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Timing of defoliation affects anthocyanin and sugar decoupling in Grenache variety growing in warm seasons

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A R T I C L E I N F O

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A B S T R A C T

Warming trends over the winegrowing regions lead to an advance of grapevine phenology, decreased yield and increased sugar content with a lower polyphenol content. We hypothesized that different leaf removal timings may counteract these effects. A two-year experiment was conducted in La Rioja (Spain) with *Vitis vinifera* L. cv. Grenache trained in an open-vase system. Trial consisted in 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 at veraison (LLR) compared to an untreated control (Control). Both leaf removal treatments tended to decrease sugar content with no effect on yield, these effects being highly affected by the year. Defoliation accounted for a decreased flavanol and stilbene contents 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. ELR was only effective for delaying ripening by means of impairing the sugar:anthocyanin decoupling during the 2021 growing season which was related to lower % of kaempferol. Altogether, results suggested that defoliation should still be applied under currently warming trends in some viticulture regions.

1. Introduction

Climate change has been reported to have detrimental impact on historic viticulture regions, with effects on grapevine phenology and advanced harvest dates (Duchêne and Schneider, 2005; Petrie and Sadras, 2008; Webb et al., 2012). Under optimal growing conditions, it is well-established the direct relationship between sugar content and anthocyanin synthesis in grapes through the sugar-regulated gene expression of some flavonoid synthesis genes (LDOX and DFR), which possess 'sucrose boxes' in their promoters (Gollop et al., 2001; Gollop et al., 2002). However, elevated temperatures associated with climate change have been reported to uncouple berry phenolic composition and sugar metabolism, leading to low color and highly alcoholic wines (Arrizabalaga-Arriazu et al., 2020; Sadras and Moran, 2012). Among phenolic compounds, flavonoids are key compounds in berry and wine composition and antioxidant properties. Anthocyanins are responsible for color (Savoi et al., 2017), bitterness (Gonzalo-Diago et al., 2014; Soares et al., 2013) and mouthfeel properties (Ferrer-Gallego et al., 2015; Sáenz-Navajas et al., 2017; Ferrero-del-Teso et al., 2020), whereas flavonols act as photoprotectants, scavenging free oxygen radicals and preventing enzymatic reactive oxygen species, and contribute to wine color through co-pigmentation with anthocyanins (Waterhouse et al., 2016). Thus, it has been reported in Cabernet Sauvignon berries and wines that a higher exposure of berries to solar radiation and elevated temperatures (due to leaf and shoot removal managements) lead to this sugar:anthocyanin decoupling through decreased anthocyanin hydroxylation which reduced the color stability (Torres et al., 2020a, b). In order to delay phenology and avoid the deleterious impact on berry quality, several strategies have been essayed such as changes in vineyard location (higher latitudes and altitudes) or modifications of training systems and canopy management (higher trunks, late pruning, minimal pruning of reduced leaf area to fruit weight ratios) (Van Leeuwen and Darriet, 2016; Zheng et al., 2016; Martínez De Toda and Balda, 2013; Parker et al., 2014).

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Table 1
Weather conditions during the growing seasons of 2020–2021 and 2021–2022 in Alfaro (La Rioja, Spain).

	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	
	Mean Daily Temperature (°C)												**Mean**
2020–21	13.27	9.98	6.36	5.78	9.71	9.79	11.44	15.8	20.21	22.61	22.51	19.23	13.89
2021–22	14.38	8.66	6.22	5.43	8.74	9.21	11.86	19.2	23.52	25.21	24.94	19.99	14.78
	Minimum Daily Temperature (°C)												**Mean**
2020–21	2.68	–0.48	–1.14	–4.54	2.44	–0.05	2.06	4.06	10.96	9.11	10.15	10.3	3.8
2021–22	4.25	0.84	–1.34	–3.22	–0.95	1.44	–0.66	9.49	10.58	10.2	13.41	9.06	4.43
	Maximum Daily Temperature (°C)												**Mean**
2020–21	23.8	21.24	14.18	18.58	20.96	23	23.94	29.27	34.82	39.8	39.22	30.19	26.58
2021–22	24.37	17.17	16.56	17.93	20.72	17.46	24.56	32.66	39.39	39.49	38.78	33.25	26.86
	Days with temperature over 30 °C (no.)												**Total**
2020–21	0	0	0	0	0	0	0	0	9	22	16	1	48
2021–22	0	0	0	0	0	0	0	8	15	23	24	7	77
	Days with temperature over 35 °C (no.)												**Total**
2020–21	0	0	0	0	0	0	0	0	0	8	4	0	12
2021–22	0	0	0	0	0	0	0	0	5	13	7	0	25
	Radiation (MJ/m2)												**Mean**
2020–21	12.04	6.9	5.26	7.47	9.4	15.75	17.2	22.27	25.05	25.91	24.45	16.44	15.68
2021–22	13.05	7.57	4.5	8.9	11.89	10.44	18.8	24.7	26.43	28.15	23.22	18.32	16.33
	Precipitation (mm)												**Total**
2020–21	29.1	29.2	28.3	48.8	14.9	15.21	15.8	23.6	42.3	3.3	3.2	70.4	324.1
2021–22	24.8	77.4	19.3	14.5	4.9	35.3	48.8	10.7	6.4	15.5	9	20.3	286.9
	Reference ET (ETo. mm)												**Total**
2020–21	65.81	30.1	27.72	34.08	48.23	86.17	97.99	141.11	164.6	202.76	180.84	96.44	1175.85
2021–22	74.33	43.79	23.84	34.32	55.57	60.35	107.11	169.69	198.36	218.12	181.45	126.06	1292.99

Weather data were obtained from the SIAR weather station #21 (Corella, Navarra, Spain) located close to the research site.

Leaf removal consists in the elimination of basal leaves close to the clusters, and it is a common practice in medium to high vigor vineyards. However, it is important when and how it is performed. Thus, an early leaf removal (ELR) conducted before flowering is used to regulate yield components and improve grape quality (Diago et al., 2010). ELR at pre-flowering reduces fruit-set and berry weight, leading to smaller and looser clusters, increasing the skin-to-berry and seed-to-berry ratio (Tardaguila et al., 2010). In addition, defoliated shoots generally have a higher final leaf-to-fruit ratio when leaf removal is performed pre-flowering (Poni et al., 2006), because leaves are the main carbohydrate source at the pre-flowering stage and, therefore, the primary regulator of subsequent fruit-set (Coombe, 1962) and a temporary foliar stress can reduce cell division rates during the green stage of berry growth; affecting the final berry size (Palliotti et al., 2010). However, there is a general consensus about removing leaves several weeks after flowering that had no detrimental impact on yield, and frequently improved fruit quality (Caspari et al., 1998; Koch et al., 2012; VanderWeide et al., 2020), whereas a late leaf removal (LLR), applied after veraison, tends to have little effect on yield but it may postpone grape ripening (Palliotti et al. 2013; Caccavello et al. 2017). Poni et al. (2013) demonstrated that after applying LLR at veraison in Sangiovese cultivar, technologically defined ripeness was delayed without affecting berry phenolic substances or color. In this regard, Lanari et al. (2013) found that LLR negatively affected the concentration of anthocyanin and phenolic substances in Montepulciano grapevines, but not in Sangiovese. Thus, Bobeica et al. (2015) reported that sugar accumulation in berries of potted Sangiovese and Cabernet Sauvignon vines subjected to leaf removal was maintained in detriment of phenolic substances. LLR has been generally reported to be less effective than ELR in modifying the chemical composition of grapes and subsequently the quality of the produced wine (Sternad-Lemut et al., 2013). Nevertheless, defoliation performed at veraison might reduce anthocyanin content and increased the impact of sunburn (Pastore et al., 2013), especially in warm climates when berries are too exposed (Torres et al., 2020a).

Grenache cultivar ("Garnacha Tinta") is a Spanish ancient red variety, widely cultivated in the world (although there is a decreasing trend towards its cultivation), which in 2015 covered about 163000 ha (OIV, 2017). It is predominantly grown in Spain and France, accounting for the 87% of its world vineyard area (OIV, 2017). This variety is extremely drought resistant and adapts easily to different soil types given that tightly regulates stomatal conductance to avoid fluctuations in midday leaf water potential (i.e., isohydric behavior) (Chaves et al. 2010; Martínez-Vilalta and Garcia-Forner, 2017). In fact, previous research has underlined its ability for rapidly adapting to environmental conditions through the stomata regulation compared to other varieties (Gallo et al., 2020). Nevertheless, favorable conditions at the end of ripening joint to warm temperatures lead to increased total soluble solids (TSS) in Grenache. Furthermore, recent research reported that climate change scenarios could account for decreases in acidity and total anthocyanins in Grenache and Tempranillo varieties, the effect being higher for Grenache (Ramos and Martínez de Toda, 2020). Given its varietal characteristics, there is a need to seek a better management for maintaining its production under the current environmental conditions.

Open-vase ("Globelet") conduction system is the traditional way of cultivation in La Rioja (Spain), however, the effects of canopy management strategies might be affected by grapevine training. As far as we know there are no studies dealing with the comparison between an early and a late leaf removal on Grenache open-vase-trained grapevines under current warming conditions. We hypothesized that a severe defoliation after fruit set might promote delayed ripening in Grenache grapevines; whereas a severe defoliation at veraison, when berries have a degree 11.5°, may reduce the accumulation of sugars. Therefore, this study aims: i) 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 affect phenolic composition; and ii) to determine the implications of leaf removal on the sugar:anthocyanin decoupling over two seasons with contrasting patterns of solar exposure and precipitation.

2. Material and methods

2.1. Plant material, experimental design and weather conditions

The experiment was conducted with *Vitis vinifera* L. Grenache cultivar in a commercial rainfed vineyard in Alfaro, La Rioja, Spain (42.143 N, −1.902 W). Grapevines were planted in 2012 on 110 R rootstock with a space of 1.5 m × 1.5 m with a row orientation of NW-SE and trained in an open-vase system with three arms and five buds.

