Phenolic profile of grapevine cv. Tempranillo skins is affected by timing and severity of early defoliation

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Abstract

Aim of study: To investigate the effects of three early leaf removal treatments on the phenolic compounds of cv. ‘Tempranillo’ (Vitis vinifera L.) grape skins.

Area of study: The experiment was conducted in a vineyard located in Requena, Valencia (South-eastern Spain) over two consecutive seasons.

Material and methods: Four treatments were investigated over two seasons in drip-irrigated vines: Control (C), non-defoliated and three defoliation treatment, applied at different phenological stages and intensities where all leaves from the first 6 nodes were eliminated just before flowering (ED) and at fruitset (LD). The fourth defoliation treatment was performed at the same time of ED but only the leaves facing east of the eight first nodes were removed (EED). At harvest, thirty-eight phenolic compounds were quantified by HPLC in the grape skins, including anthocyanins, flavanols, flavonols, hydroxycinnamic acids and their tartaric derivatives.

Main results: A general increase of the skin phenolic compounds concentration was found in response to the defoliation treatments. The largest and more significant effects were observed for LD in 2009 with relative increases with respect to the un-defoliated vines of 14.8, 86.0, 119.0, and 75.9% for anthocyanins, flavanols, flavonols and hydroxycinnamates, respectively. On the other hand, EED did not clearly modify any polyphenolic compound. In addition, the response of phenolic families analyzed to defoliation treatments was different. Malvidine derivatives were not altered by any of the treatments, while the contents of quercetin and kaempferol derivatives and ferulic and coumaric acids, increased in both years when LD was applied.

Research highlights: The defoliation effects on specific phenolic substances were dependent on timing, severity, and the season. Skin phenolic compounds increase in response to defoliation treatments and flavonols and hydroxycinnamates were the most affected families.

Additional key words: anthocyanins, flavanols, flavonols, hydroxycinnamic acids

Abbreviations used: B1, B2 and B3 (procyanidins B1, B2 and B3); C (Control); CA [(+)-catechin]; CF (caffeic acid); CFT (caftaric acid); CI (cool night index); COU (coumaric acid); COUT (couteic acid); Cy (cyanidin); DI (Dryness index); Dp (delphinidin); DpA, CyA, PtA, PaNA and MvA (respective acetylgulcose forms); DpC, CyC, PtC, PaNC and MvC (respective p-coumaroylgulose forms); DpG, CyG, PtG, PaNG and MvG (respective 3-glucoside forms); EC [(−)-epicatechin]; ED (Hand leaf removal applied just before flowering); EED (Hand leaf removal applied just before flowering but only the leaves facing east of the eight first nodes were removed); FE (ferulic acid); FET (fertaric acid); HI (Huglin index); Ih (isohamnetine); Kp (kaempherol); KP (kaempherol-3-rutinoside); LD (Late hand defoliation applied at fruit set); Mv (malvidine); My (myricetin); MyG, KpG and IhG (respective 3-glucoside forms); Pn (peonidin); Pt (pe-tunidine); Qc (quercetin); QcGL (quercetin-3-galactoside); QcGR (quercetin-3-glucuronide); QcR (quercetin-3-rutinoside); TA (titratable acidity); TPC (total phenolic content); TTR (t- resveratrol); TSS (total soluble solids).

Authors’ contributions: Conceived and designed the research: DM and EV. Substantial contributions to the conception or designs of interpretation of data for the work: EV, MV, JRC, DSI. Coordinated the research project: JRC, DSI, EV. Analyzed the data and wrote the paper: DM and EV. Conception of design: EV, DSI. Critical revision and final approval of the version to be published: EV, MV, DSI.

Citation: Moreno, D; Intrigliolo, DS; Vilanova, M; Castel, JR; Gamero, E; Valdés, E (2021). Phenolic profile of grapevine cv. Tempranillo skins is affected by timing and severity of early defoliation. Spanish Journal of Agricultural Research, Volume 19, Issue 3, e0905. https://doi.org/10.5424/sjar/2021193-17089

Supplementary material (Tables S1 and S2) accompanies the paper on SJAR’s website

Received: 26 Jun 2020. Accepted: 30 Jul 2021.

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Funding agencies/institutions Project / Grant
INIA-FEDER RTA2008-0037
GOBEX GR10006
AEI-FEDER AGL2014-54201-C4-4-R; AGL2017-83738-C3-3-R
Junta de Extremadura and European Regional Development Fund (ERDF) Grant to D. Moreno contract in CICYTEX

Competing interests: The authors have declared that no competing interests exist.

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Introduction

In modern viticulture, canopy management plays a key role, and it is widely recognized as an important factor in the composition of the resulting grapes and wines. Traditionally, leaves have been removed around the area of the clusters between fruit set and ripening in cool climate viticulture regions around the world to manipulate and to reduce the canopy density in order to increase canopy air circulation and improve bunch microclimate (Poni et al., 2010). Limiting carbohydrate supply, induced by early leaf removal applied around bloom, causes a reduction in fruit set (Poni et al., 2009; Acimovic et al., 2016), lowering cluster compactness, decreasing yield (Tardaguila et al., 2010), affecting the content of primary and secondary metabolites in grape berries (Lemut et al., 2013a; VanderWeide et al., 2018; Yue et al., 2019), improving fruit technological maturity and polyphenolic composition at harvest (Gatti et al., 2012; Silvestroni et al., 2018), and improving wine aroma (Vilanova et al., 2012; Moreno et al., 2017). However, it has been reported that the effect of early defoliation depends on timing, intensity and site specific characteristics (Uriarte et al., 2012). Severe defoliation (i.e. more than five leaves) has to be carried out to clearly affect vine performance (Tardaguila et al., 2008).

