the dark at 22 °C and crushed using liquid nitrogen. Skin (2 g approximately) was extracted by 20 mL of methanol containing 0.1% HCl on a magnetic stirrer for 1 h. After that, extracts were kept in the dark at 4 °C for 24 h, and then filtered through Whatman No. 40 filter paper. The extraction was repeated three times. All three fractions were collected, combined, and dried to a dry state in a rotary evaporator IKA RV8 (IKA®-Werke GmbH & CO. KG, Staufen, Germany) under low pressure at 40 °C. The evaporated residue was diluted in 10 mL of ultrapure water, and such solutions were used for further analyses. One gram of seeds was extracted by 10 mL of MeOH/H_2O (80/20) containing 0.1% HCl. The further procedure was the same as for the skin. Extracts were filtered through a 0.45-µm membrane PTFE syringe filter before analysis.

2.6. Determination of the Total Phenolic Content (TPC)

The total phenolic content of seeds and skin was determined using the partially modified spectrophotometric method according to Folin–Ciocalteu described by Pavlović et al. [34]. A standard curve was constructed with 20, 40, 60, 80, and 100 mg gallic acid per liter. Based on that, the total phenolic content was expressed as gram equivalents of gallic acid (GAE) per kilogram of frozen sample (FW, skin) or kilogram of dry sample (DW, seeds).

2.7. Determination of Radical-Scavenging Activity (RSA)

Radical-scavenging activity was estimated using DPPH and a partially modified method from the literature [35]. The calibration curve Trolox was expressed as a function of the DPPH inhibition percent. The results were given as mmol of Trolox equivalent per kilogram of sample (mmol TE/kg).

2.8. Determination of the Total Anthocyanin Content (TAC)

Total anthocyanin content was determined by the pH-differential method. Test solutions were prepared by diluting extracts in buffer solutions pH 1.0 (HCl/KCl, 0.025 mol/L) and pH 4.5 (CH_3COOH/CH_3COONa, 0.4 mol/L). After 30 min of incubation at room temperature, absorbances of both solutions were measured at two wavelengths, 520 and 700 nm. Ultrapure water was used as a blank. Total anthocyanin content was expressed as gram equivalents of malvidin-3-glucoside per kg of frozen skin sample [36].

2.9. UHPLC-DAD MS/MS Analysis of Phenols

Quantification of polyphenols was carried out using ultra-high-performance liquid chromatography (UHPLC; Dionex Ultimate 3000, ThermoFisher Scientific, Bremen, Germany) with a diode array ultraviolet detector (DAD), coupled to triple quadrupole mass spectrometry (UHPLC-DAD MS/MS; TSQ Quantum Access Max, ThermoFisher Scientific, Bremen, Germany). The column used for separation was Syncronis C18 (1.7 um, 100 × 2.1 mm) from ThermoFisher Scientific. Chromatographic conditions: Mobile phase at a flow rate of 0.3 mL/min, consisted of 0.1% acetic acid in water (A), and LC–MS grade acetonitrile (B) was performed as gradient elution program: 0.0–1.0 min 5% B, 1.0–14.0 min from 5% to 95% (B), 14.0–14.1 min from 95% to 5% (B), and 5% (B) for 6 min. A mass spectrometer was equipped with an ion source based on electrospray ionization at 200 °C, with a spray electric potential of 5 kV and capillary temperature of 300 °C. The mass spectrometer recorded the masses in negative ionization mode in the range 100–1000 *m/z*. In order to quantify polyphenols, for every standard, the molecular ion peak and two most intensive fragments from the MS^2 spectra were generated [37]. Xcalibur software (v.2.2) was used to control the instruments. Polyphenols were identified by direct comparison with commercial standards. The content of each compound was calculated by integrating the peak areas, and was expressed as mg/kg [37].

