TIMING OF LEAF REMOVAL EFFECTS ON *VITIS VINIFERA* L. CV. GRENACHE DIFFERED ON TWO CONTRASTING SEASONS

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Abstract:

Context and purpose of the study – Warming trends over the winegrowing regions lead to an advance of grapevine phenology, diminution of yield and increased sugar content and must pH with a lower polyphenol content, especially anthocyanins. Canopy management practices are applied to control the source sink balance and improve the cluster microclimate to enhance berry composition. We hyphothesized that an early leaf removal might promote a delayed ripening through severe defoliation after fruitset; whereas, a late leaf removal at mid-ripening would reduce sugar accumulation.

Material and methods - The experiment was conducted with *Vitis vinifera* L. Grenache cultivar over the 2021 and 2022 seasons in a commercial vineyard in Alfaro, La Rioja, Spain. Grapevines were trained in a Globet system with three arms and 5 two-bud spurs. Experimental design was conducted as a complete block design with two leaf removal treatments differing in the moment of manipulation: i) severe leaf removal treatment conducted after fruit set (ELR); and ii) severe leaf removal after veraison (LLR) compared to an untreated control (UNT). Both leaf removal treatments consisted in the elimination of the basal leaves before the second internode. Each treatment had five replicates of 10 grapevines. Harvest commenced when berries reached commercial maturity (approximately, 26°Bx) and yield components were studied. At harvest, sample of berries were collected for berry mass and chemistry, color development and polyphenol profile determinations by UHPLC-MS/MS.

Results – Sugar content at harvest was increased during the warmer season, however, no effect of defoliation treatments was observed. Leaf removal and year factor affected pH and total acidity, without interactive effects. Respecting their polyphenol profiles, leaf removal treatments accounted for a decreased flavanols and stilbene content in berries at harvest. An ELR increased anthocyanin and phenolic acid contents at harvest, while warming during 2022 accounted for decreased contents of all the monitored groups of flavonols. The ELR was effective for delaying ripening by means of impairing the sugar:anthocyanin decoupling during the 2021 growing season, however, no difference between treatments was assessed during the warmer season of 2022. These results suggested that leaf removal strategies, even under currently warming trends, could still be useful at some circumstances to obtain an improvement of some berry quality traits and its low incidence in yield.

Keywords: Anthocyanins, Decoupling, Grapevine, Polyphenols, Warming trends.

1. Introduction

Elevated temperatures associated with warming trends over the winegrowing regions lead to an advance of grapevine phenology, diminution of yield and increased sugar content and must pH with a lower polyphenol content, especially anthocyanins, which lead to low color and highly alcoholic wines (Arrizabalaga-Arriazu et al., 2020; Sadras and Moran, 2012).

Canopy management practices are applied to control the source sink balance and improve the cluster microclimate to enhance berry composition. Leaf removal consists in the elimination of basal leaves close to the clusters, however, its effects depend on how and when it is performed (Sternad-Lemut et al., 2013). We hypothesized that an early leaf removal (ELR) might promote a delayed ripening through severe defoliation after fruit set; whereas, a late leaf removal (LLR) at mid-ripening would reduce sugar accumulation. Therefore, a two-year study was performed to evaluate the optimal timing of leaf removal among two phenological stages (after fruit set and in late veraison) as a tool for delaying ripening and reduce the accumulation of sugars without affecting phenolic composition; and to determine the implications of leaf removal on the sugar:anthocyanin decoupling over two season with contrasting patters of solar exposure and precipitation.

2. Material and methods

Plant material and growing conditions

Plant materials - The experiment was conducted with *Vitis vinifera* L. Grenache cultivar over the 2021 and 2022 seasons in a commercial vineyard in Alfaro, La Rioja, Spain. Grapevines were trained in a Globet system with three arms and 5 two-bud spurs. Experimental design was conducted as a complete block design with two leaf removal treatments differing in the moment of manipulation: i) severe leaf removal treatment conducted after fruit set (ELR); and ii) severe leaf removal after veraison (LLR) compared to an untreated control (UNT). Both leaf removal treatments consisted in the elimination of the basal leaves before the second internode. Each treatment had five replicates of 10 grapevines. Harvest commenced when berries reached commercial maturity (approximately, 26°Bx) and yield components were studied.

