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Influence of leaf removal and reflective mulch on phenolic composition and antioxidant activity of Merlot, Teran and Plavac mali wines (*Vitis vinifera* L.)

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ABSTRACT

Two years study was conducted to evaluate if leaf removal and red geotextile reflective mulch affect phenolic composition of wines from three red wine grape cultivars, Merlot, Teran and Plavac mali (*Vitis vinifera* L.). Leaf removal (LR) and reflective mulch (RM), made from weave of aluminum platelets protected by a transparent film and sewn together with red polypropylene threads, were tested separately and combined (LR + RM) on vines of Merlot, Teran and Plavac mali in 2009 and 2010 years, and compared with untreated control. All treatments were applied at veraison. LR had the strongest influence on phenolic composition of experimental wines, particularly in terms of increasing gallic acid, catechin, malvidin-3-glucoside, delphinidin-3-glucoside and peonidin-3-glucoside content. LR + RM resulted in higher gallic acid and malvidin-3-glucoside concentrations. RM treatment had the least effect on phenolic composition of wines but still affected higher epicatechin and gallic acid content. The total phenols, anthocyanins and flavan-3-ols contents in wines exhibited strong correlation with antioxidant activity.

1. Introduction

Phenolic compounds, which are present in grape berries and wines, play very important role in wine quality and sensory properties. They strongly contribute to the color and mouthfeel of red wines (Kennedy, 2008; Ribéreau-Gayon et al., 2000) and help protect against chronic diseases such as arteriosclerosis and cancer, and degenerative diseases involving oxidative damage due to their antioxidant action (Cimino et al., 2007; Fernández-Pachón et al., 2004; Minussi et al., 2003).

Grape and wine phenolics are classified into two groups: the flavonoids and nonflavonoids. The flavonoids include many different classes of phenols, but flavan-3-ols, flavonols and anthocyanins usually account for 80–90% of the phenolic contents in conventional red wines (Jeong et al., 2008) while nonflavonoids include hidroxybenzoic acids, hydroxycinnamic acids and stilbenes (Paixão et al., 2007; Ribéreau-Gayon et al., 2000). Grape berries nonflavonoids are found mainly in the pulp (with the exception of stilbenes), while flavonoids are located in the skins, seeds and stems (Paixão et al., 2007). The phenolic composition of wines is conditioned by different factors, such as cultivar, ecological conditions, agrotechnical and canopy management in vineyard and vinification techniques (Adams, 2006; Downey et al., 2006; Jackson and Lombard, 1993).

Investigations into the effects of light on flavonoid biosynthesis in grapes have taken a range of approaches, including defoliation in different stages of vine development (Hunter et al., 1995; Poni et al., 2006; Tardaguila et al., 2010). Increased cluster zone temperature through direct heating by incident radiation will increase the rate of metabolic processes in the grapes. This leads to increase in development and metabolite accumulation (Dokoozlian and Kliewer, 1996; Ebadi et al., 1995; Jones, 1992).

The positive aspects of leaf removal through increased fruit exposure to sunlight are well known. Leaf removal can increase soluble solids and reduce total acidity, as well as increase total phenols content in grapes (Smart et al., 1990). Besides other canopy management techniques, leaf removal is a common practice, especially in cooler regions, used to improve canopy microclimate and fruit ripening. Increased sunlight exposure causes the elevation of berry temperature, and both factors are closely related with fruit composition and quality. It is well known that at temperatures above 30 °C many metabolic processes stop or are significantly reduced (Jones,

Abbreviations: LR, leaf removal; RM, reflective mulch; LR + RM, combination of leaf removal and reflective mulch.

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Table 1
Growing season average temperature and precipitation of observed vintages 2009 and 2010.

	Average T (°C)		Precipitation (mm)	
Experimental site	2009	2010	2009	2010
Zagreb	18.3	16.8	432.9	688.4
Istrian peninsula	19.6	18.5	331.1	686.1
Pelješac peninsula	19.7	18.8	989.7	929.8

1992). Generally, the consensus developed is that increasing light increased the flavonoid content of grapes (Dokoozlian and Kliewer, 1996). The increase in final grape composition is not just the effect of sun exposure, but also changes in leaf-to-yield ratio, remained leaves assimilation rates, changes in source-sink balance and finally changes in berry size and hence skin-to-pulp ratio (Poni et al., 2006). On the other hand, several works reported lowered anthocyanins content in grape berries exposed to direct sunlight (Crippen and Morrison, 1986; Spayd et al., 2002), as a consequence of increased berry temperature which inhibited anthocyanins synthesis or even enhanced their degradation. Furthermore, anthocyanins concentration has been reported to be higher in the berries positioned in the shadow, when compared to the exposed ones in the work of Bergqvist et al. (2001) and Spayd et al. (2002).

Therefore, there are also many works who reached a general consensus that excessive light and thermal exposure of bunches should be avoided, especially in warm climates (Downey et al., 2006; Price et al., 1995). Prolonged exposure of bunches to high light and temperature leads to aroma degradation and reduced titratable acidity through excessive malic acid degradation (Marais et al., 1999). Summer heat stress may cause berry sunburn (Chorti et al., 2010) and enhance photoinhibition of basal leaves (Pallioti et al., 2013). Gatti et al. (2015) stated that early leaf removal should be preferred under specific environmental conditions leading to a high risk of bunch rot and/or sunburn disorders.