2.10. Statistical Analysis

Data were processed and analyzed by standard statistical methods using software packages MS Excel 2007 (MS Office 2007, Microsoft, Redmond, WA, USA) and Statistica v.9.0 (StatSoft Inc., Tulsa, OK, USA). Results of TPC and RSA were expressed as three measurements' means ± standard deviation (SD). Data were subjected to factorial ANOVA (grape yield, leaf area) and main effects ANOVA (phenolic compounds). Differences between the treatments were tested by F test and Duncan's multiple range test.

3. Results and Discussion

3.1. Climatic Conditions

Total rainfall, calculated from April to September, was 697.6 mm in 2014, 286.8 mm in 2015, and 483.9 mm in 2016 (Table 1). During 2014, a much higher rainfall amount was observed compared with the other two years and the 50-year average. The highest rainfall amount was observed in 2015 and was lower than the 50-year average. The summer months' rainfall amount of 2016 (249.6 mm) and 2014 (247.0 mm) was much higher in regard to the 50-year average (151.2 mm), while the same amount of 2015 (122.6 mm) was lower compared with the other two years and the 50-year average. The mean air temperature was greater than the 50-year average in all three years of investigation (18.3 °C in 2014, 19.4 °C in 2015, and 18.9 °C in 2016). During the summer months, the average air temperature (21.2 °C in 2014, 22.4 °C in 2015, and 21.6 °C in 2016) was also higher compared with the 50-year average (20.8 °C). The absolute maximum air temperature during the investigated period ranged from 28.5 °C August 2014 to 29.4 °C in August 2016, and up to 32.2 °C in August 2015. During the studied period, a high air temperature was observed in August, mainly in the veraison stage, when defoliation of variant III was taking place. The high air temperature did not represent a limiting factor for Prokupac variety growth and development during the study, in all trial variants.

Table 1. Mean temperature (T) and rainfall sum (P) recorded from April to September during the three studied years (2014, 2015, and 2016).

Month	2014		2015		2016		AVG 1961–2010	
	T (°C)	P (mm)	T (°C)	P (mm)	T (°C)	P (mm)	T (°C)	P (mm)
April	12.5	190.8	11.5	53.9	14.7	62.0	11.4	48.9
May	15.9	129.0	17.7	51.2	15.9	122.7	16.1	57.5
June	19.9	114.2	19.5	99.1	21.6	77.1	19.6	55.6
July	21.8	93.2	23.8	3.2	22.5	102.4	21.4	50.1
August	21.9	39.6	23.9	20.3	20.8	70.1	21.3	45.5
September	18.0	130.8	20.1	59.1	17.9	49.6	17.3	44.9
AVG/SUM	18.3	697.6	19.4	286.8	18.9	483.9	17.8	302.5

3.2. Grape Yield

Table 2 shows the effect of defoliation on grape yield per vine. Defoliation significantly affected grape yield, so defoliated variants had lower grape yields than the control. Such a decrease was not significant in 2014 for variant III, while in 2016, there were no significant differences. A significant difference was observed between 2015 (2.219 kg) on the one hand, and 2014 (1.829 kg) and 2016 (1.682 kg) on the other hand. Early defoliation was reported by Moreno et al. [12] to decrease the grape yield of the Tempranillo variety, which was also observed in this study for Prokupac.

Table 2. Grape yield per vine (kg).

Year of the Study	Experiment Variants			
	Control	**Var. I**	**Var. II**	**Var. III**
2014	2.279 [b]	1.569 [a]	1.598 [a]	1.871 [a,b]
2015	2.661 [b]	2.058 [a]	1.971 [a]	2.187 [a]
2016	1.759 [a]	1.571 [a]	1.856 [a]	1.542 [a]
AVG 2014–16	2.233 [b]	1.733 [a]	1.808 [a]	1.867 [a]
F [1]	experiment variants	year	interaction	
	**	***	ns	

[a,b] Values were 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 *F* test: ns means $p > 0.05$; ** means $0.001 < p \leq 0.01$; *** means $p \leq 0.001$; control—no defoliation; var.I—early defoliation at flowering stage when 50% of flowers were open; var.II—early defoliation at the stage when grape size was 3 to 5 mm; var.III—late defoliation at the onset of grape ripening.