The experiment was performed following a complete block design with two leaf removal treatments differing in the moment of

Table 2
Reproductive growth and yield components 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.

Year	Treatment	Number of clusters	Yield (kg/vine)	Cluster mass (g)	Fertility	Number of berries
2021	Control	9.38 ± 0.36	1.19 ± 0.15	126.86 ± 16.66	1.56 ± 0.06	69.85 ± 9.25 b
	ELR	9.32 ± 0.42	1.12 ± 0.12	118.98 ± 11.76	1.59 ± 0.06	62.66 ± 6.84 b
	LLR	8.86 ± 0.45	1.20 ± 0.16	132.43 ± 16.62	1.51 ± 0.07	74.50 ± 8.04 b
2022	Control	8.86 ± 0.31	1.75 ± 0.18	191.78 ± 10.67	1.48 ± 0.05	165.35 ± 11.85 a
	ELR	9.04 ± 0.33	1.60 ± 0.19	176.14 ± 15.15	1.51 ± 0.06	147.55 ± 14.79 a
	LLR	9.50 ± 0.38	1.73 ± 0.12	185.82 ± 15.52	1.58 ± 0.06	157.15 ± 11.76 a
Main effects	Control	9.12 ± 0.24	1.46 ± 0.14	159.31 ± 14.28	1.52 ± 0.04	117.60 ± 17.42
	ELR	9.18 ± 0.26	1.36 ± 0.13	147.56 ± 13.13	1.55 ± 0.04	105.11 ± 16.10
	LLR	9.18 ± 0.30	1.46 ± 0.13	159.13 ± 13.93	1.55 ± 0.05	115.82 ± 15.32
	2021	9.19 ± 0.40	1.17 ± 0.13 b	126.10 ± 14.29 b	1.56 ± 0.04	69.01 ± 7.83
	2022	9.13 ± 0.34	1.69 ± 0.16 a	184.57 ± 13.26 a	1.52 ± 0.03	156.68 ± 12.39
ANOVA	Treatment	ns	ns	ns	ns	ns
	Year	ns	* **	* **	ns	* **
	T x Y	ns	ns	ns	ns	* **

Values represent means ± SE (n = 5) separated by Tukey HSD test (p ≤ 0.05). Different letters within a column 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 0.1% probability levels, respectively.

manipulation: i) severe leaf removal treatment conducted after fruit set (ELR); and ii) severe leaf removal at veraison (LLR) compared to an untreated control without any leaf removal (Control). Both leaf removal treatments consisted in the elimination of 30% of the leaves of the main shoot from the cluster area up to the second internode above the last cluster (removed leaf area: 0.674 m^2) (Supplementary Figure 1). ELR was conducted after fruit set in both seasons (17-June-21) and (21-June-22) whereas leaf removal on LLR grapevines was performed at mid-ripening when the berry probable sugar content reached 11.5° (approximately, 20°Bx), August 23 and August 16 for 2021 and 2022, respectively. Each treatment replicate had five replicates of 10 vines previously selected by their similar anatomic characteristics. Harvest commenced when berries reached commercial maturity (approximately, 26°Bx) on average in all treatments on October 7 and September 12 in 2021 and 2022, respectively.

Weather data (Table 1) were obtained from the Spanish System of Agro-climatic information for Irrigation Management, SIAR weather station #21 (Corella, Navarra, Spain) located close to the research site. The number of days with temperatures above 30 °C and above 35 °C were counted for the 2021 and 2022 growing seasons.

2.2. Yield components and sugar and anthocyanin contents during ripening

The fertility of grapevines was assessed by counting the number of clusters per shoot. At harvest 300 berries per treatment-replicate were randomly collected for other determinations. Then, grapevines were harvested and clusters were counted and weighed on a top-loading balance to determine cluster mass. Berry samples were collected on August 27, September 14, October 1 and 7 in 2021 and August 24 and September 12 in 2022 to evaluate berry ripening. A sample of 100 berries was weighed to determine berry mass and used for berry chemical characterization during ripening. After being gently pressed by hand, the juice obtained 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 0.1 N sodium hydroxide to an endpoint of pH 8.3 and reported as $g \cdot L^{-1}$ of tartaric acid.

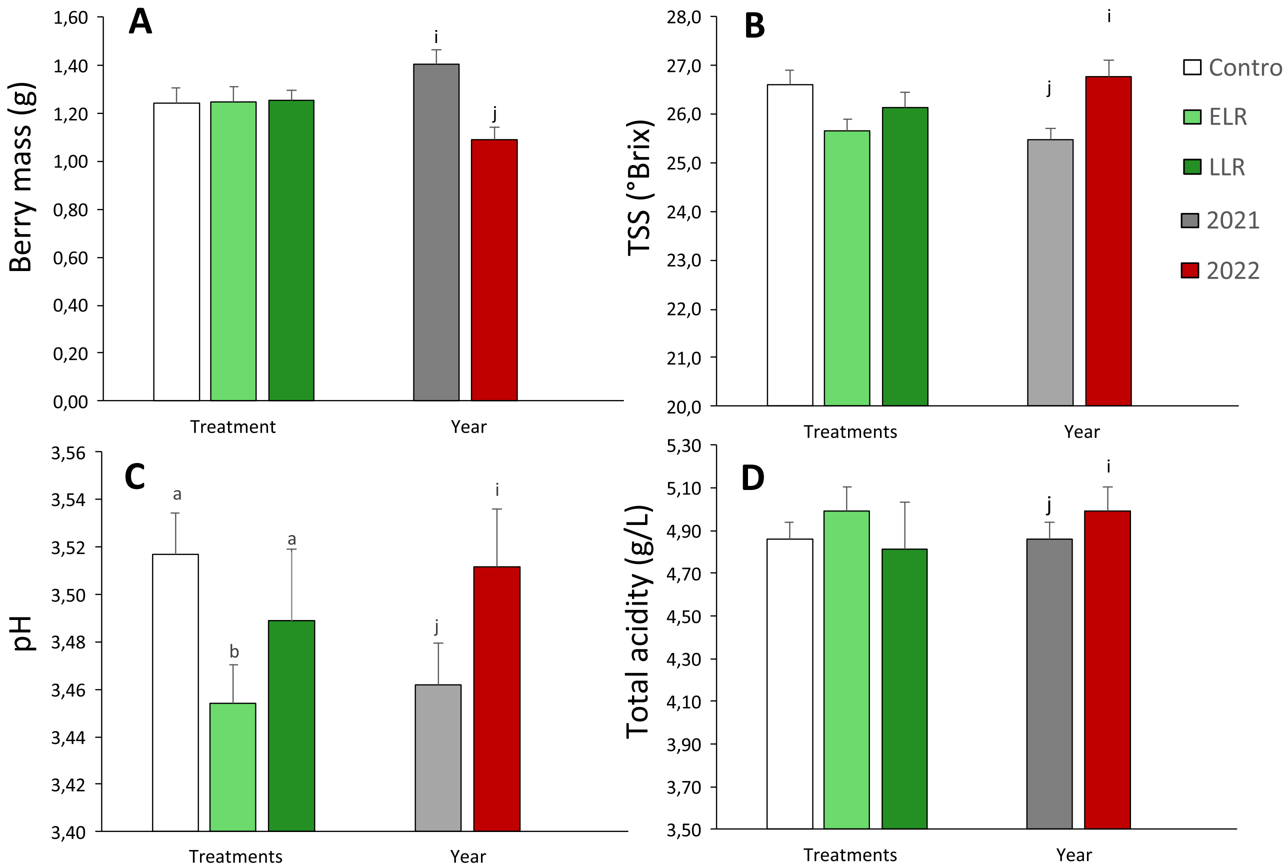
Another subsample of 200 berries was used for determining total anthocyanin content by the Cromoenos™ method which provides a fast and accurate estimation of phenolics and color (Kontoudakis et al., 2010). This method uses specific equipment and reagents provided by the manufacturer (Bioenos, Spain). Briefly, berries were placed in a blender (Oster, USA) and aliquots of 40 mL of the extracts were introduced in the thermoextractor till the temperature reached 80 °C (at roughly 2 min), then, 1 mL of the sample was centrifuged (13,400 rpm; 2 min) in a Mikro 200/200 R centrifuge (Hettich, Germany). An aliquot of 60 μL of the supernatant was diluted with 2% HCl (*v/v*) to 4 mL and the absorbances at 520 and 280 nm were measured in a spectrophotometer (Fisher Scientific, USA). Then, the estimation of total anthocyanins (mg/L) was obtained using the calibration provided by the manufacturer.

2.3. Preparation of phenolic fractions (PF)

Extraction of phenolic compounds from grapes were prepared according to a method adapted from Alegre et al. (2020). At harvest, an aliquot of 50 mL from the berries crushed in the blender was used for the color and polyphenolic determinations. Samples (50 mL) were weighed and 15% (*w/w*) of alcohol and 2.5 mg of potassium metabisulfite were added. Samples were sonicated for 45 min at 23 °C. Then, grape extracts were centrifuged at 3500 rpm, 10 °C for 12 min, after which they were separated from the alcohol in a rotary evaporator system (8 mbar, 26 °C, 40 min). Samples were again centrifuged (3500 rpm, 10 °C, 12 min) and the resulting dealcoholized extracts (containing no more than 2% ethanol) were passed through a 1 g C18 prepared cartridge (Waters-Sep Pak-C18, 1.6 mL). For cartridge conditioning, methanol followed by milli-Q water with 2% ethanol was employed. Then, the whole sample was loaded, and washed with milli-Q water pH 3.5 to remove sugars, amino acids, acids and ions. Cartridges were finally dried by letting air pass through them and phenolic fractions (PF) were recovered with 6 mL of ethanol.