Reflective mulches might also increase sunlight in the fruit zone, and consequently improve berry ripening and disease suppression in cooler climate (Hostetler et al., 2007a, 2007b). There are only few published works lately reporting about effects of reflective mulches or geotextiles on fruit composition and yield (Hostetler et al., 2007a, 2007b; Osrečak et al., 2015; Sandler et al., 2009; Vanden Heuvel and Neto, 2006). Reflective mulch studies demonstrated various effects on yield, fruit composition and wine quality.

The aim of this research was to evaluate if leaf removal and red geotextile reflective mulch could affect phenolic composition of wines from three red cultivars, Merlot, Teran and Plavac mali (Teran is cultivar typical for Istrian peninsula, while Plavac mali is grown at the south coastal part of Croatia) and to provide some useful information for adjusting vineyard practices and thus optimizing phenolic quality of red wines.

2. Materials and methods

2.1. Experimental site and plant material

Research was performed in two consecutive years (2009 and 2010) on cultivars Merlot, Teran, and Plavac mali. Considering biological diversity of investigated cultivars, this experiment was conducted on three different locations. Merlot is grown in continental region of Croatia (University of Zagreb, Faculty of Agriculture, lat. 45°51′ N, long. 16°0′ E), Teran on Istrian peninsula (lat. 45°37′ N, long. 13°65′ E) and Plavac mali on Pelješac peninsula (lat. 42°96′ N, long. 17°36′ E) (Table 1).

All three cultivars were Royat cordon trained, leaving 12 buds per vine. Fruit-bearing wire was set to 40 cm above ground, with addition of two sets of catch wires at 40 cm intervals from the fruit-bearing wire. Maximum canopy height was 140 cm. Vine spacing was 0,8 m between vines x 2 m between rows (6250 vines/ha).

Merlot and Teran were grafted on SO4 (*Vitis berlandieri* Planch. x *Vitis riparia* Michx.) rootstock, while Plavac mali on P1103 (*Vitis berlandieri* Planch. x *Vitis rupestris* Scheele) rootstock. Because of the above, experiments were analyzed and results presented separately for each cultivar.

Veraison was estimated based on the color beginning of the berries, which represent stage 35 according to modified Eichorn and Lorenz system (Coombe, 1995). Before treatments, vines were shoot-thinned and cluster-thinned in aim to equalize vegetative and generative potential.

Shoot thinning to 10 shoots/vine (12 shoots per meter of cordon length) was performed at stage 12 according to modified Eichorn and Lorenz system (Coombe, 1995). Cluster thinning to 15 clusters/vine (18 clusters per meter of cordon length) was performed at stage 27 according to modified Eichorn and Lorenz system (Coombe, 1995). Grapes were harvested manually at full ripeness, when the total soluble solids of 100 randomized collected berries remained constant for a few days. Each treatment and replicate was harvested separately.

2.2. Experimental design

The experiment was completely randomized block design, with four treatments in three replications for each cultivar. Each replication consisted of 3 continuous vines, with a two untreated vines as a guard between them, so there were 9 grapevines in each treatment. Experiment had a total of 36 units (plots). The treatments were as follows:

- Leaf removal (LR) – removal of 5 basal leaves from each shoot at veraison, so there were 8–10 remaining leaves per each shoot
- Reflective mulch (RM) – made from a special weave of thin aluminum platelets protected by a transparent film and sewn together with red polypropylene threads, so it can provide multidirectional light reflection, placed on the soil, directly beneath the vine rows, at veraison. Reflective mulch was 0,5 m width and placed from both sides of the vine row, so the total width was 1 m.
- Leaf removal and reflective mulch (LR + RM) – reflective mulch together with defoliation
- Control (C) – without any treatments

2.3. Microvinification

Clusters were separately destemmed and crushed for each experimental plot, and putted into 15-L stainless steel tanks. Finally, 12 microvinifications (4 treatments x 3 replicate) per cultivar was conducted. Must samples were collected immediately after pressing, for sugar and titratable acidity analysis. Each lot was sulfited with 100 mL 5% sulfurous acid/100 L. All 36 fermentations were carried out with *Saccharomyces cerevisiae* Lallemand 254 to completion. Simultaneously with fermentation, the grapes were macerated for 8 days with temperature of 25–28 °C, by adjusting the room temperature. The fermentation cap was kept submerged by the stainless steel screen. The pomace was manually punched down and mixed daily. At the end of fermentation, the wine was racked, and samples were frozen for further analysis. Sugar content in musts was determined by refractometer (expressed in °Brix) and titratable acidity of the must (g/L) was estimated using the coloration pattern volumetric method according to the O.I.V. method (OIV, 2001).

Table 2
Reflective mulch (RM) and leaf removal (LR) effect on grapes soluble solids, grapes titratable acidity, and wine total phenols, total anthocyanins and flavan-3-ols of three cultivars.