A lower grape yield in early defoliated variants was caused by a decreased grape cluster size, number of berries per cluster, and changed microclimatic conditions in the cluster zone. Similar effects of early defoliation on the grape yield of the Tempranilo variety were reported by Moreno et al. [12] and Tardaguila et al. [9]. Leaf removal due to early defoliation decreased the grape yield of the Sangiovese variety [16]. On the other hand, Tardaguila et al. [9] found that defoliation did not affect the grape structure and yield, if applied after grape formation, which was not observed in this study.

3.3. Leaf Area

The total leaf area of primary and secondary shoots (m^2) is presented in Table 3. Measurements were carried out four times each year. The first measurement involved the control and variant I with early defoliation at 50% open flowers. The F test showed significant effects of the variant and investigation year ($p < 0.001$), as well as of the interaction between them ($p < 0.01$). Variant I had a significantly lower leaf area than the control based on Duncan's test. The second measurement involved the control and variant I and II, and the effects of both observed treatments and their interaction were significant ($p < 0.001$). The highest leaf area (0.847 m^2) was observed in the control than in variant I (0.743 m^2), and the lowest one in variant II (0.467 m^2). Differences between these values were all significant (Table 3). The third measurement was as the previous one, but here the effects of the observed treatments were significant ($p < 0.001$), while the effect of their interaction was not. Again, the highest assimilation area was observed in the control, followed by variants I and II, with all differences being significant.

The fourth measurement included all variants, and was carried out during the veraison stage. Effects of the observed treatments were significant ($p < 0.001$), while the interaction was not. There was not any significant difference in the total leaf area between the control and variant I, and these two variants showed significantly higher values than the other two. Variant II had a significantly larger leaf area in regard to variant III. The data reported by Tardaguila et al. (2008) showed that the removal of the first five leaves of the main shoots in berry formation (diameter 3–4 mm) and veraison did not cause any significant decrease in the total leaf area per vine, and that was explained by the observed more intensive primary and secondary shoot growth on defoliated vines, which compensated for the removed leaf mass. It was partially confirmed by our results when early defoliation during bloom was in question, but in later defoliation terms, removed leaf mass could not be compensated for, and was probably caused by intrinsic properties of the Prokupac variety.

Table 3. Total primary and lateral leaf area (m^2) by measurement term and year of study.

Leaf Area by Measurement Term and Year	Experiment Variants			
	Control	**Var. I**	**Var. II**	**Var. III**
First measurement term				
2014	0.479 [b]	0.332 [a]	– [2]	–
2015	0.339 [b]	0.206[a]	–	–
2016	0.570 [b]	0.305 [a]	–	–
Average 2014–16	0.462 [b]	0.281 [a]	–	–
Significance [1] based on *F* test	variants (a)	years (b)	interaction (axb)	
	***	***	**	
Second measurement term				
2014	0.835 [c]	0.681 [b]	0.526 [a]	–
2015	0.712 [b]	0.765 [b]	0.318 [a]	–
2016	0.996 [c]	0.784 [b]	0.559 [a]	–
Average 2014–16	0.847 [c]	0.743 [b]	0.467 [a]	–
Significance [1] based on *F* test	variants (a)	years (b)	interaction (axb)	
	***	***	***	
Third measurement term				
2014	1.174 [b]	0.896 [a]	0.821 [a]	–
2015	1.113 [b]	1.004 [b]	0.746 [a]	–
2016	1.362 [c]	1.158 [b]	0.973 [a]	–
Average 2014–16	1.216 [c]	1.019 [b]	0.847 [a]	–
Significance [1] based on *F* test	variants (a)	years (b)	interaction (axb)	
	***	***	ns	
Fourth measurement term				
2014	0.907 [a]	0.819 [a]	0.718 [a]	0.733 [a]
2015	1.447 [c]	1.418 [b,c]	1.209 [b]	0.947 [a]
2016	1.861 [c]	1.677 [b]	1.576 [b]	1.299 [a]
Average 2014–16	1.405 [c]	1.305 [c]	1.168 [b]	0.993 [a]
Significance [1] based on *F* test	variants (a)	years (b)	interaction (axb)	
	***	***	ns	