2.4. Conventional oenological parameters, CIELab coordinates, and anthocyanin-derived pigments

Spectrophotometric analysis was used to determine conventional oenological parameters, wine color characteristics using CIELab parameters (OIV, 2021) and anthocyanin-derived pigments (Harbertson et al., 2003). Total polyphenol index (TPI) was estimated as absorbance at 280 nm (Ribéreau-Gayon, 1970), HUE was determined as the ratio of the absorbance at 420 and 520, and color intensity (CI) as the sum of absorbance at 420, 520 and 620 nm (Glories, 1984). The absorbances at 420, 520, and 620 nm were determined using distilled water as a reference. CIELab chromatic parameters were expressed according to those of the International Commission on Illumination. L* stands for lightness (0 = black, 100 = white), a* indicates the red (positive)/green



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ANOVA	Treatment (T)	Year (Y)	T × Y
Berry mass	ns	***	ns
TSS	ns	*	ns
pH	.	*	ns
Total acidity	ns	***	ns

Fig. 1. Berry mass (A) and chemistry (B, C and D) of berries from 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. Values are means ± S.E. ($n = 10$–15) separated by each main factor (i.e. treatment and year). Within each main factor, different letters mean significant difference for that parameter ($p \leq 0.05$). ns,·, * , and * ** indicate non-significance and significance at 10%, 5% and 0.1% probability levels, respectively.

(negative) coordinate, and b* represents the yellow (positive)/blue (negative) coordinate. The concentrated PFs were reconstituted in a pH 3.7 solution prepared with 5 g/L tartaric acid and milli-Q water to finally have the PFs in a hydroalcoholic solution of 12% (*v/v*). Determination of monomeric (MP), small polymeric pigments (SPP), and large polymeric pigments (LPP) in PF was carried out as described by Harbertson et al. (2003).

2.5. UHPLC–MS/MS quantification of low molecular weight phenolic compounds

The determination of anthocyanins, flavanols, flavonols, hydroxycinnamic acids and stilbenes was carried out at the Analysis Service of the ICVV according to Royo et al. (2021). Briefly, PF samples were filtered with 2 µm CHROMAFIL AO-20/15 MS filters (Düren, Germany) and transferred to injection vials. Then, samples were analyzed with a Shimadzu Nexera liquid chromatograph (Shimadzu Corporation, Kyoto, Japan), coupled to an AB Sciex 3200QTRAP® mass spectrometer (Sciex, USA) equipped with an electrospray ionization source (ESI Turbo V™ Source). The analytical column used was a Waters AcQuity BEH C18 (100 mm × 2.1 mm i.d., 1.7 µm,) equipped with a VanGuardTM Pre-Column Acquity BEH C18 (5 × 2.1 mm, 1.7 µm) from Waters (Milford, MA,). Mobile phase solvents were Milli-Q water, LC–MS grade acetonitrile and LC–MS grade formic acid. The elution gradient was: 0–0.5 min, 1% B isocratic; 0.5–1.5 min, 1–8% B; 1.5–4 min, 8% B isocratic; 4–5 min, 8–12% B; 5–5.5 min, 12% B isocratic; 5.5–6 min, 12–14% B; 6–7 min, 14% B isocratic; 7–9 min, 14–22% B; 9–12 min, 22–30% B; 12–13.5 min, 30–90% B; 13.5–14.5 min, 90% B isocratic; 14.5–15 min, 90–1% B;

Table 3
HUE and color intensity (CI), total polyphenol index (TPI), CIELab parameters, and Harbertson indices (monomeric, MP); small polymeric pigments, SPP; and large polymeric pigments, LPP) obtained from the PFs 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.

Year	Treatment	HUE	CI	TPI	a*	b*	L*	SPP	LPP	MP
2021	Control	0.444 ± 0.012	2.174 ± 0.070 b	32.46 ± 0.73 b	59.14 ± 0.99	−2.14 ± 0.50	50.96 ± 0.95 b	0.073 ± 0.001	0.048 ± 0.003	0.709 ± 0.043 b
	ELR	0.412 ± 0.003	3.273 ± 0.244 a	41.37 ± 0.51 a	63.61 ± 0.27	3.59 ± 1.56	40.10 ± 2.06c	0.082 ± 0.001	0.054 ± 0.001	1.117 ± 0.045 a
	LLR	0.468 ± 0.035	2.644 ± 0.402 b	30.47 ± 3.30 b	55.91 ± 4.92	−1.07 ± 2.84	45.80 ± 3.71 bc	0.068 ± 0.008	0.050 ± 0.009	0.715 ± 0.125 b
2022	Control	0.569 ± 0.011	1.091 ± 0.045c	21.62 ± 0.63c	−42.56 ± 1.20	8.23 ± 0.57	71.98 ± 1.29 a	0.050 ± 0.002	0.015 ± 0.004	0.546 ± 0.054 b
	ELR	0.540 ± 0.014	1.186 ± 0.018c	21.38 ± 0.80c	−44.75 ± 1.02	10.63 ± 0.39	75.16 ± 0.49 a	0.051 ± 0.003	0.017 ± 0.006	0.645 ± 0.018 b
	LLR	0.570 ± 0.012	1.090 ± 0.065c	20.92 ± 0.70c	−42.49 ± 1.34	7.99 ± 0.39	71.76 ± 1.89 a	0.054 ± 0.006	0.012 ± 0.006	0.571 ± 0.047 b
Main effects	Control	0.507 ± 0.022	1.632 ± 0.185	27.04 ± 1.86	8.29 ± 16.97 a	3.04 ± 1.76 b	61.47 ± 3.58	0.061 ± 0.004	0.032 ± 0.006	0.627 ± 0.042
	ELR	0.492 ± 0.022	1.969 ± 0.354	28.88 ± 3.30	−4.11 ± 17.74 b	7.99 ± 1.31 a	62.01 ± 5.80	0.062 ± 0.005	0.031 ± 0.007	0.822 ± 0.080
	LLR	0.532 ± 0.022	1.673 ± 0.298	24.50 ± 2.06	−5.59 ± 16.23 b	4.59 ± 1.84 b	62.03 ± 4.59	0.059 ± 0.005	0.026 ± 0.008	0.625 ± 0.059
	2021	0.442 ± 0.020 b	2.602 ± 0.303	34.35 ± 2.61	59.48 ± 2.66 a	−0.28 ± 1.87 b	46.59 ± 2.90	0.073 ± 0.004 a	0.050 ± 0.005 a	0.822 ± 0.107
	2022	0.559 ± 0.013 a	1.122 ± 0.048	21.31 ± 0.67	−43.27 ± 1.21 b	8.95 ± 0.70 a	72.97 ± 1.44	0.052 ± 0.004 b	0.015 ± 0.005 b	0.587 ± 0.044
ANOVA	Treatment	ns	ns	*	* **	* *	ns	ns	ns	* *
	Year	* **	* **	* **	* **	* **	* **	*	*	* **
	T x Y	ns	*	* *	ns	ns	* *	ns	ns	*

Values represent means ± SE (n = 5) separated by Tukey HSD test ($p \leq 0.05$). Different letters within a column 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 5%, 1%, and 0.1% probability levels, respectively.

15–18 min, 1% B isocratic. Ionization was achieved using the electrospray (ESI) interface operating in the positive mode $[M]^+$ for the analysis of anthocyanins, and in the negative mode $[M]^-$ for the rest of the phenolic compounds (Supplementary Figure 2). Data were acquired through multiple reaction monitoring (MRM). The retention time and MRM transitions for quantification and identification, including the individual declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP), for each phenolic compound, are shown in Supplementary Table 1. The dwell time established for each transition was optimized through the chromatogram with the Scheduled MRM tool by means of the retention time, MRM detection window of 60 s and a target scan time of 1 s. Data acquisition was carried out with Analyst ® 1.6.2 software (AB Sciex, USA). Compounds were identified by comparing their chromatographic behavior and mass spectra with those of authentic standards (Supplementary Table 2) and the literature data. Then, ratios of mono/tri and di/tri hydroxylated anthocyanins and flavonols were calculated by using the glucoside derivative of each mono-, di- or tri-hydroxylate compound, respectively.

2.6. Statistical analyses

Statistical analyses were conducted with R studio version 3.6.1 (RStudio Team, 2020). Yield components, berry mass, primary and secondary metabolites and phenolic composition were analyzed by using 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, 2016). 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). Then, the relationship between the slope of the total anthocyanins and TSS regressions and the percentage of kaempferol in each treatment was analyzed. Finally, a principal component analysis (PCA) was conducted and visualized with the same software, by using the "factoextra" package (Kassambara & Mundt, 2020).

3. Results and discussion

3.1. Weather data, yield components and berry composition at harvest

Weather data for the 2020–21 and 2021–22 growing seasons are shown in Table 1. The latter season was warmer than the 2020–21 growing season, with 1 °C more on average on the mean daily temperature, and 29 and 13 days more with temperature over 30 °C and 35 °C, respectively. Radiation was also higher and precipitation lower, leading to an ET_0 of about 120 mm higher (Table 1). Accordingly, several studies have pointed out rising temperatures and extreme events on irrigation patterns on the long-term climate records for most wine-production regions of the world (Webb et al., 2013; Barnuud et al., 2014; Fraga et al., 2016). It is well established that elevated temperatures have a detrimental impact on grapevine phenology and consequently, on berry quality (Fraga et al., 2016; Mosedale et al., 2016). However, it was recently pointed out that viticulture adaptation to climate change needs to take into account the importance of microclimate and requires further research on a smaller scale (Resco et al., 2016).