Phenolic compounds belong to an important group of pigments that are widely spread throughout the plant kingdom. These compounds, which include flavonoids (i.e., anthocyanins, flavonols and flavanols) and non-flavonoids (i.e., hydroxycinnamic acids and stilbenes), have been described as important indicators of grape quality. The main anthocyanins identified in Vitis vinifera L. grapes and wines are the 3-O-glucosides, 3-O-acetyl glucosides and 3-O-pcoumaroyl glucosides of five anthocyanidins (delphinidin-Dp, cyanidin-Cy, petunidin-Pt, peonidin-Pn and malvidin-Mv). Flavanols are also largely localized to the grape skins, where they are found as flavonol glycosides of quercetin, kaempferol, myricetin and isorhamnetin. Flavan-3-ols, such as catechin and epicatechinin, and flavan-3,4-diol dimers such as B1, B2 and B3, are present in the skin and mainly in grape seeds. Lastly, hydroxycinnamic acids such as coumaric, caffeic and ferulic acids, and their tartaric esters or diesters caffeoyltartaric acid, p-coumaric acid (coumaryltartaric acid), and ferulic acid (feruloyltartaric acid) are commonly accumulated in berry skin and the flesh of white and red vinifera and non-vinifera varieties (Downey et al., 2006).

The phenolic content and profile of grapes depends on the cultivar and, for a determinate cultivar, on the growing area, climatic conditions, and viticultural practices (Downey et al., 2006). As the different polyphenolic compounds are synthesized at different times, and as they have different chemical reactivity and play different roles in the organoleptic characteristics of wines, a detailed and thorough study of their response to the different viticultural and oenological practices is necessary.

Studies have shown that the timing of defoliation (Poni et al., 2006; Lemut et al., 2013b) as well as the specific percentage of leaves removed (Acimovic et al., 2016) may affect phenolic berry composition differently. Contrary to anthocyanins, limited data exist regarding the effect of this technique for other skin phenolic compounds.

Color is an important factor for evaluating the quality of red wine, and it is linked to the accumulation of anthocyanins in the grape berry skin. However, it is not only the anthocyanin concentration and profile that is responsible for wine color: copigmentation phenomena can account for 30% to 50% of color in young wines (Boulton, 2001). Some authors suggest that the copigmentation reactions of anthocyanins are the first phase in the formation of stable polymeric pigments during wine aging (González-Manzano et al., 2008). Copigmentation results from molecular interactions between anthocyanin pigments and other organic molecules, called cofactors. The principal cofactors in young red wines are flavonoids and non-flavonoid phenolic compounds, such as the flavonols, flavan-3-ols, oligomeric proanthocyanidins, cinnamic acids and hydroxycinnamoyl derivatives (Boulton, 2001). Given the importance of these phenolic substances on the stability and intensity of the color of red wines through copigmentation phenomena, the study and monitoring of agronomic factors and viticultural practices that increase their content and improve the cofactor concentration is a major objective for the production of high quality wines, especially in terms of their color.

A previous work on ‘Tempranillo’ vines grown in South-eastern Spain (Risco et al., 2014) studied the effects of early defoliation on grape performance, cluster micro-climate and berry soluble solids, acids and general phenolic composition. In the same way, the aim of this research was to provide more information about the effect of defoliation on anthocyanins, flavanols, flavonols, and hydroxycinnamic phenolic compounds from grape skins in a ‘Tempranillo’ vineyard grown in the semiarid conditions of SE Spain.

Material and methods

Plant material and site description

The experiment was conducted in a ‘Tempranillo’ vineyard (rootstock 161-49) located in Requena, Valencia, SE Spain (lat: 39° 29’ N, long: 1° 13’ W; elevation 750 m asl) for two consecutive seasons (2008 and 2009).
The same experimental set up was also used in Risco et al. (2014) to report the grape performance results. The vineyard was planted in 1991 at a spacing of 2.45 × 2.45 m (1666 plants/ha). Vines were trained to a vertical shoot position (VSP) training system oriented in a north-south direction. All treatments were drip-irrigated to replace half of the estimated crop water needs. By the end of the season, irrigation applications were 130 and 174 mm in 2008 and 2009 respectively. The soil at the site was a Typic Calciorthid with clay loam to light clay texture and the climate semi-arid continental with annual rainfall of 430 mm, with 65% falling during the vine dormant period.

**Climatic conditions**

Weather conditions were measured with an automated meteorological station located in the plot. Climatic indices were calculated (Table S1), related to water balance and thermal conditions: Huglin index (HI) (Huglin, 1978), cool night index (CI) and dryness index (DI) (Tonietto & Carbonneau, 2004). These indices are commonly used in viticulture-climate studies, helping to define the representative variability of viticulture climates worldwide.

**Leaf removal treatments**

A randomized block design was set up with each treatment replicated across four blocks. Four treatments were studied:

- **Control:** no leaves were removed.
- **ED:** Early defoliation. Leaf removal was applied on the 29th and 25th of May in 2008 and 2009 respectively, just before flowering (phenological stage H, Baggiolini, 1952). All leaves from the first six nodes, including leaves from lateral shoots at these node positions were removed. The portion of the total leaf area removed were 79% and 93% in 2008 and 2009, respectively.
- **LD:** Late defoliation. Leaf removal was performed at fruit set (phenological stage J, Baggiolini, 1952) on the 17th and 6th of June in those years, respectively. All leaves from the first 6 nodes were removed. The portions of the total leaf area removed were 58 and 84% in 2008 and 2009 respectively.
- **EED:** As in the ED treatment, leaf removal was performed just before flowering in phenological stage H, but only the leaves facing east of the eight first nodes were removed (four leaves). The portion of the total leaf area removed were 55% and 60% in 2008 and 2009, respectively.

In all leaf defoliation treatments, the main leaves from the secondary shoot were also removed. The amount of leaf area (LA) removed was estimated using allometric relationships between shoot length and leaf area obtained in four shoots per experimental vine using the methodology proposed by Mabrouk & Carbonneau (1996).

**Harvest and sampling**

All treatments were harvested on the same day, on the 1st and 20th of September in 2008 and 2009, respectively. The clusters were harvested according to a technological maturation index (TSS/TA), using a threshold ratio between 5 and 6.5. Analysis for total soluble solids (TSS, °Brix), and titratable acidity (TA, g/L) were performed according to the official methods of the Organisation Internationale de la Vigne et du Vin (OIV, 1990). At harvest, 500 g of healthy grapes were randomly sampled from each experimental plot. From them, samples composed of 150 berries were obtained, weighed (analytical balance, Mettler Toledo PL602-S), and stored three months at -20 °C until analysis.