[a,b,c] Values were 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 means $p > 0.05$; ** means $0.001 < p \leq 0.01$; *** means $p \leq 0.001$; [2] – stands for "measurement has not been carried out"; control—no defoliation; var.I—early defoliation at flowering stage when 50% of flowers were open; var.II—early defoliation at the stage when grape size was 3 to 5 mm; var.III—late defoliation at the onset of grape ripening veraison.

3.4. Grape Seeds and Skin Phenolic Composition, Total Antocyanins Content and Radical Scavenging Activity

The content of phenolic compounds in grape seeds (Table 4) was significantly affected by the investigated defoliation variants, according to the F test, only in the case of quercetin 3-*O*-galactoside ($p < 0.01$). In grape seeds of variant I, 16 phenolic compounds were identified and quantified, 12 in variant III, 11 in the control sample, while the lowest number of phenolic compounds (nine) was found in the seeds of variant II.

Flavan-3-ols were the prevalent phenols in seeds (Table 4), which was in accordance with previous reports [38,39]. The highest concentration of catechin, 1605.08 mg/kg of dry weight (DW), was observed in berry seeds of variant II, and were not significantly higher in regard to variant III and the control but were also higher than the data reported by Pantelić et al. [36] for the Prokupac variety. An increased flavan-3-ols content of grape seeds could be connected with higher levels of wine bitterness according to Robichaud et al. [40]. Epigallocatechin gallate was not identified in any sample from the investigated variants, which contrasted with the results of the same study.

Table 4. The average content of phenolic compounds in grape seeds (mg/kg DW).

Phenolic Compounds [§]	Experiment Variants				F [1]
	Control	Var. I	Var. II	Var. III	
Hydroxybenzoic acids					
gallic acid	258.64	199.14	226.84	165.13	ns
protocatechuic acid	0.81	0.38	0.47	0.58	ns
p-hydroxyphenylacetic acid	6.93	1.83	– [2]	11.83	ns
gentisic acid	–	0.14	–	0.16	ns
ellagic acid	618.70	542.27	520.10	613.38	ns
vanillic acid	–	–	–	0.90	–
Hydroxycinnamic acids					
caffeic acid	–	0.95	–	–	–
Coumarins					
aesculin	–	–	–	–	–
aesculetin	–	–	–	–	–
Flavan-3-ols					
catechin	959.06	1215.53	1605.08	881.94	ns
gallocatechin gallate	5.61	5.06	4.91	8.37	ns
epigallocatechin gallate	–	–	–	–	–
Flavonols					
kaempferol	–	0.12	–	–	–
kaempferol 3-*O*-glucoside	–	–	–	–	–
quercetin 3-*O*-galactoside	0.31 [a]	0.33 [a]	0.64 [b]	0.44 [a]	**
rutin	–	–	–	–	–
galangin	–	1.34	–	–	–
Flavanones					
naringenin	0.09	–	–	0.20	ns
hesperetin	0.12	0.13	0.08	–	ns
pinocembrin	–	3.28	–	–	–
Flavones					
luteolin 7-O-glucoside	0.29	0.29	0.33	0.16	ns
chrysin	–	4.92	–	–	–
Dihydrochalcone derivatives					
phlorizin	9.79	9.02	12.79	7.62	ns
Stilbenes					
resveratrol	–	–	–	–	–
Stilbenoids					
oxyresveratrol	–	–	–	–	–
polydatin	–	–	–	–	–