Defoliation treatments did not explain variations in yield components; however, a higher yield, cluster mass and berry number were recorded in the second season with no effect on the number of clusters (Table 2). Fertility was unaffected by treatments or years. These results suggest no carry over effects of defoliation on yield components. Although some authors have reported a carryover effect on berries per cluster and yield (Martínez-Lüscher and Kurtural, 2021), the intensity of defoliation applied in our study was less severe, and therefore defoliation implications on carbohydrate reserves and root mass were not limiting. Similarly, Acimovic et al. (2016) did not find differences in

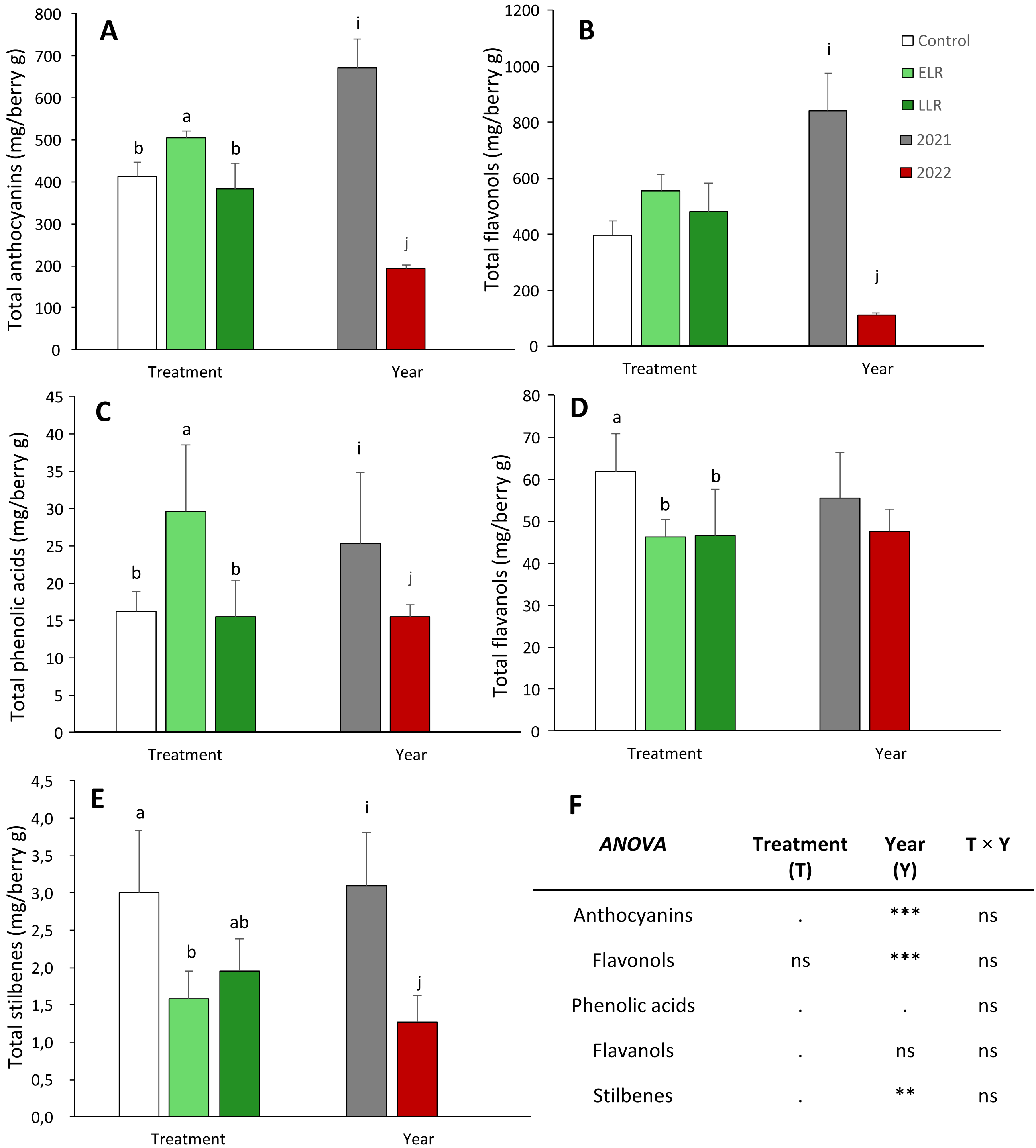

Fig. 2. Anthocyanin (A), flavonol (B), phenolic acid (C), flavanol (D), and stilbene (E) contents in phenolic fractions (PFs) of Grenache grapevines subjected to early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain), in 2020–21 and 2021–22 seasons. Values are means ± S.E. (n = 10–15) separated by each main factor (i.e. treatment and year). Within each main factor, different letters mean significant difference for that parameter ($p \leq 0.05$). ns,·, * *, and * ** indicate non-significance and significance at 10%, 1% and 0.1% probability levels, respectively.

fertility when applying defoliation on the six basal nodes (as we performed). These authors demonstrated that defoliation on the first year impacted the number of flowers per cluster and the number of berries per clusters of the following year when it is severe (i.e., eight-to-ten basal nodes), however, no carryover effect was observed in the less severe leaf removal treatments. These authors explained that this effect should be related with the lower root mass and fruit load that allowed a faster recovery of starch reserves given that root mass may be the only factor explaining changes in yield in the successive season (Torres et al., 2021). These discrepancies might be explained by the different severity of defoliation treatments across both studies, suggesting that under our experimental conditions, leaf removal did not affect root mass. Atmospheric factors, such as temperature, precipitation and radiation, strongly control grapevine growth and development, primarily by affecting photosynthetic rate (Santos et al., 2011). Previous research has shown that warming trends associated with climate change led to lower yields (Droulia and Charalampopoulos, 2021). Nonetheless, under our experimental conditions, yield during the warmer season was higher.

Table 4
Anthocyanin compounds obtained from the PFs 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.

Year	Treatment	Petun-3-G	Petun-3-G-6-Ac	Petun-3-G-6-Cum	Delphid-3-G	Delphin-3-G-6-Ac	Delphin-3-G-6-Cum	Peon-3-G	Peon-3-G-6-Ac	Peon-3-G-6-Cum	Cyanid-3-G	Cyanid-3-G-6-Ac	Cyanid-3-G-6-Cum	Malvid-3-G	Malvid-3-G-6-Ac	Malvid-3-G-6-Cum
2021	Control	38.81 ± 3.96	0.57 ± 0.06	0.62 ± 0.06	35.42 ± 4.30 b	0.66 ± 0.07	1.12 ± 0.14	75.01 ± 8.03 a	1.97 ± 0.22	2.31 ± 0.23	13.31 ± 1.65	0.12 ± 0.01	0.56 ± 0.07	440.11 ± 44.33	16.42 ± 1.50	7.97 ± 0.65
	ELR	54.71 ± 2.45	0.73 ± 0.05	0.81 ± 0.07	57.42 ± 3.64 a	0.91 ± 0.07	1.60 ± 0.15	89.33 ± 9.68 a	2.14 ± 0.19	2.59 ± 0.21	19.43 ± 2.15	0.16 ± 0.02	0.69 ± 0.06	539.17 ± 25.46	16.94 ± 0.29	8.70 ± 0.28
	LLR	39.46 ± 9.35	0.60 ± 0.07	0.69 ± 0.05	37.93 ± 10.03 b	0.76 ± 0.10	1.18 ± 0.12	52.11 ± 4.76 b	1.71 ± 0.18	2.19 ± 0.17	11.50 ± 2.91	0.12 ± 0.02	0.55 ± 0.06	414.24 ± 58.96	15.13 ± 1.00	7.84 ± 0.36
2022	Control	8.35 ± 0.54	0.12 ± 0.01	0.08 ± 0.01	8.14 ± 0.77c	0.20 ± 0.01	0.25 ± 0.01	20.65 ± 2.46c	0.48 ± 0.04	0.30 ± 0.03	3.64 ± 0.55	0.03 ± 0.00	0.07 ± 0.01	141.60 ± 7.04	4.23 ± 0.13	1.06 ± 0.07
	ELR	11.18 ± 0.38	0.15 ± 0.01	0.10 ± 0.01	11.87 ± 0.56c	0.28 ± 0.01	0.34 ± 0.01	23.38 ± 1.80c	0.50 ± 0.05	0.34 ± 0.02	4.86 ± 0.36	0.04 ± 0.00	0.11 ± 0.01	154.10 ± 4.56	4.01 ± 0.33	1.05 ± 0.06
	LLR	9.24 ± 0.77	0.12 ± 0.01	0.08 ± 0.01	9.84 ± 0.88c	0.22 ± 0.02	0.27 ± 0.03	22.00 ± 4.85c	0.46 ± 0.07	0.28 ± 0.03	4.69 ± 1.27	0.04 ± 0.01	0.08 ± 0.02	128.62 ± 10.47	3.66 ± 0.30	0.93 ± 0.10
Main effects	Control	23.58 ± 5.42	0.34 ± 0.08	0.35 ± 0.09	21.78 ± 4.99	0.43 ± 0.08	0.69 ± 0.16	47.83 ± 9.89	1.22 ± 0.27	1.30 ± 0.35	8.47 ± 1.81	0.08 ± 0.02	0.32 ± 0.09	290.86 ± 54.06	10.32 ± 2.15 a	4.52 ± 1.19 a
	ELR	27.50 ± 7.19	0.36 ± 0.10	0.36 ± 0.12	28.95 ± 7.59	0.51 ± 0.11	0.81 ± 0.21	48.11 ± 11.44	1.11 ± 0.28	1.18 ± 0.38	10.32 ± 2.53	0.09 ± 0.02	0.32 ± 0.10	298.50 ± 63.80	8.86 ± 2.13 ab	3.92 ± 1.26 ab
	LLR	20.58 ± 6.09	0.30 ± 0.08	0.31 ± 0.10	20.37 ± 5.98	0.43 ± 0.10	0.61 ± 0.16	33.29 ± 5.85	0.93 ± 0.22	0.99 ± 0.32	7.24 ± 1.71	0.07 ± 0.02	0.26 ± 0.08	235.73 ± 52.09	7.96 ± 1.92 b	3.52 ± 1.14 b
	2021	43.33 ± 5.97 a	0.62 ± 0.06 a	0.69 ± 0.07 a	42.10 ± 7.05	0.76 ± 0.09 a	1.27 ± 0.16 a	72.67 ± 9.57	1.94 ± 0.19 a	2.35 ± 0.20 a	14.48 ± 2.42 a	0.13 ± 0.02 a	0.59 ± 0.06 a	460.07 ± 46.39 a	16.21 ± 1.10 a	8.14 ± 0.49 a
	2022	9.59 ± 0.77 b	0.13 ± 0.01 b	0.09 ± 0.01 b	9.95 ± 0.57	0.23 ± 0.02 b	0.29 ± 0.03 b	22.01 ± 3.10	0.47 ± 0.03 b	0.31 ± 0.03 b	4.40 ± 0.80 b	0.04 ± 0.01 b	0.09 ± 0.01 b	141.44 ± 8.64 b	3.96 ± 0.27 b	1.01 ± 0.08 b
ANOVA	Treatment	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	*	*
	Year	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **
	T x Y	ns	ns	ns	.	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns

Values represent means ± SE (n = 5) separated by Tukey HSD test (p ≤ 0.05). Different letters within a column 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.