Berry quality and polyphenolic profile determinations - At harvest, a 100-berry sample was collected for berry mass and chemistry. Berries were weighted and berry mass was obtained, then, they were gently pressed by hand, then, the juice was used to determine total soluble solids (TSS), pH and titratable acidity (TA). TSS was determined using a high precision temperature compensating refractometer (RF Mogul, USA). Must pH and TA were determined with an autotitrator (Crison, Spain). TA was estimated by titration with 0.1N sodium hydroxide to an endpoint of 8.3 pH and reported as g/L of tartaric acid.
Another 200-berry sample was crushed in a blender and 50 mL of the resultant mush was used for polyphenol profile determinations after obtaining phenolic fractions (PF) following Alegre et al. (2020). PF were filtered with 2 µm CHROMAFIL AO-20/15 MS filters (Düren, Alemania) and transferred to injection vials (Royo et al., 2021). Then, samples were analyzed with an Acquity Ultra Performance Liquid Chromatographic system (Waters, MA, USA) coupled to a Xevo TQ MS System (Waters, UK) operating under MassLynx XS software. The chromatographic separation of the phenolic compounds was carried out in a Waters Acquity HSS T3 column, 1.8 µm, 2.1 × 150 mm (Waters). The samples were analyzed with an Acquity Ultra Performance Liquid Chromatographic system (Waters, MA, USA) coupled to a Xevo TQ MS System (Waters, UK) operating under MassLynx XS software. A reverse phase (RP) Acquity UPLC BEH C18, 1.7 µm, 2.1 × 150 mm column (Waters), protected with an Acquity UPLC BEH C18, 1.7 µm, 2.1 × 5 mm precolumn (Waters) was employed.

Statistical analysis - Statistical analyses were conducted with R studio version 3.6.1 (RStudio Team, 2020). Berry mass, primary and secondary metabolites and phenolic composition were analyzed by using the two-way analysis of variance (ANOVA) after assessing the normality of the data with year and treatment as main factors. Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Tukey's honest significance difference (HSD) test was executed using "agricolae" 1.2–8 R package (de Mendiburu, 2017).
Linear regressions between total anthocyanins and TSS were calculated and the significance of the analysis was

estimated with the same software for each treatment within the year and for each year separately. For both analyses, the slopes were compared at $P<0.05$ using analysis of covariance (ANCOVA).

3. Results and discussion

3.1. *The effects of timing of leaf removal are dependent upon the weather conditions of the growing season.*

The two season assessed in current study differed in their mean temperature and precipitation pattern. Thus, the 2021-22 was warmer than the 2020-21 growing season, with 1 °C more on average on the mean daily temperature, 13 days more with temperature over 35°C and 37.2 mm less of precipitation. Berry mass and chemistry were affected by both leaf removal treatments and year, but no interaction between them was observed (Table 1). Berry mass was diminished during the 2022 growing season, presumably because the higher exposure of berries during the second season could lead to a reduction in sugar allocation (Torres et al., 2020). The warmer season also accounted for increased TSS in berries, however, no effect of defoliation treatments was observed (Table 1). LLR has been proposed to reduce sugar accumulation rates and postpone harvest dates (Poni et al., 2018), but these effects are highly dependent upon the environment and grapevine variety, especially on rainfed vines (Buesa et al., 2019). Regarding acidity related parameters, ELR treatment tended to decrease pH and 2022 increased it. TA was also affected by year factor, which was increased during the 2022 without any effect of leaf removal treatments. Similarly, Torres et al. (2020) found no difference on the must TA of vines subjected to leaf removal. Increased pH and TA in 2022 could be explained by the lower berry size that year, which could concentrate the main acids present in berries, despite the possible degradation of malic acid under elevated temperatures (Sweetman et al., 2014).

The total contents of the different phenolic compounds measured in this work are shown in Table 2, where no interaction between leaf removal treatments and year was observed. Anthocyanin content at harvest was increased with ELR and decreased in 2022. Anthocyanin accumulation in grape skins was significantly higher at 20 °C than at 30 °C and the most sensitive stage for temperature was from one to three weeks after coloring began (Yamane et al., 2006), in accordance with the lack of effect of LLR in our study. On the other hand, increased anthocyanin contents with ELR were reported in studies conducted with different varieties (Pastore et al., 2017; Sternad Lemut et al., 2013). The other polyphenolic compound families showed the same trend with decreased contents of flavonols, phenolic acids and stilbenes during the 2022 growing season (Table 2) suggesting that warming trends accounted for decreased synthesis and accumulation of these compounds (Sternad-Lemut et al., 2013; Bavaresco et al., 2008). Regarding defoliation effects, both leaf removal treatments accounted for decreased proanthocyanidins and stilbenes, while ELR increased phenolic acids.