[§] Results are expressed as the average of the three years of the study (2014–2016). [a,b] Values were grouped based on Duncan's multiple range test ($\alpha = 0.05$), where different letters within the same row denotes significant differences between treatments. [1] Significance based on F test: ns means $p > 0.05$; ** means $0.001 < p \leq 0.01$; [2] – stands for "not detected". Control—no defoliation; var.I—early defoliation at flowering stage when 50% flowers were open; var.II—early defoliation at the stage when grape size was 3–5 mm; var.III—late defoliation at onset of grape ripening veraison.

Six hydroxybenzoic acids were quantified in the grape seeds of the examined variants. The gallic acid concentration ranged from 165.13 (variant III) to 258.64 mg/kg DW (control). The range of protocatechuic acid (0.38–0.81 mg/kg DW) and p-hydroxyphenylacetic acid (1.83–11.83 mg/kg DW) was lower than ones reported previously [6]. p-Hydroxyphenylacetic acid was not detected in variant II. The concentration of ellagic acid was the highest in seeds of variant III (613.38 mg/kg DW), higher than the value reported for Prokupac by [36]. In all examined variants, a high content of ellagic acid was determined, which opposed the opinion that ellagic acid presence was only a characteristic for

muscat varieties [41]. Gentisic acid was detected only in defoliation variants (variants I and III), while vanillic acid was identified only in the late defoliation variant (variant III—0.90 mg/kg DW).

One hydroxycinnamic acid (caffeic acid) was detected, but only in variant I. Chlorogenic and ferulic acids were not identified in seeds, unlike Pantelić et al. [36]. Of the three identified flavonols, kaempferol and galangin were found only in variant I with early defoliation. Quercetin 3-*O*-galactoside was quantified in all variants, and variant II had a significantly higher content than the others ($p < 0.01$), which followed the tendency observed by Song et al. [42]. On the other hand, it was not in accordance with data of Ristić et al. [43], who reported the highest flavanols concentration found in the seeds of bunches from the shadow due to increased seed weights.

Table 5 gives the phenol content of the grape berry skin (three-year average, 2014–2016) of the investigated variants. Increased exposure of grape clusters to sunlight caused by leaf removal could be connected with improved grape quality, which reflects an increased content of anthocyanins and phenolic compounds of grape skin, according to Smart and Robinson [44]. In this study, an increased content of skin phenolic compounds in defoliated variants was observed only for flavonols and coumarins. In the skin of the control variant, 19 phenolic compounds were identified and quantified, in variants I and III 16 compounds, while in variant II only 14 compounds were found. Bešlić et al. [45] reported that early defoliation of the Prokupac variety increased the total polyphenols content of the skin, which was partially confirmed by this study. Flavonols were the predominant phenols quantified in the grape berry skin of all variants, and that was similar with previous reports [36,38,46]. Quercetin 3-O-galactoside, rutin, kaempferol, and kaempferol 3-O-glucoside were found in he skin of the all experimental variants. Flavonols' concentration in the skin was significantly higher in variant I with defoliation at flowering in regard to control ($p < 0.05$), which agreed with Downey et al. [15], who reported up to 10 times higher phenols content in the same vine cultivar if bunches were exposed to sunshine compared with shaded parts of the vine. The total amount of compounds from the flavonols group is increased by early defoliation according to Diago et al. [47], which was confirmed by this study.

The effect of the treatment was significant (*F*-test: $p < 0.05$) for quercetin3-O-galactoside and rutin. Content of quercetin3-O-galactoside was highest in variants I and III (73.92 and 68.36 mg/kg FW, respectively), which was significantly higher than in the control (32.93 mg/kg FW), while it did not differ significantly from variant II (56.39 mg/kg FW). The rutin content of variant I (13.89 mg/kg FW) was significantly larger than in the other three variants ($p < 0.05$). Myricetin was not found in any trial variant, which opposed the statement that it is a characteristic compound of red grape varieties [48]. Pantelić et al. [36] also reported myricetin in the Prokupac variety, but it could not be confirmed by this research.