**Determination of berry skin low molecular weight phenolic compounds**

**Extraction of berry skin phenolics**

Berry skins of 50 berries from each plot (in duplicate) were separated from the pulp and seeds with a scalpel and weighed using a high-precision analytical scale (CSB 600C, Cobos, Barcelona). The skins were brought to full dryness in a Virtilis Genesis 25L0L lyophilizer (Virtis Company, Gardiner, NY, USA). The lyophilized skins obtained were weighted and homogenized. Extraction of phenolic compounds from 0.5 g of homogeneous grape skin powder with 4 mL methanol/formic acid (95:5 v/v) was done according to Gao et al. (1997). The extracts were centrifuged for 10 min at 1962 × g. The supernatant was collected in a laboratory flask and stored in the fridge at 4 °C. The extraction process was repeated until the skin were colorless. The supernatants were combined in a flask and the volume was brought to 50 mL with methanol:formic acid. For each sample, two extractions were performed.

**Analysis of berry skin phenolic compounds by HPLC**

The acid-alcohol extracts, which were previously passed through a 0.25 μm pore size nylon membrane (Chromafil GF/PET-20/25 filters), were analyzed with an HPLC Agilent Model 1200 LC instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with a UV–Visible diode-array detector (DAD), fluorescence spectrophotometer detector (FLD), and the Chemstation software package for LC 3D Systems (Agilent Technologies, Palo Alto, CA, USA) to control the instrument and for data acquisition and data analysis.
The analysis was carried out as described, following a previously reported method (Moreno et al., 2015). For identification and quantification of compounds, 10 µL extracts were injected directly into the HPLC. Absorbance at 280, 320, 360 and 520 nm was measured with the DAD detector and excitation at 280 and emission at 320 nm was measured with the FLD detector. Phenolic compounds were identified according to the retention times of pure compounds available. The total amount of polyphenols families was given in mg of malvidine-3-glucoside, myricetin-3-glucoside, (+)-catechin and caffeic acid per kg of berry fresh weight for anthocyanins, flavonols, flavanols and hydroxycinnamic acids, respectively, and total phenolic content (TPC) was calculated from the sum of the concentrations (mg of gallic acid per kg of berry fresh weight) for each compound individually.

Forty-two phenolic compounds were identified and quantified in 'Tempranillo' grape skins, including anthocyanins, flavonols, and hydroxycinnamic acids, as well as flavanol monomers and dimmers. The HPLC analyses were performed in duplicate. Thus, each result is the mean value from 32 determinations (2 HPLC/extract*2 extract/sample*2 samples/block*4 blocks/treatment).

**Statistical analysis**

The effect of treatment, year and treatment × year interaction was evaluated by a one- (treatment) or two-way (treatment, year) analysis of variance ANOVA. Means were compared using Tukey’s HSD test (*p*<0.05). The data analyses were performed using XLstat-Pro (Addinsoft, Paris, 2009).

**Results**

**Climate index study**

The HI were 2109 and 2271 for 2008 and 2009 vintages, respectively (Table S1). This implies that in the area of study, the HI corresponded to warm temperate (HI+1; 2100<HI<2400). The CI for this location reached values from 14.5 °C (2008 vintage) to 14.7 °C (2009 vintage) classifying the area of study as temperate nights (CI-1; 14<CI<18). In terms of drought, different DI values were obtained in the two seasons, with the 2008 vintage classified as sub-humid (DI-1; 50<DI<150) and 2009 as moderately dry (DI+1; -100<DI<50).

**Effect of early defoliation**

**Berry components**

The data on the effect of early defoliation, year and their interaction on berry components and yield in 'Tempranillo' cultivar is reported in Table S2 [suppl]. The effect of the treatment was significant on berry weight, pulp and seed fresh weight, and yield, however all parameters were affected by year except for the pulp and seed fresh weight. It is important to note the yield decrease reported in 2009 respect with the previous season, especially in the defoliated treatments (17.1%, 46.5%, 39.0% and 28.1% for C, ED, LD and EED respectively). When the effect of treatments was investigated on a yearly basis, it is to mention that in 2008, with respect to C, all early leaf removal treatments (ED, EED and LD) significantly reduced the berry weight, with the lowest value for LD. These decreases in berry weight were due to a reduction in weight of all the berry constituents, pulp, seeds and skins. Nevertheless, in 2009, no effect on berry weight was observed in LD and EED, and skin weight was not modified by any of the treatments imposed. Regarding the skin-to-berry-ratio (%), no significant differences were detected for any of the two years under study. Finally, with the exception of EED in 2008, a significant decrease in yield was observed for all defoliated treatments respect to C in both years. Besides, these decreases were higher in 2009. Thus, the reduction in yield registered compared with C in LD and ED reached 22.5% and 26.1% in 2008 and 50.0% and 45.6% in 2009, respectively. In 2009, the reduction in yield observed in EED compared with the C treatment was of only 27.2%. The entire grapevine performance data set is reported in Risco et al. (2014).

**Polyphenolic compounds**

**Polyphenolic families:** As depicted in Fig. 1, the content of polyphenolic families analyzed was different on both seasons and a statistically significant year effect was observed for all families except for flavonol. With respect to the experimental treatments carried out, in both years, a general trend to increase the concentration of polyphenolics from defoliated treatments respect to the Control was found. Statistically significant differences were found on the biannual mean with exception of total anthocyanin (Fig. 1). Because the effects of defoliation were different according to the applied treatment, polyphenolic groups and seasons, it was interesting to analyse the effect of the treatments on different families in each year. In 2008, with respect to the C, LD did not significantly affect the total anthocyanin family content in the skins of cv. ‘Tempranillo’ berries; however, flavanol, flavonol and hydroxycinnamic compounds concentrations increased significantly (27.3%, 105.7% and 38.3% respectively, *p*<0.01). There were not significant differences between LD and ED, either EDD or C treatments for any polyphenolic group analyzed.