3.2. *Sugar and anthocyanin decoupling are affected by solar exposure caused by defoliation*

Relationships between berry anthocyanin and sugar contents are reported in Table 3. In 2021, ELR modulated relationship between sugar and anthocyanin accumulation as highlights the ANCOVA conducted ($p \leq 0.05$; Table 3). However, in 2022, regressions of each treatment were not different and the thermal decoupling of these traits was remarkable with almost half of the anthocyanin content for the same sugar contents in 2022 compared to 2021. In accordance with these results, previous research reported that defoliation at veraison decreased TSS with a potential diminution of anthocyanins while LLR did not lead to significant changes on berry sugars and anthocyanins (Pastore et al., 2017; Tessarin et al., 2014).

4. Conclusions

Current study revealed that increasing the cluster exposure to temperature and radiation strongly affects primary and secondary metabolisms. ELR could reduce this decoupling when environmental conditions are not too extreme, but no effect was observed during the warmer and drier season. Therefore, these results suggested that leaf removal strategies, even under currently warming trends, could still be useful at some circumstances to obtain an improvement of some berry quality traits and its low incidence in yield.

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6. Litterature cited

Alegre, Y., Arias-Pérez, I., Hernández-Orte, P., Ferreira, V., 2020. Development of a new strategy for studying the aroma potential of winemaking grapes through the accelerated hydrolysis of phenolic and aromatic fractions (PAFs). Food Res. Int. 127, 108728.

Arrizabalaga-Arriazu, M., Gomès, E., Morales, F., Irigoyen, J.J., Pascual, I., Hilbert, G., 2020. High temperature and elevated carbon dioxide modify berry composition of different clones of grapevine (*Vitis vinifera* L.) cv. Tempranillo. Front. Plant Sci. 11, 603687.

Bavaresco, L., Gatti, M., Pezzutto, S., Fregoni, M., Mattivi, F., 2008. Effect of Leaf Removal on Grape Yield, Berry Composition, and Stilbene Concentration. Am. J. Enol. Vitic. 59, 3

Buesa, I., Caccavello, G., Basile, B., Merli, M.C., Poni, S., Chirivella, C., Intrigliolo, D.S., 2019. Delaying berry ripening of Bobal and Tempranillo grapevines by late leaf removal in a semi-arid and temperate-warm climate under different water regimes. Aust. J. Grape Wine Res. 25, 70-82.

Pastore, C., Allegro, G., Valentini, G., Muzzi, E., Filippetti, I., 2017. Anthocyanin and flavonol composition response to veraison leaf removal on Cabernet Sauvignon, Nero d'Avola, Raboso Piave and Sangiovese *Vitis vinifera* L. cultivars. Sci. Hortic. 218, 147–155.

Poni, S., Gatti, M., Palliotti, A., Dai, Z., Duchêne, E., Truong, T.-T., et al., 2018. Grapevine quality: a multiple choice issue. Sci. Hortic. 234, 445–462.

Sweetman, C., Sadras, V.O., Hancock, R.D., Soole, K.L., Ford, C.M., 2014. Metabolic effects of elevated temperature on organic acid degradation in ripening *Vitis vinifera* fruit. J. Exp. Bot. 65, 5975–5988,

Royo, C., Ferradás, Y., Martínez-Zapater, J.M., Motilva, M.J., 2021. Characterization of Tempranillo negro (VN21), a high phenolic content grapevine Tempranillo clone, through UHPLC-QqQ-MS/MS polyphenol profiling, Food Chem. 360, 130049.

Sadras, V.O., Moran, M.A., 2012. Elevated temperature decouples anthocyanins and sugars in berries of Shiraz and Cabernet Franc. Aust. J. Grape Wine Res. 18, 115–122

Sternad Lemut, M., Trost, K., Sivilotti, P., Arapitsas, P., Vrhovsek, U., 2013. Early versus late leaf removal strategies for Pinot Noir (*Vitis vinifera* L.): Effect on colour-related phenolics in young wines following alcoholic fermentation. J Sci Food Agric. 93, 3670-81.

Torres, N., Martínez-Lüscher, J., Porte, E., Kurtural, S.K., 2020. Optimal Ranges and Thresholds of Grape Berry Solar Radiation for Flavonoid Biosynthesis in Warm Climates. Front. Plant Sci. 11, 931.