Regarding flavonols and stilbenes, previous reports indicated that leaf removal is capable of positively affecting their content in grapes and wines [11,49], and this study confirmed that for flavonols. Flavan-3-ols were identified in the grape skin of all trial variants, excluding epigallocatechin gallate in variant III. Defoliation did not affect the concentration of hydroxybenzoic and hydroxycinnamic acids, in contrast to the results for the Tempranillo variety reported by Moreno et al. [12]. Among four quantified hydroxybenzoic acids, only ellagic acid was detected in every analyzed sample. We observed higher values of ellagic acid, especially in the control (2.66 mg/kg FW), than values for Prokupac (0.73 mg/kg FW), as stated by Pantelić et al. [36]. Gallic acid was detected only in the control and variant III, protocatechuic acid only in the control and variant II, while gentisic acid was identified only in the control and variant I. The effect of defoliation on the accumulation of hydroxycinnamic acids was reported by Diago et al. [50], but we could not confirm it. Mattivi et al. [51] found that resveratrol decreases due to defoliation, which we confirmed, finding resveratrol only in the skin of the control variant (1.84 mg/kg FW). There was a much higher amount of resveratrol in the grape skin of Prokupac (13.42 mg/kg FW) reported by Pantelić et al. [36] than found in this research. Sabbatini [52] found an influence of defoliation on the increase of skin phenols, which was partially confirmed by this study.

Table 5. The average content of phenolic compounds in grape skin (mg/kg FW).

Phenolic Compounds [§]	Experiment Variants				F [1]
	Control	Var. I	Var. II	Var. III	
Hydroxybenzoic acids					
gallic acid	0.15	– [2]	–	0.10	ns
protocatechuic acid	0.26	–	0.03	–	ns
p-hydroxyphenylacetic acid	–	–	–	–	–
gentisic acid	0.21	0.19	–	–	ns
ellagic acid	2.66	1.37	1.45	1.03	ns
vanillic acid	–	–	–	–	–
Hydroxycinnamic acids					
caffeic acid	0.28	0.19	0.07	0.12	ns
Coumarins					
aesculin	0.04 [a]	0.15 [b]	–	0.08 [a,b]	*
aesculetin	–	–	–	–	–
Flavan-3-ols					
catechin	12.78	14.12	15.38	11.38	ns
gallocatechin gallate	0.54	0.63	0.57	1.53	ns
epigallocatechin gallate	0.13	0.36	0.15	–	ns
Flavonols					
kaempferol	1.86	2.51	1.13	2.26	ns
kaempferol 3-*O*-glucoside	1.62	4.06	2.45	3.76	ns
quercetin 3-*O*-galactoside	32.93 [a]	73.92 [b]	56.39 [a,b]	68.36 [b]	*
rutin	3.86 [a]	13.89 [b]	5.44 [a]	5.83 [a]	*
galangin	–	–	–	–	–
Flavanones					
naringenin	0.08	0.17	–	0.08	ns
hesperetin	3.41	4.71	2.38	3.15	ns
pinocembrin	–	–	–	–	–
Flavones					
luteolin 7-*O*-glucoside	25.45	65.52	42.64	62.27	ns
chrysin	–	–	–	–	–
Dihydrochalcone derivatives					
phlorizin	0.68	0.17	0.12	0.24	ns
Stilbenes					
resveratrol	1.84	–	–	–	–
Stilbenoids					
oxyresveratrol	–	–	0.04	0.07	ns
polydatin	8.38	2.15	–	1.01	ns

[§] Results are expressed as the average for the three years of the study (2014–2016). [a,b] Values were 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 means $p > 0.05$; * means $0.01 < p \leq 0.05$; [2] – stands for "not detected". Control—no defoliation; var.I—early defoliation at the flowering stage when 50% of flowers were open; var.II—early defoliation at the stage when the egrape size was 3 o 5 mm; var.III—late defoliation at the onset of grape ripening veraison.