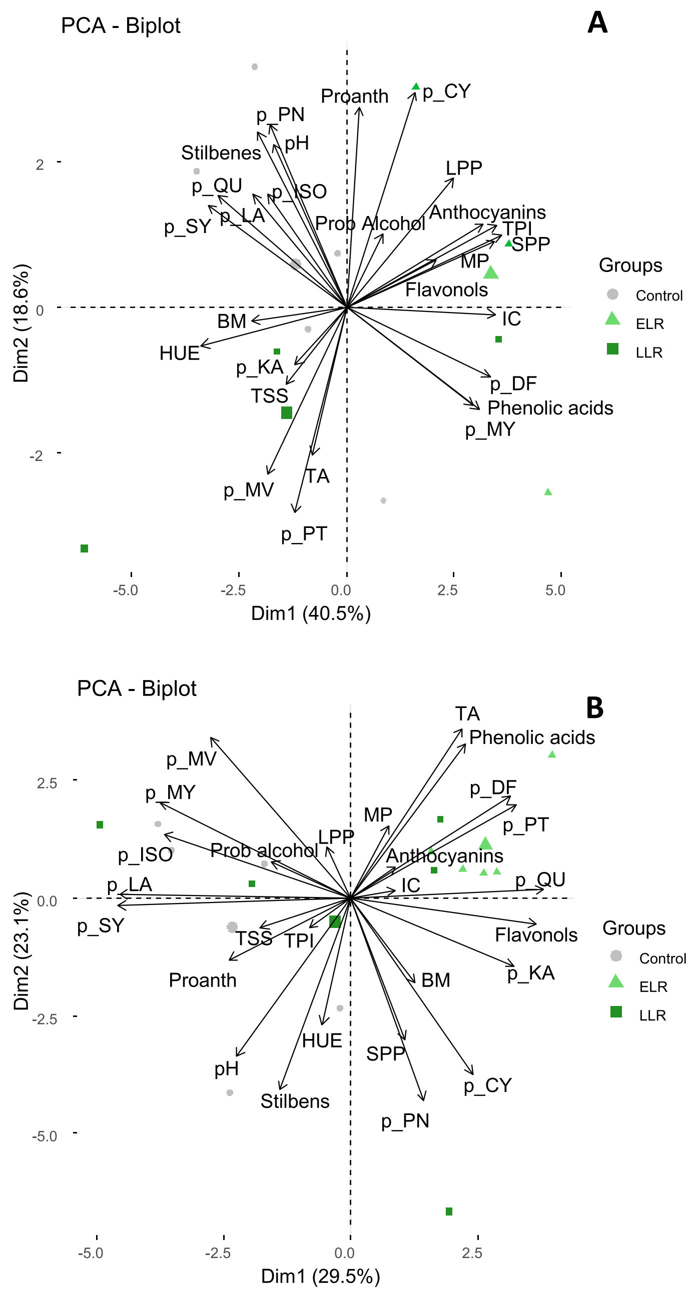

Fig. 3. Principal component analysis of the berry mass and chemistry, conventional enological parameters, CIELab coordinates, anthocyanin-derived pigments and polyphenolic total contents and proportions of the main anthocyanin and flavonol families for the 15 samples of Grenache grapevines subjected to early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain) collected in 2021 (A) and 2022 (B), respectively.

This can be explained by the higher precipitation recorded during the spring of 2022, which could reload with water the soil profile. Studies on Cabernet Sauvignon demonstrated that yield at harvest is highly dependent on the amount of water received by the grapevines early in the season (Yu et al., 2020) (i.e., spring), specially, in rain-fed vineyards (Pérez-Álvarez et al., 2021).

Berry mass and chemistry were affected by both leaf removal treatments and year, but no interaction between them was observed (Supplementary Figure 3). Berry mass was mainly affected by the year and so smaller berries were collected during the 2022 growing season (Fig. 1A and E). Overexposure of berries could lead to reduced berry mass when berries were directly exposed to sunlight through a reduction in sugar allocation (Torres et al., 2020a), this effect being dependent upon the severity of leaf removal (Acimovic et al., 2016; Martínez-Lüscher and Kurtural, 2021).

Year was also the main factor that influenced the sugar content of musts (Fig. 1B and E). Therefore, the warmer and drier year accounted for higher TSS in berries. Leaf removal, especially after veraison, has been proposed as an adaptation tool to warming trends by reducing sugar accumulation rates and postponing the harvest dates (Poni et al., 2018a, 2018b). However, in accordance with a previous study conducted on rainfed vines, the effects of leaf removal are highly dependent upon the environment and grapevine variety (Buesa et al., 2019).

Regarding acidity related parameters, pH ranged between 3.45 and 3.57 and ELR treatment tended to decrease it. It was also observed increased pH values during the warmer growing season (Fig. 1C and E). Titratable acidity (TA) was also affected by year factor, and an increased TA in all the treatments was recorded in the harvest on 2022 but no effect was observed due to leaf removal treatments (Fig. 1D and E). Similarly, Torres et al. (2020a) found no difference on the must TA of

Table 5
Flavonol compounds obtained from the PFs 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.

Year	Treatment	Isorh Gal	Isorh Gluc	Isorh Glucur	Kaemp Gal	Kaemp Gluc	Kaemp Glucur	Myric Gal	Myric Gluc	Myric Glucur	Querc Gal	Querc Gluc	Querc Glucur	Laric Gluc	Syring Gal	Syring Gluc
2021	Control	0.311 ± 0.048 b	8.39 ± 1.22	0.124 ± 0.015	3.38 ± 0.51 b	14.13 ± 2.20	0.968 ± 0.148	6.15 ± 0.96	146.43 ± 23.06	8.61 ± 1.26	56.71 ± 8.45	295.39 ± 36.27	74.28 ± 11.65	2.91 ± 0.39	0.025 ± 0.006	2.86 ± 0.42
	ELR	0.523 ± 0.072 a	12.29 ± 1.97	0.158 ± 0.010	5.80 ± 0.57 a	23.84 ± 2.68	1.357 ± 0.138	10.20 ± 0.67	221.49 ± 16.22	11.41 ± 0.72	84.48 ± 10.30	407.30 ± 47.24	89.35 ± 10.74	3.69 ± 0.39	0.036 ± 0.001	3.08 ± 0.34
	LLR	0.486 ± 0.077 a	12.06 ± 2.15	0.135 ± 0.013	5.38 ± 0.74 a	23.02 ± 3.18	1.319 ± 0.204	7.95 ± 2.08	185.02 ± 40.57	9.02 ± 1.74	78.18 ± 13.71	366.00 ± 50.32	77.43 ± 16.77	3.67 ± 0.58	0.024 ± 0.008	3.28 ± 0.29
2022	Control	0.080 ± 0.008c	2.96 ± 0.23	0.049 ± 0.004	1.47 ± 0.17c	6.06 ± 0.67	0.392 ± 0.036	0.66 ± 0.06	10.48 ± 0.45	0.83 ± 0.05	5.93 ± 0.66	30.60 ± 3.16	18.70 ± 0.94	3.21 ± 0.19	0.003 ± 0.000	2.75 ± 0.14
	ELR	0.098 ± 0.009c	3.62 ± 0.26	0.062 ± 0.005	2.23 ± 0.14 bc	9.19 ± 0.55	0.655 ± 0.024	0.83 ± 0.03	12.51 ± 0.13	1.05 ± 0.02	8.76 ± 0.55	45.71 ± 2.61	25.92 ± 0.66	3.08 ± 0.18	0.005 ± 0.001	2.22 ± 0.19
	LLR	0.094 ± 0.018c	3.21 ± 0.68	0.049 ± 0.009	1.68 ± 0.28c	7.31 ± 1.28	0.389 ± 0.055	0.64 ± 0.09	10.06 ± 1.09	0.77 ± 0.08	6.30 ± 1.09	33.27 ± 7.12	17.11 ± 0.79	2.91 ± 0.32	0.003 ± 0.001	2.39 ± 0.22
Main effects	Control	0.196 ± 0.045	5.68 ± 1.08	0.087 ± 0.015	2.43 ± 0.41	10.09 ± 1.73 b	0.680 ± 0.120	3.41 ± 1.02	78.45 ± 25.13	4.72 ± 1.43	31.32 ± 9.36	162.99 ± 47.35	46.49 ± 10.78	3.06 ± 0.21	0.017 ± 0.005	2.80 ± 0.21
	ELR	0.258 ± 0.075	6.87 ± 1.61	0.098 ± 0.016	3.57 ± 0.63	14.68 ± 2.62 a	0.919 ± 0.127	4.34 ± 1.56	90.87 ± 34.75	4.94 ± 1.72	37.15 ± 12.99	181.31 ± 61.83	49.71 ± 11.15	3.31 ± 0.20	0.017 ± 0.005	2.54 ± 0.22
	LLR	0.241 ± 0.071	6.53 ± 1.70	0.081 ± 0.016	3.06 ± 0.68	13.20 ± 2.92 a	0.738 ± 0.173	3.38 ± 1.43	75.67 ± 32.49	3.86 ± 1.50	33.25 ± 12.87	158.05 ± 57.81	39.73 ± 11.74	3.19 ± 0.30	0.011 ± 0.005	2.72 ± 0.22
	2021	0.417 ± 0.072	10.46 ± 1.76 a	0.136 ± 0.014 a	4.59 ± 0.74	19.20 ± 3.18 a	1.170 ± 0.169 a	7.75 ± 1.39 a	177.42 ± 28.47 a	9.49 ± 1.29 a	70.14 ± 11.03 a	345.17 ± 44.55 a	79.25 ± 11.93 a	3.33 ± 0.43	0.028 ± 0.006 a	3.03 ± 0.23 a
	2022	0.091 ± 0.012	3.26 ± 0.43 b	0.053 ± 0.007 b	1.79 ± 0.24	7.52 ± 1.02 b	0.479 ± 0.069 b	0.71 ± 0.07 b	11.02 ± 0.46 b	0.88 ± 0.08 b	6.99 ± 0.94 b	36.53 ± 3.01 b	20.58 ± 1.92 b	3.07 ± 0.23	0.004 ± 0.001 b	2.45 ± 0.12 b
ANOVA	Treatment	ns	ns	ns	* *	.	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Year	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	* **	ns	* **	*
	T x Y	*	ns	ns	.	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Values represent means ± SE (n = 5) separated by Tukey HSD test ($p \leq 0.05$). Different letters within a column 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%, 1%, and 0.1% probability levels, respectively.