Tessarin, P., Boliani, A. C., Botelho, R. V., Rusin, C., Versari, A., Parpinello, G.P., Rombolà, A.D., 2014. Effects of late defoliations on chemical and sensory characteristics of cv. Uva Longanesi wines. J. Soil Sci. Plant Nut. 14, 1021-1038.

Table 1: Berry mass, TSS, pH and total acidity of must obtained from berries of Grenache grapevines subjected to an early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain), in 2020-21 and 2021-22 seasons.

Main effects		Berry mass (g)	TSS (Brix)	pH	Total acidity (g/L)
Treatment	Control	1.41 ± 0.06	26.6 ± 0.3	3.52 ± 0.02 a	4.9 ± 0.1
	ELR	1.39 ± 0.06	25.6 ± 0.3	3.45 ± 0.02 b	5.0 ± 0.1
	LLR	1.42 ± 0.04	26.1 ± 0.3	3.49 ± 0.03 a	4.8 ± 0.2
Year	2021	1.72 ± 0.06 a	25.5 ± 0.2 b	3.46 ± 0.02 b	4.5 ± 0.1 b
	2022	1.09 ± 0.05 b	26.8 ± 0.3 a	3.51 ± 0.02 a	5.3 ± 0.1 a
ANOVA	Treatment	ns	ns	.	ns
	Year	***	*	*	***
	T x Y	ns	ns	ns	ns

Values represent means ± SE (n=10-15) separated by Tukey HSD test ($p \leq 0.05$) of the main effects of treatments and year. Within a column, different letters indicate significant differences as affected by the main factors canopy management (Control, ELR, LLR), growing season (2021, 2020) and their interactions (T × Y). ns, ., *, and *** indicate non-significance and significance at 10%, 5%, and 0.1% probability levels, respectively.

Table 2: Total anthocyanins, flavonols, proanthocyanidins, phenolic acids and stilbenes in phenolic fractions obtained from berries of Grenache grapevines subjected to an early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain), in 2020-21 and 2021-22 seasons.

Main effects		Anthocyanins (mg/berry g)	Flavonols (mg/berry g)	Proanthocyanidins (mg/berry g)	Phenolic acids (mg/berry g)	Stilbenes (mg/berry g)
Treatment	Control	412.2 ± 34.9 b	397.1 ± 50.0	61.8 ± 9.1 a	16.2 ± 2.8 b	3.0 ± 0.8 a
	ELR	503.9 ± 18.0 a	554.4 ± 62.3	46.3 ± 4.2 b	29.6 ± 8.9 a	1.6 ± 0.4 b
	LLR	383.4 ± 60.9 b	478.7 ± 103.4	46.6 ± 10.9 b	15.6 ± 4.8 b	2.0 ± 0.4 ab
Year	2021	672.1 ± 67.1 a	840.7 ± 135.3 a	55.4 ± 10.85	25.3 ± 9.4 a	3.1 ± 0.7 a
	2022	194.2 ± 8.5 b	112.9 ± 8.5 b	47.7 ± 5.2	15.6 ± 1.5 b	1.3 ± 0.4 b
ANOVA	Treatment	.	ns	.	.	.
	Year	***	***	ns	.	**
	T x Y	ns	ns	ns	ns	ns

Values represent means ± SE (n=10-15) separated by Tukey HSD test ($p \leq 0.05$) of the main effects of treatments and year. Within a column, different letters indicate significant differences as affected by the main factors canopy management (Control, ELR, LLR), growing season (2021, 2020) and their interactions (T × Y). ns, ., ** and *** indicate non-significance and significance at 10%, 1%, and 0.1% probability levels, respectively.

Table 3: Relationship between the must anthocyanin content and the total soluble solids (TSS) of the Grenache grapevines subjected to an early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain) separate by leaf removal treatment in 2021 and 2022.

Year	Treatment	y	a		R	p-value	ANCOVA
2021	Control	-1976.7	132.1	a	0.77	***	
	ELR	-4016.8	224.4	b	0.93	***	*
	LLR	-1605.5	115.8	b	0.51	*	
2021	Control	-1415.1	98.9		0.97	***	
	ELR	391.1	26.3		0.51	ns	ns
	LLR	-612.3	69.1		0.92	***	

"y" indicates the intercept, "a" the slope of the regression, "r" the correlation coefficient, "p-value" the significant according to the Pearson correlation and "ANCOVA" the significance of the regressions according to the ANCOVA test performed for each year. Different letters indicate significant differences between regressions. ($p \leq 0.05$).