In 2009, there were clear increases of flavanol, flavonol and hydroxycinnamic contents on LD (86.0%,...
Defoliation effects on ‘Tempranillo’ grape skin phenolics

Defoliation effects on ‘Tempranillo’ grape skin phenolics

111.9% and 75.9%, respectively) and also on ED (86.0%, 87.1% and 47.3%, respectively) with respect to the C. Significant increases of TPC were found on LD. The LD was the most effective treatment for increasing phenolic compounds concentration of the cv. ‘Tempranillo’ skins.

Anthocyanin compounds: Fifteen compounds were identified, quantified and grouped according to the corresponding anthocyanidin derivatives: Dp, Cy, Pt, Pn and Mv, as shown in Table 1. The results show that Mv, (61.3%), Pt (17.4%) and Dp (14.7%) derivatives were the predominant anthocyanin substances in grape skins of ‘Tempranillo’, regardless of the season and the treatment, while Cy and Pn exhibited the lowest values in all treatments under our experimental conditions. Table 1 also shows that anthocyanidin-monoglucosides were predominant forms, followed by coumaroyl glucosides. However, the acetyl glucoside group had the lowest value. Malvidine-3-glucoside (43.5% with respect to the total anthocyanin content average value) was the major individual anthocyanin compound.

Table 1 reflects the effect of treatment, year and their interaction on these compounds. The factor treatment was statistically significant on nine individual anthocyanin compounds and on ΣDp, ΣCy, ΣPt and ΣPn. It should be highlighted that ΣMv (the most abundant group) were not affected by the early defoliation treatments, neither by the year and treatment × year effect.

In general, the contents of ED and EED were similar to C in 2008 and 2009, and LD was the most effective treatment for increasing the anthocyanin compounds in both seasons. Focusing on this treatment (LD), with respect to C, significant differences were registered for DpG, CyA, and PtG in 2008 and CyC and PnC in 2009. Thus, in 2008, LD increased the concentrations of ΣDp, ΣCy and ΣPt derivatives in ‘Tempranillo’ grape skins. However, only ΣDp and ΣPt increased in 2009. Nevertheless, it was noticeable that MvG compounds were not altered by any of the treatments in any of the two years studied. The anthocyanin profile was modified by LD defoliation treatment in both years. Considering the biannual mean, LD increased the content of DpG, DpA, ΣDp, CyA, CyC,

Figure 1. Effect of early leaf removal on ‘Tempranillo’ grape-skin content of total anthocyanin, flavanols, flavonols, hydroxycinnamic compounds and total phenolic content (TPC)
Table 1. Effect of early leaf removal on grape-skin anthocyanin concentration (mg/kg berry fresh weight) under three defoliation regimes

| Compound | Treatment (T) | Year | Interannual mean | Statistical significance |
|----------|---------------|------|------------------|-------------------------|
|          |               | 2008 | 2009             |                         |
| Pt derivates |              |      |                  |                         |
| DpG      | Control       | 131.49 | 84.94            | 108.22                  |
|          | ED            | 151.02 | 90.81            | 120.91                  |
|          | LD            | 161.04 | 112.26           | 136.65                  |
|          | EED           | 134.99 | 81.32            | 108.15                  |
| DpA      | Control       | 4.07  | 4.33             | 4.20                    |
|          | ED            | 4.50  | 4.47             | 4.49ab                  |
|          | LD            | 4.87  | 5.37             | 5.13b                   |
|          | EED           | 4.32  | 4.36             | 4.35a                   |
| DpC      | Control       | 1.71  | 7.98             | 4.84                    |
|          | ED            | 1.46  | 9.02             | 5.24                    |
|          | LD            | 1.63  | 8.38             | 5.00                    |
|          | EED           | 1.84  | 11.80            | 6.82                    |
| ΣDp      | Control       | 137.27 | 97.24           | 117.26                  |
|          | ED            | 158.98 | 104.30          | 130.64                  |
|          | LD            | 167.55 | 126.02          | 146.79                  |
|          | EED           | 141.17 | 97.48           | 119.32                  |
| CyG      | Control       | 16.50 | 12.95            | 14.73                   |
|          | ED            | 16.96 | 13.00            | 14.99                   |
|          | LD            | 20.40 | 17.11            | 18.75                   |
|          | EED           | 14.27 | 11.04            | 12.66                   |
| CyA      | Control       | 0.67  | nd               | 0.34                    |
|          | ED            | 1.24  | nd               | 0.62                    |
|          | LD            | 1.35  | nd               | 0.68                    |
|          | EED           | 1.00  | nd               | 0.50                    |
| CyC      | Control       | 3.47  | 3.44             | 3.46                    |
|          | ED            | 4.15  | 4.88             | 4.52                    |
|          | LD            | 4.90  | 4.99             | 4.95                    |
|          | EED           | 3.62  | 3.86             | 3.74                    |
| ΣCy      | Control       | 20.65 | 16.39            | 18.52                   |
|          | ED            | 22.37 | 17.89            | 20.13                   |
|          | LD            | 26.65 | 22.10            | 24.38                   |
|          | EED           | 18.90 | 14.90            | 16.90                   |
| PtG      | Control       | 116.03 | 94.63           | 105.33                  |
|          | ED            | 129.04 | 100.60          | 114.82                  |
|          | LD            | 138.26 | 123.00          | 130.63                  |
|          | EED           | 115.56 | 95.68           | 105.62                  |
| PtA      | Control       | 4.60  | 6.61             | 5.61                    |
|          | ED            | 4.58  | 7.20             | 5.89                    |
|          | LD            | 5.20  | 7.70             | 6.45                    |
|          | EED           | 4.96  | 7.35             | 6.16                    |
| PtC      | Control       | 29.20 | 28.84            | 29.02                   |
|          | ED            | 32.58 | 32.53            | 32.56                   |
|          | LD            | 32.84 | 32.25            | 32.55                   |
|          | EED           | 31.81 | 30.70            | 31.26                   |
| ΣPt      | Control       | 149.83 | 130.08          | 139.95                  |
|          | ED            | 166.21 | 140.33          | 153.27                  |
|          | LD            | 176.30 | 162.95          | 169.63                  |
|          | EED           | 152.33 | 133.72          | 143.03                  |
| Pn derivates |              |      |                  |                         |
| PnG      | Control       | 34.95 | 24.96            | 29.96                   |
|          | ED            | 29.47 | 25.89            | 27.68                   |
|          | LD            | 35.12 | 30.99            | 33.06                   |
|          | EED           | 27.08 | 23.81            | 25.44                   |
Table 1 (cont.). Effect of early leaf removal on grape-skin anthocyanin concentration (mg/kg berry fresh weight) under three defoliation regimes