	Grapes		Wine		
	Soluble solids (Brix)	Titratable acidity (g/L)	Total phenols (mg/L)	Total anthocyanins (mg/L)	Flavan-3-ols (mg/L)
Merlot					
Treatment	2009				
Control	24.76	5.03	1830.00	393.39	110.03
LR	24.85	4.47	1949.22	396.16	134.32
RM	25.63	4.80	1678.86	363.31	82.03
LR + RM	24.20	4.53	1774.40	341.55	91.36
Signif	ns	ns	ns	ns	ns
	2010				
Control	24.26	5.40	1337.90	188.37	68.18
LR	24.14	5.20	1558.97	225.49	74.68
RM	24.14	5.27	1537.70	222.08	68.45
LR + RM	23.63	5.10	1608.29	187.73	67.67
Signif	ns	ns	ns	ns	ns
Year					
2009	24.86 a	4.71 b	1808.12 a	373.60 a	104.43 a
2010	24.04 b	5.24 a	1510.72 b	205.92 b	69.75 b
Signif	*	*	*	*	*
Treatment*Year	ns	ns	ns	ns	ns
Teran					
Treatment	2009				
Control	19.67	9.57	856.44 b	182.61	43.49
LR	21.76	8.30	1260.00 a	306.04	93.24
RM	19.92	8.87	924.51 b	244.48	63.45
LR + RM	21.28	8.50	1200.40 a	210.99	72.64
Signif	ns	ns	*	ns	ns
	2010				
Control	16.44	12.43	766.43 b	158.72 b	51.51
LR	17.85	11.00	1152.87 a	253.01 a	58.65
RM	18.00	11.80	1206.87 a	256.43 a	62.43
LR + RM	17.46	11.33	1216.77 a	242.13 a	61.22
Signif	ns	ns	*	*	ns
Year					
2009	20.64 a	8.81 b	1060.33	236.03	68.21
2010	17.43 b	11.89 a	1085.73	227.57	58.45
Signif	*	*	ns	ns	ns
Treatment*year	ns	ns	*	ns	ns
Plavac mali					
Treatment	2009				
Control	24.72	4.63	1982.72	267.73	168.30
LR	24.60	4.23	2120.28	314.45	218.25
RM	24.46	4.00	1960.10	300.37	163.25
LR + RM	24.16	4.07	2099.44	267.52	187.33
Signif	ns	ns	ns	ns	ns
	2010				
Control	20.27 b	4.43 a	2237.30	239.57	159.37
LR	22.00 a	3.47 b	2507.55	253.01	183.57
RM	22.86 a	3.50 b	2571.40	256.43	211.82
LR + RM	22.00 a	4.04 b	2449.53	242.13	152.74
Signif	*	*	ns	ns	ns
Year					
2009	24.48 a	4.23 a	2040.58 b	287.52 a	184.28
2010	21.78 b	3.95 b	2441.45 a	247.78 b	176.87
Signif	*	*	*	*	ns
Treatment*year	*	ns	ns	ns	ns

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

2.4. Spectrophotometric measurements

Total phenolic content was done with Folin-Ciocalteu method (Singleton and Rossi, 1965). Results were expressed as mg gallic acid equivalents per liter of wine (mg GAE/L).

Total flavan-3-ol content was determined by reaction of flavonoids with vanillin reagent in the acid medium (Ough and Amerine, 1988). Results were expressed as mg (+)-catechin equivalents per liter of wine (mg CAT/L).

Total anthocyanin content was determined by sodium hydrogen sulfite blanching method (Ribéreau-Gayon and Stonestreet, 1965). Results were expressed as mg malvidin-3-*O*-glucoside equivalents per liter of wine (mg MAE/L).

All spectrophotometric measurements were performed on Specord 40 UV–vis spectrophotometer (Analytik Jena, Germany).

2.5. HPLC analysis

Levels of individual phenolic compounds were measured by a HPLC system (Agilent 1100 Series, Palo Alto, USA), according to method developed by Tomaz and Maslov (2016). These compounds were separated on Luna C18 column (Phenomenex, SAD) (250 × 4,6 mm i.d.) with gradient elution. The gradient consisted

of two eluents: (A) water/phosphoric acid (99.5/0.5; v/v) and (B) acetonitrile/water/phosphoric acid (50/49.5/0.5; v/v/v). Flow rate was 1 mL/min. The gradient conditions were as follows: 100% A for 2 min, from 2 min to 7 min 20% B, from 7 min to 25 min 40% B, followed by hold to 31 min, from 32 min to 40 min 100% B. Equilibrium time to original conditions was 10 min. Compounds were detected by DAD and FLD detectors. Peaks were identified by comparisons UV–vis spectra of each peak with the corresponding spectra of standard compounds and by comparisons of their retention times. Quantification was based on peak areas using external standards. Linear calibration curves for standards (peak area vs. concentration) were constructed. Wine samples were defrost, filtered by PTFE membrane filters (45 μm) and directly injected to HPLC system. Injected volume was 20 μL.

2.6. Antioxidant activity

For the determination of the reducing power of wines, a protocol based on the ferric reducing/antioxidant power (FRAP) assay was used. The FRAP reagent consists of acetate buffer (300 mM, pH 3.6), TPTZ (10 Mm in HCl 40 Mm) and $FeCl_3 \cdot 6H_2O$ (20 mM) (10:1:1, v/v/v). A total of 3 mL of FRAP reagent was mixed with 300 μl Mili-Q water and 100 μl of sample. Absorbance was measured after 8 min at 593 nm. An aqueous solution of $FeSO_4 \cdot 7H_2O$ in the 0–1 mM range was used for calibration. Results are expressed as mmol of Fe^{+2}/g of fraction or extract (Benzie and Strain, 1996).