Table 6
Phenolic acids obtained from the PFs 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.

Year	Treatment	Caffeic acid	Caftaric acid	Coutaric acid	*trans*-Ferulic acid	Fertaric Acid	Galic acid
2021	Control	0.072 ± 0.012	10.10 ± 2.43	1.61 ± 0.40	0.038 ± 0.003	6.34 ± 0.84	0.091 ± 0.017
	ELR	0.065 ± 0.014	28.13 ± 11.91	3.38 ± 0.80	0.035 ± 0.002	9.46 ± 0.56	0.056 ± 0.006
	LLR	0.053 ± 0.014	9.05 ± 3.87	1.92 ± 0.69	0.031 ± 0.002	5.53 ± 1.83	0.063 ± 0.016
2022	Control	0.029 ± 0.004	5.82 ± 1.19	3.38 ± 0.53	0.015 ± 0.003	4.73 ± 0.48	0.118 ± 0.021
	ELR	0.027 ± 0.003	8.53 ± 0.57	4.33 ± 0.15	0.014 ± 0.002	5.02 ± 0.47	0.088 ± 0.019
	LLR	0.048 ± 0.024	5.87 ± 0.83	3.58 ± 0.56	0.024 ± 0.011	4.79 ± 0.43	0.128 ± 0.031
Main effects	Control	0.050 ± 0.009	7.96 ± 1.46	2.49 ± 0.43 ab	0.026 ± 0.004	5.54 ± 0.53	0.105 ± 0.014
	ELR	0.041 ± 0.008	15.88 ± 5.54	3.98 ± 0.35 a	0.022 ± 0.004	6.68 ± 0.80	0.076 ± 0.012
	LLR	0.050 ± 0.014	7.07 ± 1.62	2.96 ± 0.48 b	0.027 ± 0.006	5.07 ± 0.74	0.104 ± 0.021
	2021	0.065 ± 0.012 a	14.73 ± 6.97 a	2.18 ± 0.64 b	0.035 ± 0.003 a	6.97 ± 1.24 a	0.074 ± 0.015 b
	2022	0.035 ± 0.014 b	6.74 ± 1.02 b	3.76 ± 0.46 a	0.018 ± 0.007 b	4.85 ± 0.43 b	0.111 ± 0.024 a
ANOVA	Treatment	ns	ns	*	ns	ns	ns
	Year	*	*	* *	* *	* *	*
	T x Y	ns	ns	ns	ns	ns	ns

Values represent means ± SE (n = 5) separated by Tukey HSD test ($p \leq 0.05$). Different letters within a column 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 5%, and 1% probability levels, respectively.

vines subjected to leaf removal. Must acidity is mainly explained by the concentration of malic and tartaric acids, the former being susceptible to degradation under elevated temperatures (Sweetman et al., 2014) as we found in our experiment (data not shown). However, we observed increased pH and TA in 2022, these differences being explained by the lower berry size that year, which could concentrate the main acids present in berries.

3.2. Phenolic profiles and color characteristics

To characterize PF color, CIELab parameters, conventional enological parameters and anthocyanin derived-molecules were analyzed. Conventional enological parameters analysis shows a significant interaction between leaf removal treatments and year for CI and TPI (Table 3). During the first season, ELR tended to increase CI and TPI, whereas no differences were recorded between treatments during the second season. Moreover, treatments did not affect PFs HUE, which was significantly higher during the second season.

CIELab parameters were highly affected by defoliation treatments, year and their interaction. Thus, the second year tended to decrease a* , and increase b* and L* . On the other hand, defoliation treatments decreased a* and L* , and increased b* , although differences in L* due to leaf removal treatments were not evident during the second season as highlights the significant interaction ($p \leq 0.01$). In general, lower a* (positive red) and L* (lightness/darkness) might be related to anthocyanin content and composition (Esparza et al., 2009). Increases of b* coordinate in ELR and during 2022 expressed trends to yellow color in accordance with Sternad Lemut et al. (2013) who found that leaf removal treatments significantly increase b* coordinate in Pinot noir compared to the untreated young wines.

On the other hand, anthocyanin-derived molecules were strongly affected by the year, with 2022 decreasing SPP, LPP and MP. This latter was also increased by ELR in 2021 as shown by the significant interaction T × Y (Table 3). SPP are formed after the reaction of anthocyanins with diverse compounds, including acetaldehyde, pyruvic acid, and flavan-3-ol monomers or dimers, among others (Casassa et al., 2015). These low molecular weight pigments stabilize color in red wine because they are more resistant to bisulfite bleaching and their color is not as pH-dependent as that of anthocyanins (Somers, 1971). Conversely, LPP are pigmented tannins that precipitate with proteins, and they reportedly contribute to perceived astringency (Casassa et al., 2015). Under our experimental conditions, elevated temperatures recorded during the 2022 growing season accounted for decreased polymeric pigments (SPP and LPP). However, we did not identify a preferential formation of LPP over SPP, which had been related to a higher astringency (Casassa et al. 2013). On the other hand, the increase of MP in ELR PF might be related to the higher CI (He et al. 2012).

The total contents of the different phenolic compounds measured in this work are shown in Supplementary Figure 4. There was no interaction between leaf removal treatments and year; however, each factor separately modulated their contents (Fig. 2). Anthocyanin content at harvest was increased in the ELR treatment and strongly decreased in the warmer year (2022, Fig. 2A and F). It is well established that anthocyanin content is responsive to environmental conditions; thus, Yamane et al., (2006) demonstrated that 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, in accordance with the lack of effect of LLR in our study. During the 2022 growing season, Grenache grapevines were subjected to 29 and 13 days with temperatures over 30 °C and 35 °C, respectively, more than in 2021 (Table 1). At high temperatures (35 °C) Mori et al. (2007) reported decreases in anthocyanin contents due to chemical and/or enzymatic degradation, as well as inhibition of anthocyanin biosynthesis. On the other hand, the increase of anthocyanin content with ELR was previously shown in studies with different varieties (Pastore et al., 2017; Sternad Lemut et al., 2013).

The analysis of the anthocyanin profiles showed the main factor affecting their composition was the year (Table 4). Thus, the warmer season (2022) accounted for decreased glucoside, acetyl-glucoside and coumaroyl-glucoside anthocyanin derivatives. A significant interaction between leaf removal treatment and year was observed on the delphinidin and peonidin-3-glucoside derivatives where LLR decreased their concentration in 2021. This treatment also accounted for a decreased acetyl- glucosides and coumaroyl-glucoside malvidin derivatives in both years. This agrees with previous research on Cabernet Sauvignon, where acetyl and coumaroyl glucoside derivatives were decreased in LR wines (Torres et al., 2020b). Additionally, the interaction between leaf removal treatments and year led to changes on the ratios between mono, di and tri-hydroxylated anthocyanins (Supplementary Table 3). Thus, decreased mono/tri and di/tri ratios were observed during 2022 and after applying ELR, whereas LLR accounted for decreased mono/tri hydroxylated anthocyanins, especially in 2021. The correlation between the berry exposure and the diminution of the tri-hydroxylated derivatives was previously reported (Martínez-Lüscher et al., 2019a; Torres et al., 2020a). This is explained by the changes in the transcriptional regulation of flavonoid 3′-hydroxylase (F3’H) and flavonoid 3′, 5′-hydroxylase (F3’5’H) with the higher exposure (Martínez-Lüscher et al., 2014) that seem to be cultivar-dependent (reviewed in Pastore et al., 2013).

Regarding flavonol content and compositions, panels C and F in

Table 7
Flavanol compounds obtained from the PFs 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.