The observed values of total polyphenolic content (TPC) in seeds did not vary significantly between trial variants and years (Table 6). A similar tendency was observed for the radical scavenging activity (RSA).

Table 6. Total polyphenolic content and radical scavenging activity of grape seeds.

Year of the Study	Experiment Variants				F [1]
	Control	Var. I	Var. II	Var. III	
	Total polyphenolic content (g GAE/kg)				
2014	79.84 ± 0.36 [2]	64.41 ± 0.46	78.05 ± 2.01	75.92 ± 1.19	ns
2015	85.65 ± 1.15	85.53 ± 2.35	104.47 ± 0.09	65.32 ± 0.99	ns
2016	61.29 ± 1.38	46.99 ± 0.29	64.42 ± 0.29	57.53 ± 0.19	ns
AVG 2014–16	75.59 ± 0.96	65.64 ± 1.03	82.31 ± 0.80	66.26 ± 0.79	ns
	Radical scavenging activity (mmol TE/kg)				
2014	489.86 ± 15.48	427.08 ± 1.58	504.66 ± 4.69	522.35 ± 7.82	ns
2015	538.42 ± 0.22	524.46 ± 17.72	620.14 ± 13.93	437.31 ± 9.29	ns
2016	514.50 ± 11.72	482.54 ± 14.50	429.44 ± 9.52	474.40 ± 11.70	ns
AVG 2014–16	530.28 ± 8.97	495.53 ± 11.27	534.68 ± 9.38	493.71 ± 9.60	ns

[1] Significance based on the F test: ns means $p > 0.05$; [2] Values are presented as the average value ± standard deviation. Control—no defoliation; var.I—early defoliation at the flowering stage when 50% of flowers were open; var.II—early defoliation at the stage when the grape size was 3 to 5 mm; var.III—late defoliation at the onset of grape ripening veraison.

Values of TPC in the grape skin, given in Table 7, varied significantly between trial variants in all years of the study according to the F test ($p < 0.05$). The skin of variant I with early defoliation had significantly higher TPC (16.91 ± 0.09 g GAE/kg) in regard to the control and variant II ($p < 0.05$). The highest TPC was found in 2014 at variant I (21.03 ± 0.04 g GAE/kg), while the lowest one in 2016 at variant II (11.00 ± 0.03 g GAE/kg). Skin RSA was also the highest in variant I (100.51 ± 3.15 mmol TE/kg), and were not significantly higher than in variant II (82.07 ± 1.13 mmol TE/kg) and the control (78.86 ± 1.44 mmol TE/kg).

Table 7. Total polyphenolic content, total anthocyanins content, and radical scavenging activity of grape skin.