2.7. Statistical analysis

All analyses were performed separately by cultivar using 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. Correlations between variables were estimated by Pearson's correlation coefficients. Data were analyzed using SAS statistical software, version 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Soluble solids and titratable acidity

Grape soluble solids and titratable acidity were unaffected by applied treatments for Merlot and Teran (Table 2). Only Plavac mali grapes in 2010 exhibit significant differences between treatments, where the lowest level of soluble solids and the highest level of titratable acidity was measured in control grapes. There was also a significant interaction between year and treatments on Plavac mali grape soluble solids, due to the fact that in 2009, when soluble solids and temperature were higher, no effects were observed, while they were observed in cooler 2010, with low soluble solids content. The year effect on soluble solids and titratable acidity content was significant across all cultivars.

3.2. Total phenols, anthocyanins and flavan-3-ols

Only Teran wines exhibit significant differences between treatments. Teran control wines had the lowest total phenol content, while LR and LR + RM affected higher total phenols in both years. All applied treatments increased Teran total anthocyanins content, but only in 2010 year. Flavan-3-ols content in all experimental wines were unaffected by treatments applied.

There was an effect of experimental year on Merlot and Plavac mali total phenols and anthocyanins content, as well as on Merlot flavan-3-ols content (Table 2). There was no year effect on total phenols, anthocyanins and flavan-3-ols of Teran wines. The interaction between year and treatments was observed only for Teran total phenols content.

3.3. Wine phenolic composition

3.3.1. Merlot

Control wines of Merlot had the lowest content of caffeic acid, gallic acid and malvidin-3-glucoside in both years ( ). LR + RM treatment increased the content of gallic acid and malvidin-3-glucoside in Merlot wines in both years. There was no effect of applied treatments for *p*-coumaric acid, *trans*-resveratrol, petunidin-3-glucoside and peonidin-3-glucoside. Other individual phenolic compounds varied among treatments and years. There was an effect of year on all individual phenolic compounds except quercetin, epicatechin and cyanidin-3-glucoside. The concentration of all individual phenolic compounds except gallic acid, isorhamnetin and epicatechin increased in 2010 over 2009. There was no interaction between year and treatment for *p*-coumaric acid, *trans*-resveratrol, isorhamnetin and petunidin-3-glucoside.

3.3.2. Teran

Teran control wines had the lowest content of isorhamnetin, epicatechin, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside (Tables 5 and 6). RM treatment increased *p*-coumaric acid content, while LR treatment increased isorhamnetin content in wines, either in both years. There was no effect of applied treatments on *trans*-resveratrol and quercetin. Other phenolic compounds concentration was inconsistent across treatments and years. There was an effect of experimental year on all phenolic compounds except gallic acid, catechin, cyanidin-3-glucoside and peonidin-3-glucoside. There was no interaction between year and treatment only for quercetin content in wine.

3.3.3. Plavac mali

Plavac mali control wines had the highest caftaric acid content in both years but also the lowest gallic acid content (Tables 7 and 8). LR treatment increased caffeic acid content and LR + RM treatment gallic acid content in wines in both years. There was no treatments effect neither interaction between year and treatment for *trans*-resveratrol, quercetin, myricetin, isorhamnetin and epicatechin-gallate. There was no effect of experimental year on gallic acid, cyanidin-3-glucoside and malvidin-3-glucoside. The concentration of all individual phenolic compounds except isorhamnetin and cyanidin-3-glucoside increased in 2010 over 2009.

3.4. Antioxidant activity

Only Teran wines exhibit significant differences between treatments, where control wines had the lowest and LR wines the highest antioxidant activity (Table 9). There was an effect of experimental year on Merlot and Plavac mali, but not on the Teran antioxidant activity. On the other hand, interaction between year and treatment was observed only for Teran antioxidant activity.

3.5. Correlations

Correlation analysis was used to explore the relationships among the different antioxidant variables measured for all the wine samples (Table 10). The total phenols, total anthocyanins and flavan-3-ols contents of wine samples exhibited strong correlations with antioxidant properties. Among the individual

Table 3
Reflective mulch (RM) and leaf removal (LR) effect on Merlot wine phenolic composition (mg/L).

	Caft	Caff	Coum	Gall	t-resv	Querc	Myric	Isorh	Catec	Epicat	Epi-g
Treatment	2009										
Control	51.0c	3.8b	5.2	42.5c	1.0	1.6	0.6b	tr	33.2b	16.4b	12.3b
LR	71.3a	5.8a	5.7	57.8b	1.8	1.9	0.9a	tr	68.8a	23.5a	16.3a
RM	63.0ab	6.4a	6.1	58.7b	1.2	2.1	0.9a	tr	68.6a	23.6a	16.9a
LR + RM	62.2b	6.6a	6.6	69.4a	1.2	2.2	0.8a	tr	73.9a	27.8a	19.6a
Signif	*	*	ns	*	ns	ns	*	–	*	*	*
	2010										
Control	24.4	3.5b	4.6	60.1d	0.4	1.0b	0.1	0.1	28.4	20.7b	13.6b
LR	23.4	6.2a	4.1	84.0b	0.5	1.7ab	0.1	0.1	23.5	19.2b	17.8a
RM	30.5	5.2ab	4.8	74.2c	0.5	2.1a	0.1	0.1	26.1	36.6a	7.9c
LR + RM	29.6	4.5b	3.6	93.2a	0.5	2.0a	0.1	0.1	25.4	15.3b	15.4ab
Signif	ns	*	ns	*	ns	*	ns	ns	ns	*	*
Year											
2009	61.9a	5.6a	5.9a	57.1b	1.3a	1.9	0.8a	tr b	61.1a	22.9	16.3a
2010	27.0b	4.9b	4.3b	77.9a	0.5b	1.7	0.1b	0.1a	25.9b	23.0	13.7b
Signif	*	*	*	*	*	ns	*	*	*	ns	*
T*Y	*	*	ns	*	ns	*	*	ns	*	*	