| Compound ¹ | Treatment (T) ² | Year       | Interannual mean | Statistical significance ³ |
|------------|----------------|------------|-----------------|--------------------------|
|            |                | 2008       | 2009            | T            | Year | T×Year |
| Pn A       | Control        | 0.68 a     | 0.65 a          | 0.66 a       | ns   | ns     | ns     |
|            | ED             | 0.86 a     | 0.84 a          | 0.85 a       | ns   | ns     | ns     |
|            | LD             | 0.92 a     | 0.99 a          | 0.96 a       | ns   | ns     | ns     |
|            | EED            | 0.51 a     | 0.85 a          | 0.68 a       | ns   | ns     | ns     |
| PnC        | Control        | 7.97 a     | 7.02 a          | 7.49 a       | ns   | ns     | ns     |
|            | ED             | 7.28 a     | 8.38 a          | 8.73 a       | **   | ns     | *      |
|            | LD             | 8.21 a     | 9.47 a          | 8.84 a       | ns   | ns     | ns     |
|            | EED            | 7.20 a     | 7.38 a          | 7.31 a       | ns   | ns     | ns     |
| ΣPn        | Control        | 43.60 a    | 32.62 a         | 38.11 a      | ns   | ns     | ns     |
|            | ED             | 37.61 a    | 35.11 a         | 36.36 a      | *    | *      | ns     |
|            | LD             | 44.25 a    | 41.46 a         | 42.85 a      | ns   | ns     | ns     |
|            | EED            | 34.83 a    | 32.04 a         | 33.43 a      | ns   | ns     | ns     |
| Mv G       | Control        | 404.94 a   | 344.88 a        | 374.91 a     | ns   | **     | ns     |
|            | ED             | 417.80 a   | 332.17 a        | 374.99 a     | ns   | **     | ns     |
|            | LD             | 392.94 a   | 383.37 a        | 388.15 a     | ns   | ns     | ns     |
|            | EED            | 404.29 a   | 353.84 a        | 379.07 a     | ns   | ns     | ns     |
| Mv A       | Control        | 20.21 a    | 26.85 a         | 23.53 a      | ns   | ***    | ns     |
|            | ED             | 20.62 a    | 27.32 a         | 23.97 a      | ns   | ***    | ns     |
|            | LD             | 20.74 a    | 28.19 a         | 24.46 a      | ns   | ***    | ns     |
|            | EED            | 22.44 a    | 29.33 a         | 25.88 a      | ns   | ***    | ns     |
| Mv C       | Control        | 123.56 a   | 135.18 a        | 129.37 a     | ns   | **     | ns     |
|            | ED             | 119.65 a   | 139.76 a        | 129.71 a     | ns   | **     | ns     |
|            | LD             | 121.73 a   | 135.09 a        | 128.41 a     | ns   | **     | ns     |
|            | EED            | 132.33 a   | 144.22 a        | 138.28 a     | ns   | **     | ns     |
| ΣMv        | Control        | 548.70 a   | 506.90 a        | 527.80 a     | ns   | ns     | ns     |
|            | ED             | 558.00 a   | 499.25 a        | 528.66 a     | ns   | ns     | ns     |
|            | LD             | 535.41 a   | 546.65 a        | 541.03 a     | ns   | ns     | ns     |
|            | EED            | 559.06 a   | 527.39 a        | 543.23 a     | ns   | ns     | ns     |
| Σ Monoglucosides forms | Control | 723.13 a | 576.31 a | 649.74 a | ns | *** | ns |
|            | ED             | 761.02 a   | 576.79 a        | 668.92 a     | ns   | *** | ns |
|            | LD             | 767.66 a   | 684.22 a        | 726.01 a     | ns   | *** | ns |
|            | EED            | 711.32 a   | 578.71 a        | 644.96 a     | ns   | *** | ns |
| Σ Acetyl glucoside forms | Control | 30.33 a | 38.73 a | 34.69 a | ns | *** | ns |
|            | ED             | 32.47 a   | 40.17 a         | 36.32 a      | ns   | *** | ns |
|            | LD             | 33.81 a   | 42.68 a         | 38.19 a      | ns   | *** | ns |
|            | EED            | 33.74 a   | 42.32 a         | 38.00 a      | ns   | *** | ns |
| Σ Coumaroyl glucoside forms | Control | 170.16 a | 186.30 a | 178.28 a | ns | ** | ns |
|            | ED             | 169.34 a | 199.32 a | 184.34 a | ns | ** | ns |
|            | LD             | 174.03 a | 195.54 a | 184.67 a | ns | ** | ns |

Data are average values (n = 32). Within each column: different letters indicate significant difference among treatments after Tukey test (p<0.05).

¹ Dp: delphinidine. DpG: delphinidine-3-glucoside. DpA: delphinidine-3-glucoside acetate. DpC: delphinidine-3-glucoside coumarate. Cy: cyanidine. CyG: cyanidine-3-glucoside. CyA: cyanidine-3-glucoside acetate. CyC: cyanidine-3-glucoside coumarate. Pt: petunidine. PtG: petunidine-3-glucoside. PtA: petunidine-3-glucoside acetate. PtC: petunidine-3-glucoside coumarate. Pn: peonidine. PnG: peonidine-3-glucoside. PnA: peonidine-3-glucoside acetate. PnC: peonidine-3-glucoside coumarate. Mv: malvidine. MvG: malvidine-3-glucoside. MvA: malvidine-3-glucoside acetate. MvC: malvidine-3-glucoside coumarate.