Year	Treatment	Catechin	Epicatechin	Epicatechin-gal	Gallocatechin	Epigallocatechin	procyanidin B1	procyanidin B2	procyanidin B3	procyanidin B4	procyanidin B5	Procyanidin C (trimer)
2021	Control	20.06 ± 4.98	24.43 ± 2.54 a	2.41 ± 2.37 b	0.075 ± 0.014 a	0.202 ± 0.039	ND	10.82 ± 1.16	3.07 ± 0.34	5.06 ± 0.76	1.09 ± 0.12	1.13 ± 0.10
	ELR	12.80 ± 3.86	21.55 ± 1.69 a	0.04 ± 0.01c	0.038 ± 0.006 b	0.213 ± 0.065	ND	8.48 ± 1.04	2.74 ± 0.10	4.46 ± 0.88	1.02 ± 0.06	0.94 ± 0.06
	LLR	14.10 ± 4.32	11.30 ± 2.95 b	6.05 ± 1.50 a	0.027 ± 0.000 b	0.210 ± 0.015	ND	6.31 ± 2.07	1.89 ± 0.55	3.55 ± 1.26	0.90 ± 0.01	0.61 ± 0.17
2022	Control	17.44 ± 4.29	9.15 ± 0.70 b	2.97 ± 0.57 b	0.068 ± 0.011 a	0.043 ± 0.007	7.85 ± 1.32	6.56 ± 0.48	2.30 ± 0.13	2.87 ± 0.40	1.16 ± 0.09	4.73 ± 0.16
	ELR	10.40 ± 0.96	6.68 ± 0.29 b	3.48 ± 0.28 b	0.076 ± 0.006 a	0.040 ± 0.002	5.96 ± 0.70	4.73 ± 0.46	2.17 ± 0.09	1.98 ± 0.23	0.94 ± 0.06	3.83 ± 0.39
	LLR	14.11 ± 2.08	8.13 ± 1.06 b	2.67 ± 0.50 b	0.067 ± 0.007 a	0.039 ± 0.006	7.01 ± 0.83	5.66 ± 0.59	2.21 ± 0.24	2.46 ± 0.28	1.09 ± 0.16	4.32 ± 0.41
Main effects	Control	18.75 ± 3.13	16.79 ± 2.83	2.69 ± 1.15	0.071 ± 0.008	0.123 ± 0.033	3.92 ± 1.45	8.69 ± 0.92 a	2.69 ± 0.22	3.98 ± 0.55	1.13 ± 0.07	2.93 ± 0.61
	ELR	11.30 ± 1.60	12.26 ± 2.52	2.19 ± 0.58	0.062 ± 0.007	0.105 ± 0.038	3.72 ± 1.04	6.13 ± 0.77 b	2.38 ± 0.11	2.91 ± 0.54	0.97 ± 0.04	2.75 ± 0.52
	LLR	14.30 ± 1.98	9.32 ± 1.35	3.94 ± 0.84	0.052 ± 0.008	0.103 ± 0.029	4.38 ± 1.23	5.90 ± 0.85 b	2.09 ± 0.25	2.87 ± 0.53	1.02 ± 0.09	2.93 ± 0.65
	2021	16.60 ± 4.35	20.06 ± 3.40	2.75 ± 1.95	0.052 ± 0.014 b	0.208 ± 0.039 a	ND	8.95 ± 1.55 a	2.66 ± 0.40	4.50 ± 0.89 a	1.02 ± 0.09	0.93 ± 0.14 b
	2022	13.99 ± 2.92	7.99 ± 0.84	3.04 ± 0.46	0.071 ± 0.005 a	0.041 ± 0.005 b	6.94 ± 0.98	5.65 ± 0.59 b	2.22 ± 0.09	2.43 ± 0.33 b	1.06 ± 0.11	4.29 ± 0.36 a
ANOVA	Treatment	ns	* *	ns	ns	ns	ns	*	ns	ns	ns	ns
	Year	ns	* **	ns	*	* **	ns	* *	ns	* *	ns	* **
	T x Y	ns	* *	.	*	ns	ns	ns	ns	ns	ns	ns

Values represent means ± SE (n = 5) separated by Tukey HSD test ($p \leq 0.05$). Different letters within a column 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%, 1%, and 0.1% probability levels, respectively. ND, not determined.

Table 8
Stilbenes obtained from the PFs 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.

Year	Treatment	Trans-Resveratrol	Trans-Resveratrol Gluc	Cis-Resveratrol Gluc	Resveratrol
2021	Control	2.68 ± 0.74	1.65 ± 0.44	ND	4.33 ± 1.19
	ELR	1.37 ± 0.27	1.03 ± 0.20	ND	2.40 ± 0.46
	LLR	1.59 ± 0.21	0.98 ± 0.07	ND	2.56 ± 0.27
2022	Control	ND	0.69 ± 0.17	1.00 ± 0.29	1.69 ± 0.46
	ELR	ND	0.39 ± 0.06	0.37 ± 0.08	0.75 ± 0.14
	LLR	ND	0.52 ± 0.15	0.83 ± 0.33	1.35 ± 0.49
Main effects	Control	2.68 ± 0.53	1.17 ± 0.28 a	1.00 ± 0.21	3.01 ± 0.74 a
	ELR	1.37 ± 0.19	0.63 ± 0.13 b	0.37 ± 0.06	1.37 ± 0.33 b
	LLR	1.59 ± 0.15	0.69 ± 0.11 b	0.83 ± 0.24	1.81 ± 0.34 b
	2021	2.03 ± 0.57	1.30 ± 0.33 a	ND	3.32 ± 0.90 a
	2022	ND	0.53 ± 0.14 b	0.73 ± 0.27	1.26 ± 0.24 b
ANOVA	Treatment	ns	.	ns	.
	Year	ns	* *	ns	* *
	T x Y	ns	ns	ns	ns

Values represent means ± SE (n = 5) separated by Tukey HSD test ($p \leq 0.05$). Different letters within a column 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%, and 1% probability levels, respectively. ND, not determined.

Fig. 3 show that PF flavonol content was unaffected by leaf removal whereas a dramatic diminution was recorded in 2022. On the other hand, flavonol composition responded to LR treatments and year. Interaction between LR and year was significant for the galactoside derivatives of isorhamnetin and kaempferol (Table 5), where ELR and LLR tended to increase them whereas 2022 decreased them. Kaempferol glucoside was also increased by ELR and LLR. As occurred with the anthocyanin profiles, the rest of flavonol compounds were decreased during the second year (2022) despite flavonols being not as sensitive to temperature as anthocyanins are (Martínez-Lüscher et al., 2020). However, cluster exposure to solar radiation strongly affect flavonol composition due to flavonol synthesis is mainly regulated by the exposure to UV-B radiation (Martínez-Lüscher et al., 2014). In agreement with previous studies, LR treatments accounted for increased kaempferol derivatives (Pastore et al., 2017; Martínez-Lüscher et al., 2019a; Torres et al., 2020a). Thus, the interaction between year and defoliation treatments affected the ratio between mono/tri hydroxylated flavonols, whereas a higher di/tri hydroxylated ratio was reported for 2022 (Supplementary Table 3). As discussed above, this result could be explained by the impact of exposure on the transcriptomic regulation of the enzymes involved in flavonoid hydroxylation (Martínez-Lüscher et al. 2014), which lead to a lower ratio of mono/tri derivatives and a higher ratio of di/tri derivatives.

Phenolic acids showed the same trend observed in the anthocyanin content; ELR enhanced their content, while 2022 decreased it (Fig. 3D and F). Phenolic acids were mainly affected by the growing season, thus, during the 2022 year, decreased contents of caffeic, caftaric, *trans*-ferulic and fertaric acids and increased contents of coutaric and gallic acids were recorded (Table 6). Coutaric acid was also affected by leaf removal treatments, where ELR and LLR accounted for increased and decreased content, respectively. Contrarily, Nicoletti et al. (2013) found that the concentration of coutaric and caftaric acids were significantly higher in berries from post-veraison defoliated vines compared to those which were not defoliated or to fruit set defoliated ones. These discrepancies might be explained by defoliation can result in different phenolic accumulation trends related to many factors, such as site, row orientation, exposition and canopy architecture (Casassa et al., 2015).

Flavanols content in the PFs were highly affected by leaf removal treatments that accounted for their decrease (Fig. 2D and F). Regarding their composition, interaction between LR treatments and year was significant on epicatechin, epicatechin-gallate and gallocatechin (Table 7). Procyanidin B2 was decreased by both leaf removal treatments. Warmer season (2022) accounted for decreased content of epigallocatechin, and procyanidins (B2 and B4). These results partially corroborated those from Casassa et al., 2015, who reported that leaf removal at pre-flowering and veraison increased flavan-3-ols compared with control. On the other hand, the effects of LR treatments on B-type procyanidins were consistent with their results with no clear correlation to higher exposure. Finally, during the 2022 season an increment of procyanidin C was recorded (Table 7). The reaction between anthocyanins and extracted proanthocyanidins during fermentation contributes significantly to the formation of polymeric pigments, which are thought to make a significant contribution to the stabilization of wine color (Singleton and Trousdale, 1992). Therefore, the increased HUE recorded during the 2022 season (i.e., higher absorbance at 420 nm and b* coordinate value; Table 3), indicating the trend to yellow component compared to 2021 samples, suggested a higher polymerization of the proanthocyanidins with the higher exposure during the warmer season (Torres et al., 2020b). This result joined to the decreased content of anthocyanins during the warmer season, might lead to a “less young” or “more evolved” wine with the concomitant reduction of the aging potential (He et al., 2012). However, given the multiple noncovalent interactions that can occur between proanthocyanidins and other macromolecules, the final proanthocyanidin extraction might not be directly dependent upon tannin concentration (Bindon et al., 2014). Furthermore, these authors demonstrated that the selective extraction of polysaccharide affected the adsorption of proanthocyanidin by berry cell walls (Ruíz-García et al., 2014). Thus, the adsorption of proanthocyanidins by cell walls was impaired after removing pectic polysaccharides by chelator, whereas hemicellulosic fractions had a higher binding capacity for proanthocyanidins (Ruíz-García et al., 2014).

Finally, stilbenes were decreased in ELR treatment and during the 2022 year (Fig. 2F). The main stilbene compound found in the PFs was resveratrol. Leaf removal treatments (ELR and LLR) and year (2022) decreased the concentration of *trans*-resveratrol glucoside and resveratrol (Table 8). These results corroborated previous research with other grapevine varieties (Barbera, Croatina and Malvasia) where the warmest year accounted for decreased stilbene contents (Bavaresco et al., 2008).

3.3. Effect of leaf removal at different time of the season on the carbohydrate and flavonoid metabolisms

Given the strong effect that the year had on the PF color and metabolite contents, we conducted separate principal component analysis (PCA) of each year in order to assess the effect of LR treatments and visualize general patterns in the samples. Fig. 3A shows the biplot of the PCA for 2021 growing season. The first two principal components (PC) accounted for 40.5% and 18.6% of the variance, respectively. ELR samples were separated from the control and LLR along PC1, whereas a slight distinction between LLR and control was observed on PC2.