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).
Abbreviations: Caft: caftaric acid; Caff: caffeic acid; Coum: *p*-coumaric acid; Gall: gallic acid; t-resv: *trans*-resveratrol; Querc: quercetin; Myric: myricetin; Isorh: isorhamnetin; Catec: catechin; Epicat: epicatechin; Epi-g: epicatechin-gallate.

Table 4
Reflective mulch (RM) and leaf removal (LR) effect on Merlot wine anthocyanin content (mg/L).

	Dp-3-g	Cy-3-g	Pt-3-g	Pn-3-g	Mv-3-g
Treatment	2009				
Control	3.0	0.2	12.3	1.6	155.7c
LR	3.4	0.2	13.8	1.8	174.6b
RM	3.2	0.2	13.5	2.0	181.6ab
LR + RM	4.3	0.3	12.6	2.1	193.3a
Signif	ns	ns	ns	ns	*
	2010				
Control	1.9b	0.1c	8.6	0.8	61.3c
LR	3.5a	0.1c	9.7	1.0	79.3ab
RM	2.9ab	0.2b	7.9	0.8	70.4bc
LR + RM	2.0b	0.3a	7.7	1.1	93.3a
Signif	*	*	ns	ns	*
Year					
2009	3.4a	0.2	13.1a	1.9a	176.3a
2010	2.6b	0.2	8.5b	0.9b	76.1b
Signif	*	ns	*	*	*
T*Y	*	*	ns	*	*

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).
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

Table 5
Reflective mulch (RM) and leaf removal (LR) effect on Teran wine phenolic composition (mg/L).

	Caft	Caff	Coum	Gall	t-resv	Querc	Myric	Isorh	Catec	Epicat	Epi-g
Treatment	2009										
Control	39.0b	6.1a	4.6b	51.5b	0.3	0.2	0.6b	0.2b	23.6b	28.1c	22.2a
LR	51.9a	5.3a	1.0c	56.8b	0.5	1.1	1.1a	0.7a	30.2a	28.9bc	12.1b
RM	33.8b	6.1a	7.1a	53.0b	0.4	0.3	0.5b	0.2b	21.7b	32.4ab	20.7a
LR + RM	60.4a	0.54b	1.9c	81.5a	0.4	0.7	1.2a	0.7a	26.4ab	32.6a	26.9a
Signif	*	*	*	*	ns	ns	*	*	*	*	*
	2010										
Control	31.2b	6.2a	3.1b	49.7b	0.4	1.8	tr	0.2c	22.0	6.2b	11.7b
LR	76.0a	1.8b	7.8a	64.0a	0.5	1.8	tr	0.5a	25.5	13.3a	18.8a
RM	78.9a	2.3b	8.6a	65.3a	0.4	2.3	tr	0.3bc	27.2	13.8a	21.2a
LR + RM	76.9a	2.4b	8.3a	64.1a	0.5	1.9	tr	0.3b	25.9	8.8b	19.3a
Signif	*	*	*	*	ns	ns	ns	*	ns	*	*
Year											
2009	46.3b	4.5a	3.6b	60.7	0.4b	0.6b	0.8a	0.4a	25.5	30.5a	20.5a
2010	65.8a	3.2b	6.9a	60.8	0.5a	2.0a	tr b	0.3b	25.1	10.6b	17.7b
Signif	*	*	*	ns	*	*	*	*	ns	*	*
T*Y	*	*	*	*	*	ns	*	*	*	*	*

* and ns indicate significant at p = 0.05 and not significant. respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).
Abbreviations: Caft: caftaric acid; Caff: caffeic acid; Coum: *p*-coumaric acid; Gall: gallic acid; t-resv: *trans*-resveratrol; Querc: quercetin; Myric: myricetin; Isorh: isorhamnetin; Catec: catechin; Epicat: epicatechin; Epi-g: epicatechin-gallate.

Table 6
Reflective mulch (RM) and leaf removal (LR) effect on Teran wine anthocyanin content (mg/L).

	Dp-3-g	Cy-3-g	Pt-3-g	Pn-3-g	Mv-3-g
Treatment	2009				
Control	1.0b	1.6	2.2c	1.2c	79.5b
LR	3.7a	1.4	11.6a	5.6a	136.0a
RM	0.6b	1.0	2.3c	1.2c	100.7b
LR + RM	4.2a	1.1	7.4b	4.4b	148.1a
Signif	*	ns	*	*	*
	2010				
Control	2.6b	0.3c	5.5b	1.0b	73.9b
LR	5.0a	1.8a	11.6a	3.8a	113.6a
RM	3.9ab	1.3ab	11.9a	3.1a	108.8a
LR + RM	3.0b	0.9bc	9.9a	3.0a	96.2ab
Signif	*	*	*	*	*
Year					
2009	2.4b	1.3	5.9b	3.1	116.1a
2010	3.6a	1.1	9.7a	2.7	98.1b
Signif	*	ns	*	ns	*
T*Y	*	*	*	*	*

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).
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.