² C: Control, no defoliated. ED and LD: all leaves from the first 6 nodes were eliminated just before flowering and at fruit-set respectively. EED: only the leaves facing east of the 8 first nodes were removed just before flowering.

³ *, **, ***: statistical differences at p<0.05, 0.01, 0.001 respectively; ns: not significant.
ΣCy, PtG, ΣPt, PnC respect with C and increased the content of DpG, DpA, DpC, ΣDp, CyG, CyC, ΣCy, PtG, ΣPt, PnG, PnC, ΣPn respect with EED. However, no significant differences were reported between ED and LD for any compound.

**Non-anthocyanin flavonoid compounds:** Results for the non-anthocyanin flavonoid compounds are shown in Table 2, where it is observed that CA was the predominant flavan-3-ol in cv. ‘Tempranillo’ skins in both years, while B3 and B1 were the main flavan-3,4 diols in 2008 and 2009, respectively. In 2008, respect to C, the LD treatment significantly affected only one compound, increasing the B3 concentration value. However, it is noteworthy the increase of EC, B1 and B3 in LD respect to EED. In 2009, ED and LD increased the contents of all flavonol analyzed respect to C, where the contents were similar to EED, whit exception of CA. As a consequence of these results, a significant interaction treatment × year (p<0.001) was reported for CA and B1 compounds.

As shown in Table 3, MyG, QcGR, and QcG, were the most abundant flavonol compounds in ‘Tempranillo’ skins in both years. Because the treatment factor was statistically significant for all compounds, this family was the most affected by the early leaf removal treatments. ED, and mainly LD treatments exerted a positive effect in the accumulation of all individual flavonol compounds in the grape skins for these two seasons, while no effect of the EED treatment respect to control was observed in any season. In the LD treatment, the largest increases with respect to the control were found in QcR (242% in 2008) and KpR (over 300% in both seasons) compounds. In contrast, this increase never reached 40% for IhG.

**Non-flavonoid compounds:** Hydroxycinnamic compounds and t-resveratrol: The influence of early defoliation, year and their interaction on hydroxycinnamic acids (CF, COU, and FE), and on their cis- and trans-tartaric derivatives (c and t CFT, COUT and FET), and stilbenes (tR) are shown in Table 4. The results show that CF and c-FET were the predominant hydroxycinnamic compounds in ‘Tempranillo’ skins for the C treatment in 2008 and 2009, respectively. As reported in Table 4, the contents of hydroxycinnamic compounds depended mostly of the year, where the highest contents were reported for the 2008 season. With regard to the effect of the

### Table 2. Effect of early leaf removal on grape-skin flavanol concentration (mg/kg berry fresh weight) under three defoliation regimes.

| Compound | Treatment (T) | Year | Interannual mean | Statistical significance |
|----------|---------------|------|------------------|-------------------------|
|          | 2008          | 2009 |                  |                         |
| **CA**   | Control       | 0.79a| 0.42a            | 0.60a                   |
|          | ED            | 0.80a| 1.29c            | 1.05b                   |
|          | LD            | 0.74a| 1.30a            | 1.02a                   |
|          | EED           | 0.42a| 0.71b            | 0.57a                   |
| **EC**   | Control       | 0.39ab| 0.13a          | 0.26ab                  |
|          | ED            | 0.45bc| 0.35a          | 0.40bc                  |
|          | LD            | 0.59a| 0.34a            | 0.46a                   |
|          | EED           | 0.29a| 0.18a            | 0.24a                   |
| **B1**   | Control       | 5.48a| 4.28a            | 4.88a                   |
|          | ED            | 4.53a| 7.32b            | 5.93b                   |
|          | LD            | 4.85a| 7.12b            | 5.98b                   |
|          | EED           | 4.60a| 4.80a            | 4.70a                   |
| **B2**   | Control       | 0.53ab| 0.19a          | 0.36a                   |
|          | ED            | 0.61ab| 0.37a          | 0.49a                   |
|          | LD            | 0.65a| 0.45a            | 0.55a                   |
|          | EED           | 0.40a| 0.17a            | 0.28a                   |
| **B3**   | Control       | 6.54a| 2.35a            | 4.45a                   |
|          | ED            | 8.32a| 4.83b            | 6.58b                   |
|          | LD            | 10.65ab| 4.47a         | 7.56b                   |
|          | EED           | 5.91a| 2.65a            | 4.28a                   |

Data are average values (n = 32). Within each column: different letters indicate significant difference among treatments after Tukey test (p<0.05). **CA:** (+)-catechin. **EC:** (-)-epicatechin. **B1:** procyanidin B1. **B2:** procyanidin B2. **B3:** procyanidin B3. **Treatments:** see Table 1. ***, **:** statistical differences at p<0.001, 0.01, 0.001 respectively; ns: not significant.
defoliation treatments carried out, a trend to increase the concentration of eight of these compounds was observed in the ED and LD treatments with respect to the C (interannual mean value). In 2008, the increases of hydroxy-cinnamic compounds due to the application of the LD treatment was more clear than what reported for the EED regime respect to the C. On the other hand, the ED treatment did not modify the concentration of any hydroxy-cinnamic compound. In 2009, LD treatment increases, respect to the C, the concentration of C-CFT, COU, C-FET, T-FET and tR, while ED increases the concentration of C-CFT, COU and FE. However, EED treatment only increases the concentration of two compounds (C-CFT and tR). The most largely modified compounds for the LD treatment were FE (increases of 290% and 338% in 2008 and 2009, respectively) and COU (188% and 171% in the same years).