Year of the Study	Experiment Variants				F [1]
	Control	Var. I	Var. II	Var. III	
	Total polyphenolic content (g GAE/kg)				
2014	13.70 ± 0.01 ^{a} [2]	21.03 ± 0.04 ^{b}	15.24 ± 0.12 ^{a}	15.16 ± 0.19 ^{a}	*
2015	12.97 ± 0.13 ^{a}	16.77 ± 0.01 ^{b}	13.47 ± 0.29 ^{ab}	15.08 ± 0.24 ^{b}	*
2016	11.56 ± 0.21 ^{ab}	12.94 ± 0.23 ^{b}	11.00 ± 0.03 ^{a}	13.36 ± 0.10 ^{b}	*
AVG 2014–16	12.74 ± 0.12 ^{a}	16.91 ± 0.09 ^{b}	13.24 ± 0.14 ^{a}	14.53 ± 0.18 ^{ab}	*
	Total anthocyanins content (g mal 3-glu/kg)				
2014	3.46 ± 0.11	4.76 ± 0.08	3.93 ± 0.05	3.24 ± 0.15	ns
2015	4.96 ± 0.00	5.33 ± 0.13	4.96 ± 0.16	4.69 ± 0.21	ns
2016	3.80 ± 0.11	2.94 ± 0.03	3.10 ± 0.06	3.27 ± 0.12	ns
AVG 2014–16	4.07 ± 0.07	4.34 ± 0.08	4.00 ± 0.09	3.73 ± 0.16	ns
	Radical scavenging activity (mmol TE/kg)				
2014	75.09 ± 2.75	112.82 ± 1.01	84.25 ± 0.50	96.37 ± 0.76	ns
2015	79.07 ± 1.51	105.86 ± 2.07	82.60 ± 0.74	98.88 ± 2.02	ns
2016	82.43 ± 0.07	82.84 ± 6.38	79.36 ± 2.14	80.93 ± 2.14	ns
AVG 2014–16	78.86 ± 1.44	100.51 ± 3.15	82.07 ± 1.13	92.06 ± 1.61	ns

[a,b,c] Values were 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 means $p > 0.05$; * means $0.01 < p \leq 0.05$; [2] Values are presented as the average value ± standard deviation. Control—no defoliation; var.I—early defoliation at the flowering stage when 50% of flowers were open; var.II—early defoliation at the stage when the grape size was 3 to 5 mm; var.III—late defoliation at the onset of grape ripening veraison.

The F test showed no significant effect of defoliation on the skin's total anthocyanins content (Table 7). Defoliation in 2015 had no effect on TAC. The highest total anthocyanins content of the skin was found in 2015 in all trial variants. These results follow previously reported findings about

higher total anthocyanins content in warm and sunny years than in cold and rainy ones, according to Diago et al. [47]. Thus, in 2014 and 2016, there were large amounts of rainfall during summer months, with a consequent lower total anthocyanins content in all trial variants. Hunter et al. [53] found an increased anthocyanin concentration with late defoliation at veraison, which was not confirmed by this research, as well as the findings of Intrierri et al. [54], who found that all treatments had higher TAC than the control. Strong exposure to sun rays in the conditions of a warm climate can increase anthocyanins content, although it can have a negative effect on their quality if the air temperature is too high, according to Mori et al. [55]. In our investigation, high air temperature did not have any negative effect on the total anthocyanins content of grape skin; a bigger problem was the large amount of rainfall during summer months, especially in 2014 and 2016 (Table 1)

4. Conclusions

The highest assimilation area of primary and secondary shoots was observed in the control, i.e., variant with no defoliation. Based on the collected results for the Prokupac variety, significant differences between the trial variants were established regarding the content of phenols and total polyphenols, as well as radical scavenging activity. Defoliation had a significant effect on the content of many phenols in the seeds and skin: In variants with early defoliation (I and II), higher concentrations were identified. An exception was gallic acid, and its highest concentration was detected in the seeds and skin of the control variant. Grapes from all defoliated variants and the control had high values of ellagic acid content, which was said to be a characteristic feature of muscat varieties. Resveratrol was identified only in the grape skin samples of the control variant. On the other hand, myricetin, which was claimed to be a characteristic compound of red grape varieties, was not detected in any of the experiment variants. This might be attributed to the defoliation process. Defoliation significantly affected grape yield, so defoliated variants had lower grape yields compared to the control one. The weather conditions of the observed years, primarily the rainfall amount during summer months of 2014 and 2016, affected the total anthocyanins content of grape skin. The highest total anthocyanins content was found in 2015 in variant I with early defoliation. When grape phenolic composition and total anthocyanins content is considered, the best results for the whole three-year period were found in variants I and II when early defoliation was applied. On the other hand, those two variants showed the lowest grape yield per vine due to the effect of early defoliation on the grape cluster and berry structure.

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