Table 8
Reflective mulch (RM) and leaf removal (LR) effect on Plavac mali wine anthocyanin content (mg/L).

	Dp-3-g	Cy-3-g	Pt-3-g	Pn-3-g	Mv-3-g
Treatment	2009				
Control	5.3ab	0.7b	2.56ab	1.2b	121.1bc
LR	6.1a	1.0a	2.89a	2.0a	176.9a
RM	4.4b	0.7b	3.0a	0.9c	136.3b
LR + RM	4.7ab	0.6b	2.2b	0.9c	97.3c
Signif	*	*	*	*	*
	2010				
Control	5.4b	0.6b	2.9	2.4	120.1b
LR	5.9ab	0.7ab	3.1	2.5	126.0ab
RM	5.3b	0.7ab	2.9	2.1	154.7a
LR + RM	7.2a	0.8a	3.2	2.4	134.3ab
Signif	*	*	ns	ns	*
Year					
2009	5.1b	0.7	2.7b	1.3	132.9
2010	6.0a	0.7	3.0a	2.4	133.8
Signif	*	ns	*	*	ns
T*Y	*	*	*	*	*

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).
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

phenolic compounds, catechin (r = 0.582) had the strongest positive correlation ($p < 0.05$) with antioxidant activity of tested wines (Table 11). A significant correlation ($p < 0.05$) of antioxidant properties and *trans*-resveratrol, epicatechin, delphinidin-3-glucoside and malvidin-3-glucoside were also observed.

4. Discussion

4.1. Soluble solids and titratable acidity

The applied treatments had either no effect on grape soluble solids or, as was the case with Plavac mali in 2010, enhanced soluble solids. Similar results reported Guidoni et al. (2008), Kozina et al. (2008) and Zoecklein et al. (1992). As we can see, stronger influence of treatments was found in 2010, which was cooler and more humid than 2009. This is not surprising knowing that in cool climate vineyards, a small increase in heat unit accumulation may be enough to advance the onset of veraison slightly (Bledsoe et al., 1988). However, our findings are in agreement with those reported by Crippen and Morrison (1986) who stated that among fruit components measured, percent soluble solids was perhaps the least affected by either exposing/shading clusters from sunlight or artificially altering clustering temperature.

All treatments reduced grape titratable acidity for Plavac mali in 2010. These results are consistent with the previous works (Bledsoe et al., 1988; Guidoni et al., 2008; Kozina et al., 2006, 2008; Spring, 2004; Zoecklein et al., 1992) and probably reflect enhanced ripening and/or an increase in malic acid degradation with the presumed increases in daytime berry temperatures (Bergqvist et al., 2001; Reynolds et al., 1986). In the case of Merlot and Teran differences were not significant. The lack of effect was observed in warmer and drier 2009 year. The same effect was observed by Tardaguila et al. (2010), who highlighted this fact as a positive outcome due to advanced ripening induced by global warming.

Table 7
Reflective mulch (RM) and leaf removal (LR) effect on Plavac mali wine phenolic composition (mg/L).

	Caft	Caff	Coum	Gall	t-resv	Querc	Myric	Isorh	Catec	Epicat	Epi-g
Treatment	2009										
Control	40.4a	1.9d	4.4a	63.8b	0.4	1.0	0.8	0.4	40.3bc	52.3b	9.9
LR	37.1a	5.9a	3.2b	72.2b	0.6	1.4	1.4	0.5	49.5a	69.6a	12.3
RM	27.0b	5.3b	2.6b	64.2b	0.6	1.2	1.4	0.5	45.0ab	53.7b	10.8
LR + RM	27.1b	3.8c	2.4b	106.8a	0.5	1.3	0.9	0.5	34.5c	37.6c	11.5
Signif	*	*	*	*	ns	ns	ns	ns	*	*	ns
	2010										
Control	64.3a	5.2ab	4.8b	61.8c	0.8	2.2	2.3	tr	36.0c	68.7b	16.0
LR	32.0c	5.6a	6.6a	77.7b	1.1	2.6	2.6	tr	75.5b	74.2ab	16.9
RM	52.7b	4.8bc	5.2b	98.1a	1.0	2.3	2.3	tr	75.4b	80.3a	18.4
LR + RM	55.3b	4.3c	3.3b	84.9ab	1.0	2.3	2.3	tr	86.9a	70.7ab	16.8
Signif	*	*	*	*	ns	ns	ns	ns	*	*	ns
Year											
2009	32.9b	4.3b	3.1b	76.8	0.5b	1.2b	1.2b	0.5a	42.3b	53.3	11.1b
2010	51.1a	5.0a	5.2a	80.6	1.0a	2.4a	2.4a	tr b	68.5a	73.5	17.0a
Signif	*	*	*	ns	*	*	*	*	*	*	*
T*Y	*	*	*	*	ns	ns	ns	ns	*	*	ns

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).
Abbreviations: Caft: caftaric acid; Caff: caffeic acid; Coum: *p*-coumaric acid; Gall: gallic acid; t-resv: *trans*-resveratrol; Querc: quercetin; Myric: myricetin; Isorh: isorhamnetin; Catec: catechin; Epicat: epicatechin; Epi-g: epicatechin-gallate.