**Discussion**

The results of this work indicate that the application of early leaf removal on ‘Tempranillo’ grapevines growing under temperate-warm conditions decreased berry weight and yield, particularly in the more intense leaf pulling regimes. Removal of the first six basal leaves at the pre-anthesis stage has been shown to be an effective strategy for controlling yield potential via source-sink relationships (Poni et al., 2006; Acimovic et al., 2016). Furthermore, several authors attributed the changes in berry weight in response to defoliation to changes in temperature and the light regime within the cluster that probably influenced the berry sink capacity (i.e. the ability to attract photo-assimilates) (Kliwier & Antcliff, 1970). In this sense, Risco et al. (2014) reported that the microclimate conditions in the fruit zone were only influenced and improved by the LD regime imposed. On other hand, a previous research showed that the effect of defoliation on final berry size and yield at harvest is linked with the timing and extent of defoliation (Poni et al., 2006). Respect to this extent, in accordance with a previous study by Tardaguila et al. (2008), when defoliation was carried out with a lower intensity (like in the EED treatment), the final effect on the yield components was only slight.

The significant differences in berry weight and yield registered in 2009 respect to the previous season, especially in the defoliated treatments, could be in part explained by the higher proportion of leaves removed in this second year. In addition, defoliation carried out around berry flowering and set is known to induce a strong competition for assimilates between vegetative and reproductive organs: the major part of photosynthetically active foliage is removed at a time of high carbon and nitrogen requirements by the inflorescences, forcing the vine to use its reserves accumulated in the woody permanent structures (Verdenal et al., 2017). Consequently, according to previous works, during the year following defoliation, a lower vigor, bud fruitfulness and pruning weights were noted (Palliotti et al., 2011; Uriarte et al., 2012; Risco et al., 2014). Moreover, the differential responses of early defoliation across seasons may be linked to a different number of flower buttons formed year after year on the inflorescences, which in turn can modify the level of berry set induced by treatments (Poni et al., 2006).

Early leaf removal also altered berry components. The effects of defoliation on grape berry skin phenolic composition reported in this study could be due to different whole vine physiology, including those changes in berry size and total yield, resulting in a concentration effect, as demonstrated by Roby et al. (2004). In addition, the cluster microclimate was modified by defoliation as reported in our previous study (Risco et al., 2014).

In agreement with previous works, the results of this study indicate that the early defoliation modified the phenolic profile of the skins, and this effect was dependent on the year’s weather conditions, timing and intensity of defoliation, and the polyphenolic family involved (Kotseridis et al., 2012; Lemut et al., 2013a; Bogicevic et al., 2015; Moreno et al., 2015). This behavior is due to the biosynthetic pathways of phenolic substances being regulated by enzymes that are light-and temperature sensitive. Hence, the changes in microclimatic conditions, such as those imparted by early defoliation, may have a significant effect on the synthesis and accumulation of these substances (Diago et al., 2012). On the other hand, since the different phenolic compounds are not synthesized at the same developmental stage, and the different phenolic families respond in different degrees to the same variation of light and temperature (Degu et al., 2016; Gouot et al., 2020), the timing and severity of defoliation affected different polyphenolic families compounds and aspects of berry composition in a different manner.

Under our experimental conditions, the effect of defoliation on anthocyanins, flavanols, flavonols, hydroxycinnamic acids and total phenolic compounds in grape skin was most noticeable in 2009, suggesting that the environmental conditions also influenced the final response to the field practice applied in both seasons. Previous research has also shown a different season-to-season response to leaf pulling performed in several cultivars such as ‘Tempranillo’ (Diago et al., 2012; Moreno et al., 2015), ‘Chardonnay’ (Hed et al., 2015), and ‘Pinot Noir’ (Verdenal et al., 2017). One possible explanation for our results may be due to the leaf area removed as a portion of the total area was higher in 2009 for the treatments of the same severity (see Risco et al., 2014 for details). Also, the decreases in yield caused by defoliation were higher in this second year (Risco et al., 2014). In general, when a field practice results in a yield drop, this is often followed by an increase in the concentration of polyphenol substances.
The positive impact of early defoliation on grape phenolic composition has been demonstrated for 'Tempranillo' and other cultivars in several terroirs (Diago et al., 2012; Bogicevic et al., 2015; Moreno et al., 2015; Acimovic et al., 2016; Verdenal et al., 2017). In agreement with Pallioti et al. (2011), the results can be related mostly to the reduction in berry size of defoliated treatments caused by the competition for assimilates between the growing canopy and the inflorescences during early season. Bogicevic et al. (2015) demonstrated an increased synthesis of anthocyanins and proanthocyanidins in the 'Vranac' variety as a response to the variation in fruit microclimate.

### Table 3. Effect of early leaf removal on grape-skin flavonol concentration (mg/kg berry fresh weight) under three defoliation regimes.

| Compound 1 | Treatment (T) 2 | Year | Interannual mean | Statistical significance 3 |
|------------|----------------|------|-----------------|---------------------------|
|            |                | 2008 | 2009            | T | Year | T×Year |
| My derivates | MyG | Control | 21.07 a | 19.16 a | 20.11 a | *** | ** | * |
|            |    | ED | 28.54 b | 24.84 b | 26.69 b |    |    |    |
|            |    | LD | 37.36 c | 26.56 c | 31.96 c |    |    |    |
|            |    | EED | 19.14 | 18.80 | 18.97 |    |    |    |
| QcG | Control | 10.56 a | 9.31 | 9.94 a |    |    |    |
|            | ED | 17.51 b | 21.43 b | 19.47 b | *** | ns | ns |
|            | LD | 25.67 c | 23.98 b | 24.83 c |    |    |    |
|            | EED | 11.18 | 9.46 c | 10.32 a |    |    |    |
| QcR | Control | 0.52 a | 0.55 | 0.53 a |    |    |    |
|            | ED | 1.15 b | 1.51 b | 1.33 b | *** | ns | ** |
|            | LD | 1.78 b | 1.53 a | 1.66 b |    |    |    |
|            | EED | 0.72 | 0.46 | 0.59 a |    |    |    |
| QcGL | Control | 1.65 a | 1.31 a | 1.48 a |    |    |    |
|            | ED | 3.03 b | 3.15 b | 3.09 b | *** | ** | ns |
|            | LD | 4.26 b | 3.21 a | 3.73 c |    |    |    |
|            | EED | 1.83 a | 1.19 b | 1.51 a |    |    |    |
| QcGR | Control | 21.99 a | 20.11 a | 21.05 a |    |    |    |
|            | ED | 53.60 b | 43.10 a | 48.35 b | ** | ns | ns |
|            | LD | 49.40 a | 51.76 a | 50.58 b |    |    |    |
|            | EED | 23.91 a | 22.30 a | 23.10 a |    |    |    |
| KpG | Control | 3.53 a | 1.78 a | 2.65 a |    |    |    |
|            | ED | 5.05 a b | 5.46 b | 5.25 b | *** | * | ns |
|            | LD | 7.18 a | 6.37 b | 6.78 b |    |    |    |
|            | EED | 3.22 a | 1.65 a | 2.44 a |    |    |    |
| KpR | Control | 0.70 a | 0.57 a | 0.64 a |    |    |    |
|            | ED | 1.43 a | 2.00 a | 1.72 b | *** | ns | ns |
|            | LD | 3.18 b | 2.49 a | 2.83 b |    |    |    |
|            | EED | 0.90 a | 0.64 b | 0.77 a |    |    |    |
| IhG | Control | 2.64 a | 2.89 a | 2.76 a |    |    |    |
|            | ED | 3.06 a | 3.97 b | 3.52 b | *** | ns | ns |
|            | LD | 3.57 b | 3.84 a | 3.70 b |    |    |    |
|            | EED | 2.31 a | 2.24 a | 2.27 a |    |    |    |