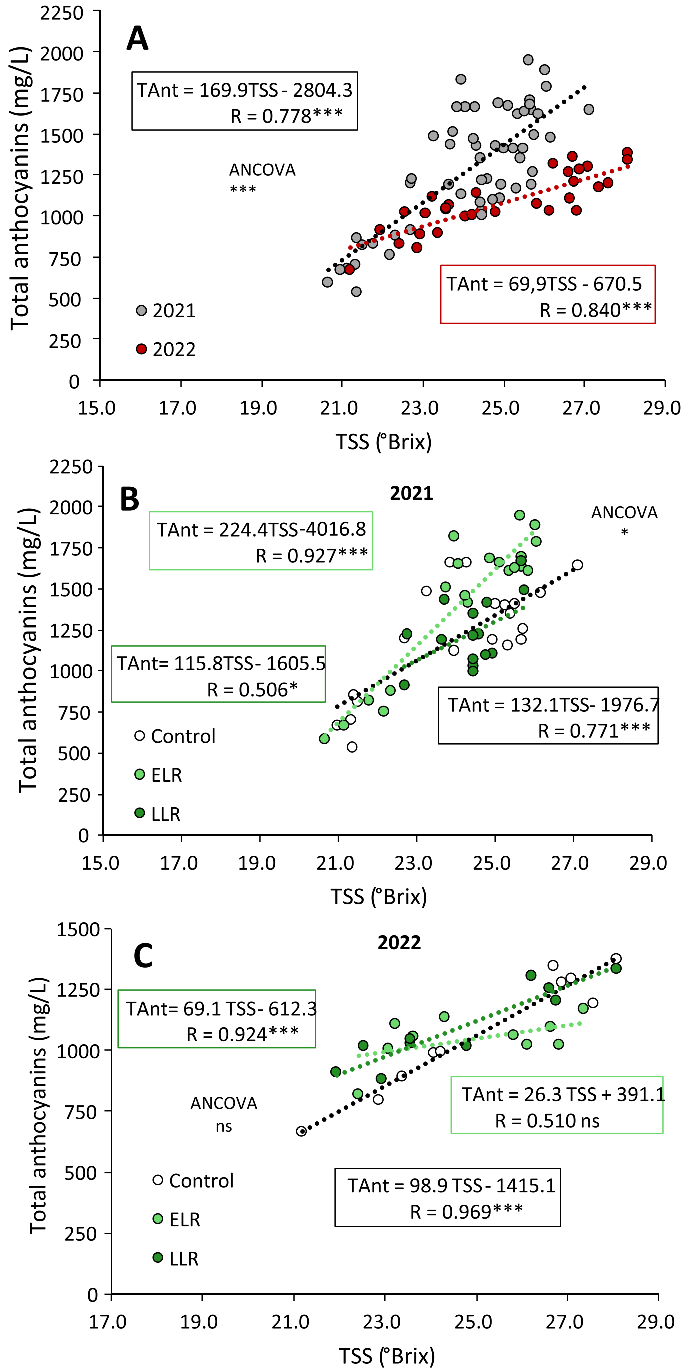

Fig. 4. Relationship between the must anthocyanin content and the total soluble solids (TSS) of the Grenache grapevines subjected to an early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain) separated by year (A) and by leaf removal treatment in 2021 (B) and 2022 (C). Dashed lines represent the linear regression according to Pearson correlation.

Separation along PC1 was explained by anthocyanins and their polymerized derived parameters (LPP and SPP), flavonols, proportion of kaempferol, quercetin and syringetin, IPT, and PF color parameters (i.e., HUE and CI). On the other hand, flavanols, stilbenes, phenolic acids, proportion of malvidin, cyanidin and peonidin and acidity-related parameters accounted for the distinction along PC2.

PCA of 2022 dataset (Fig. 3B) showed a clear distinction between ELR and Control along PC1, which explained 29.5% of the variance. Again, separation across PC1 was related to flavonols, anthocyanins and the proportions of the different flavonoid compounds.

It is well established that the changes in the light environment received by the berries through their photoreceptors are directly involved in controlling the accumulation of phenolic compounds in berry skins without affecting total soluble solids, acidity or berry size (González et al., 2015). Nevertheless, our results highlighted that the effects of LR treatments on berry composition were highly dependent upon the environmental conditions of each growing season as previous researchers reported before (Buesa et al., 2019; Ivanišević et al., 2020; Lu et al., 2022). Thus, during the warmer season (2022), control berries were highly exposed too and no clear distinction between LLR and Control was assessed. Finally, these results also highlighted the role of LR treatments in affecting the flavonoid hydroxylation patterns given the higher correlation between the proportions of the flavonoid families and the separation across the first two components (Fig. 3A and B).

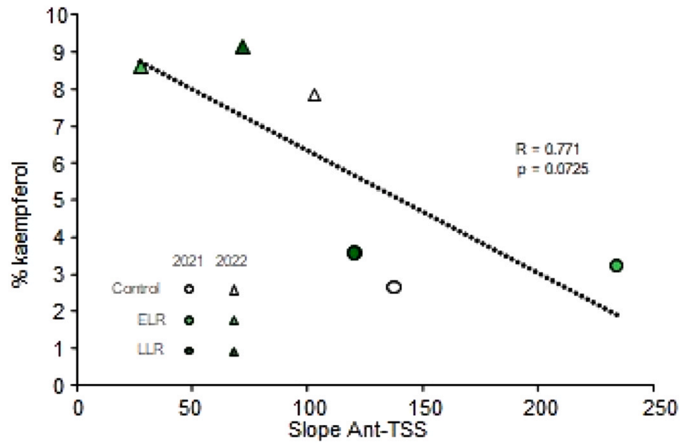

Fig. 5. Relationship between the percentage of kaempferol and the slope of the relationship between must anthocyanin content and the total soluble solids (TSS) of the Grenache grapevines subjected to early leaf removal (ELR) or late leaf removal (LLR) compared to the untreated vines (Control) collected in Alfaro, La Rioja (Spain).

Differences in the flavonoid hydroxylation patterns in response to light environment are known given its effects on the transcriptomic regulation of the F3′H and the F3′5′H and the competition between these enzymes for the same substrates (Martínez-Lüscher et al., 2014).

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

Fig. 4A shows the linear relationship between the must anthocyanin concentration and TSS during the 2021 and 2022 growing seasons. These data showed a significant decoupling between primary and secondary metabolisms in warmer and drier seasons as highlighted by the significance of both regressions ($p \leq 0.001$). Thermal decoupling can arise from changes in rate or a combination of changes in rate and after onset. Sadras and Bonada (2022) demonstrated thermal decoupling of anthocyanin and sugars in Syrah and Cabernet Franc in Barossa Valley of Australia, where the onset of anthocyanin accumulation, on a sugar scale, was delayed under elevated temperature (Sadras and Moran, 2012). Previous studies suggested that the effect of higher temperature is most critical in a constrained time window after veraison, rather than the whole ripening period (Sadras and Bonada, 2022). Our results reinforced this hypothesis as mean daily temperature at veraison was ca. 2.5 °C more in 2022 compared to 2021 (Table 1).

On the other hand, Fig. 4B and C show the effect of leaf removal treatments on the anthocyanin and sugar relationship in 2021 and 2022 growing seasons, respectively. In 2021, LR treatments affected the relationships between sugars and anthocyanins as highlighted in the ANCOVA conducted ($p \leq 0.05$). Thus, ELR berries balanced the accumulation of anthocyanins and sugars (Fig. 1B). However, during the growing season of 2022, there was no difference between the regressions of each treatment 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 (Fig. 4B and C, respectively). Our data showed that ELR might improve the balance between sugar and anthocyanin metabolisms during ripening, especially during the 2021 growing season, while LLR did not significantly affect sugar and anthocyanin metabolism. Accordingly, previous studies have shown that defoliation at veraison decreased total soluble solids but this came with a potential diminution of berry anthocyanins (Pastore et al., 2013; Pastore et al., 2017; Tessarin et al., 2014) whereas a later basal leaf removal (after veraison) did not lead to significant changes in berries (Tessarin et al., 2014; 2022).

Proportion of kaempferol has been selected as a good biomarker of the berry solar exposure (Martínez-Lüscher et al., 2019). Under our experimental conditions a linear relationship among the % of kaempferol and the slope of the linear regression of TSS and anthocyanins was shown (Fig. 5) suggesting that it could also be a good biomarker of the anthocyanin and sugar decoupling during berry ripening under different conditions. Furthermore, Fig. 5 shows that optimal solar exposure for adjusting primary and secondary metabolisms corresponded with a percentage of kaempferol around 4%.

4. Conclusion

We hypothesized that the timing of leaf removal among two phenological stages (after fruit set and in late veraison) might delay ripening and reduce the accumulation of sugars without affecting the phenolic composition. Our results revealed that increasing the cluster exposure to temperature and radiation strongly affects primary and secondary metabolisms. However, basal defoliation at post-fruit set was more effective for delaying ripening by decreasing TSS at harvest and enhancing anthocyanin content compared to leaf removal performed at veraison, especially during the less warm season. Nevertheless, both leaf removal treatments modified flavonoid metabolism as highlighted by the different proportions of anthocyanin and flavonol families at harvest. On the other hand, we assessed the effects of defoliation at different timings on sugar:anthocyanin decoupling under warmer temperatures. Our data showed that ELR could reduce this decoupling when environmental conditions are not too extreme, but no effect was observed during the warmer and drier season.

CRediT authorship contribution statement

Purificación Fernández-Zurbano: Methodology, Resources, Writing – review and editing. **Gonzaga Santesteban:** Conceptualization, Methodology, Validation, Writing – review and editing. **Ana Villa-Llop:** Investigation, Data curation. **Maite Loidi**: Investigation, Data curation. **Carlos Peñalosa**: Investigation. **Sergio Musquiz:** Investigation. **Nazareth Torres:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105729.

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