Table 9
Reflective mulch (RM) and leaf removal (LR) effect on antioxidant activity (mmol TE/L) of three cultivars.

	Merlot	Teran	Plavac mali
Treatment	2009		
Control	8.54	2.73 b	9.04
LR	9.39	4.89 a	10.60
RM	7.46	3.32 b	8.86
LR + RM	8.27	4.63 a	9.75
Signif	ns	*	ns
	2010		
Control	4.89	3.05 b	6.23
LR	4.99	4.55 a	6.62
RM	5.42	4.54 a	6.73
LR + RM	4.99	4.12 ab	6.11
Signif	ns	*	ns
Year			
2009	8.41a	3.89	9.56a
2010	5.07b	4.06	6.42b
Signif	*	ns	*
T*Y	ns	*	ns

* and ns indicate significant at p = 0.05 and not significant, respectively.
Means with different letter are significantly different within treatments and years (mean separation by Bonferroni correction at $p \leq 0.05$).

4.2. Total phenols, total anthocyanins and total flavan-3-ols

The only consistent positive effect of experimental treatments on total phenols, total anthocyanins and total flavan-3-ols was increased total phenols in Teran wines by LR and LR + RM treatments. Teran responded positively on all applied treatments by accumulation of total anthocyanins also, but only in cooler 2010. This is probably due to better light conditions in canopy microclimate, which is a decisive factor for biosynthesis of phenolic compounds (Morrison and Noble, 1990; Price et al., 1995). Similar results previously reported Di Profio et al. (2011b), Hunter et al. (1995), Mazz et al. (1999) who stated that partial defoliation mostly increased total phenols in red cultivars wines. This positive effect on total phenol concentrations suggest that increasing the light and temperature in the cluster zone through leaf removal may directly impact the rate of activity of phenylalanine ammonia lyase (PAL), a key enzyme in the biosynthesis of all phenolic compounds (Di Profio et al., 2011a; Morrison and Noble, 1990).

Merlot and Plavac mali wines total phenols and anthocyanins did not responded to applied treatments. This could be the consequence of genotype and variations in canopy porosity as suggested by Tardaguila et al. (2010). Similar to Di Profio et al. (2011b), greater responsiveness was displayed when individual phenolic compounds were examined.

RM treatment increased total phenol and total anthocyanins content only in Teran wines in 2010, opposed to other cultivars and experimental years, where total phenols and anthocyanins were unaffected by RM. These results indicate that mulch effects on fruit composition depend on soil and climatic conditions as well as on grape cultivar (Hostetler et al., 2007a). When RM was applied for a shorter period in late summer, as was done in this study, there were no effects on grape composition (Vanden Heuvel and Neto 2006). The impact of experimental treatments on flavan-3-ols content was absent. This is somehow expected because the flavan-3-ols biosynthesis in the skin of the grape berry occurs early in berry development and is completed around veraison (Downey et al., 2006; Kennedy, 2008), so treatments conducted at veraison could not affect them. Flavan-3-ols accumulation in the seed of the berry begins later than in the skin, with maximal concentrations a few weeks after veraison, but in the seed there was no observable effect of bunch exposure on either the flavan-3-ols content or composition (Downey et al., 2006).

Table 11
Pearson's correlation coefficients of antioxidant activity and individual phenolic compounds of three cultivars.

Phenolic compound	Antioxidant activity	Phenolic compound	Antioxidant activity
Caftaric acid	−0.047	Catechin	0.582*
Caffeic acid	0.086	Epicatechin	0.475*
p-Coumaric acid	−0.174	Epicat-gallate	0.030
Gallic acid	0.205	Delphinidin-3-glucoside	0.539*
trans-resveratrol	0.506*	Cyanidin-3-glucoside	−0.295
Quercetin	0.127	Petunidin-3-glucoside	−0.040
Myricetin	0.212	Peonidin-3-glucoside	−0.176
Isorhamnetin	0.109	Malvidin-3-glucoside	0.526*

* Indicate that correlation is significant at p = 0.05.

4.3. Individual phenolic compounds

Experimental treatments did not show consistent effect on hydroxycinnamic acids content in wines. The biosynthesis of hydroxycinnamates in grapes reach its peak before veraison (Adams, 2006) and it seems that hydroxycinnamates content respond positively only to early performed leaf removal (Lemut et al., 2011). On the other hand, gallic acid, as the only representative of hydroxybenzoic acids, was under great influence of LR + RM treatment, which increased gallic acid level in all cultivars and years, in regards to control. These results are in accordance with Osrečak et al. (2015) who stated positive effects of partial defoliation and reflective mulch on hydroxybenzoic acids content in white wines.

The concentrations of *trans*-resveratrol were unaffected by performed treatments. Higher *trans*-resveratrol content could be expected within LR treatments, because of UV light exposure. Results from this work are in some extent similar to Bavaresco et al. (2008) who found out that leaf removal had no effect on stilbene grape concentration under warmer and drier climatic conditions.

It seems that applied treatments did not have consistent effect on monomeric flavonols concentrations. According to Downey et al. (2006), flavonols are synthesized earlier than veraison, so treatments applied at veraison could not greatly affect on their composition.

The impact of experimental treatments on individual flavan-3-ols content was also very inconsistent, and varied among cultivars, treatments and experimental years. The only exception was epicatechin, which was increased by RM treatment when compared to the control. Opposite to this, Price et al. (1995) reported highest epicatechin levels in wines from shaded clusters and lowest in wines from exposed clusters. One of reason for those contradictory results could be, as before mentioned in chapter 4.2., time of biosynthe-

Table 10
Pearson's correlation coefficients of antioxidant activity, total phenols, total anthocyanins and flavan-3-ols of three cultivars.

	Total phenols	Total anthocyanins	Total flavan-3-ols	Antioxidant activity
Total phenols	1	0.452 *	0.885 *	0.718 *
Total anthocyanins		1	0.523 *	0.739 *
Total flavan-3-ols			1	0.817 *
Antioxidant activity				1

* and ns indicate that correlation is significant at p = 0.05 and not significant, respectively.

sis of flavan-3-ols. Furthermore, this study does not deal with the source of flavan-3-ols, so there is possibility that some of detected compounds originated from grape seeds, whose biosynthesis could not be affected by light interception. Beside that, mainly polymerized flavan-3-ols are found in wines from exposed grapes (Price et al., 1995), so polymerization could also be the reason for low concentrations of momomeric flavan-3-ols in some of the experimental wines. Finally, there is possibility of changes in monomer flavan-3-ols concentrations that occurred during maceration and fermentation, particularly polymerization with anthocyanins.

LR treatment had positive influence on malvidin-3-glucoside biosynthesis. It also enhanced delphinidin, petunidin and peonidin-3-glucoside in Teran wines, and delphinidin, cyanidin and peonidin-3-glucoside in Plavac mali wines 2009. Leaf removal enhanced individual anthocyanin concentrations in Pinot noir wines (Lemut et al., 2011) and delphinidin, petunidin, peonidin and cyanidin-3-glucosides (but not malvidin-3-glucoside) in Merlot wines (King et al., 2012). In other works changes in individual anthocyanin concentrations affected by leaf removal treatment were inconsistent (Di Profio et al., 2011b; Guidoni et al., 2008). Other treatments generally had weak or inconsistent impact on individual anthocyanins content in experimental wines. This could be due to fact that fully exposed grapes additionally heated by reflective mulch are much warmer than the ambient air temperature and although solar radiation exposure increased anthocyanin concentration, excessive berry temperature could be a limiting factor for their synthesis and accumulation (Tarara et al., 2008).

Teran individual anthocyanins content (as well as total anthocyanins content) had the strongest response to performed treatments, while Merlot response was the weakest. Teran clusters in our study were very loose when compared to other experimental cultivars. Smart and Sinclair (1976) found that the temperature of tight clusters increased above ambient more than loose clusters. So, although not measured in this work, Merlot and Plavac mali grape berry temperature, due to tight clusters, could reach a level that would inhibit anthocyanin synthesis.

Finally, Cortell and Kennedy (2006) found that extractability of anthocyanins, skin tannins and flavonols was decreased in shaded Pinot noir grapes. So, there may be other factors, such as skin thickness, cell size and cell wall properties which influence the extraction of flavonoids from the grapes during winemaking, as suggested by Ristic et al. (2007).

4.4. Antioxidant activity

LR treatment affected higher antioxidant activity in Teran experimental wines in both years, RM in second year of investigation and LR + RM in the first year. Similar pattern was evident for total phenols and total anthocyanins concentrations, which implies that those groups of phenolic compounds contribute most to antioxidant activity of experimental wines. This was proved by correlation analysis, which confirmed very strong correlation between those compounds and antioxidant properties of experimental wines, as previously reported by Cimino et al. (2007), Fernández-Pachón et al. (2004), Jiang and Zhang (2012) and Minussi et al. (2003). Among the individual phenolic compounds, catechin had the strongest positive correlation with antioxidant activity of tested wines. A positive correlation between antioxidant properties and *trans*-resveratrol, epicatechin, delphinidin-3-glucoside and malvidin-3-glucoside were also observed. This shows that those compounds can make a major contribution to the overall antioxidant capacity of wines. Jiang and Zhang (2012) also adduced catechin and epicatechin as phenolic compounds with the strongest antioxidant activity.

5. Conclusions

Plavac mali was the only cultivar which responded to applied treatments regarding soluble solids and titratable acidity content in cool conditions. Leaf removal strongly influenced phenolic composition of most experimental wines, followed by combined leaf removal and reflective mulch treatment. Reflective mulch itself had the weakest effect on wine phenolic content. So, as concluded in many previous works, it is doubtful if reflective mulch price and additional costs of its installation could justify the investment in applying reflective mulch to Croatian vineyards. Leaf removal still remains one of the most suitable and highly recommended viticultural practices, especially for red grape cultivars production. Total phenols, anthocyanins and flavan-3-ols contents in wines exhibited strong correlation with antioxidant activity, which is very important regarding human health benefits.

Obtained results suggest the possibility of seasonal and varietal differences in phenols composition regarding their response to leaf removal and reflective mulch, with Teran being the most responsive cultivar to treatments applied. This suggests that canopy management strategies should be adjusted to specific cultivars, growing season and individual vineyard site.

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