Data are average values (n = 32). Within each column: different letters indicate significant difference among treatments after Tukey test (p<0.05). 1 My: myricetine. MyG: myricetin-3-glucoside. Qc: quercetine. QcG: quercetin-3-glucoside. QcR: quercetin-3-rutinoside. QcGL: quercetin-3-galactoside. QcGR: quercetin-3-glucuronide. Kp: kaempherol. KpG: kaempherol-3-glucoside. KpR: kaempherol-3-rutinoside. Ih: isorhamnetine. IhG: isorhamnetin-3-glucoside. 2 Treatments: see Table 1. 3 *, **, ***: statistical differences at p<0.05, 0.01, 0.001 respectively; ns: not significant.

(Peña Neira et al., 2007; Guidoni et al., 2008; Gamero et al., 2018).
### Table 4. Effect of early leaf removal on grape-skin hydroxycinnamic acids and stilbenes concentration (mg/kg berry fresh weight) under three defoliation regimes.

| Compound 1 | Treatment (T) 2 | Year | Interannual mean | Statistical significance 3 |
|------------|-----------------|------|-------------------|----------------------------|
|            | 2008            | 2009 |                   | T | Year | T×Year |
| CF         | Control         | 16.92ª | 5.18ª | 11.05ª | ns | *** | ns |
|            | ED              | 17.81ª | 4.56ª | 11.19ª | ns | *** | ns |
|            | LD              | 19.14a | 5.33ª | 12.23ª | *** | *** | *** |
|            | EED             | 27.22ª | 4.86ª | 16.04ª | ns | *** | ns |
| c-CFT      | Control         | 0.22ª  | 0.73ª  | 0.48ª  | ns | *** | *** |
|            | ED              | 0.26ª  | 1.32ª  | 0.79ª  | *** | *** | *** |
|            | LD              | 0.31ª  | 1.83ª  | 1.07ª  | *** | *** | *** |
|            | EED             | 0.30ª  | 1.37ª  | 0.84ª  | ns | *** | *** |
| t-CFT      | Control         | 4.49ª  | 2.31ª  | 3.40ª  | ns | *** | ns |
|            | ED              | 5.09ª  | 2.32ª  | 3.70ª  | ns | *** | ns |
|            | LD              | 5.47ª  | 4.03ª  | 4.75ª  | ns | *** | ns |
|            | EED             | 4.91ª  | 2.62ª  | 3.76ª  | ns | *** | ns |
| COU        | Control         | 3.66ª  | 3.90ª  | 3.78ª  | ns | *** | ns |
|            | ED              | 7.16ª  | 8.63ª  | 7.90ª  | ns | *** | ns |
|            | LD              | 10.57ª | 10.59ª | 10.58ª | ns | *** | ns |
|            | EED             | 4.21a  | 4.52ª  | 4.36ª  | ns | *** | ns |
| c-COUT     | Control         | 2.09ª  | 0.59ª  | 1.34ª  | ns | *** | ns |
|            | ED              | 1.73ª  | 0.49ª  | 1.11ª  | ns | *** | ns |
|            | LD              | 2.09ª  | 0.59ª  | 1.34ª  | ns | *** | ns |
|            | EED             | 1.50ª  | 0.49ª  | 0.99ª  | ns | *** | ns |
| t-COUT     | Control         | 0.72ª  | 0.43ª  | 0.58ª  | ns | *** | ns |
|            | ED              | 1.14ª  | 0.56ª  | 0.85ª  | ns | *** | ns |
|            | LD              | 1.30ª  | 0.50ª  | 0.90ª  | ns | *** | ns |
|            | EED             | 0.84ª  | 0.38ª  | 0.61ª  | ns | *** | ns |
| FE         | Control         | 1.27ª  | 0.73ª  | 1.00ª  | ns | *** | ns |
|            | ED              | 2.26ª  | 2.54ª  | 2.40ª  | ns | *** | ns |
|            | LD              | 4.96ª  | 3.20ª  | 4.08ª  | ns | *** | ns |
|            | EED             | 1.08ª  | 0.85ª  | 0.97ª  | ns | *** | ns |
| c-FET      | Control         | 7.25ª  | 5.59ª  | 6.42ª  | ns | *** | ns |
|            | ED              | 12.39ª | 7.33ª  | 9.86ª  | ns | *** | ns |
|            | LD              | 16.48ª | 7.61ª  | 12.05ª | ns | *** | ns |
|            | EED             | 5.96ª  | 5.45ª  | 5.71ª  | ns | *** | ns |
| t-FET      | Control         | 2.20ª  | 4.38ª  | 3.29ª  | ns | *** | ns |
|            | ED              | 2.31ª  | 6.99ª  | 4.65ª  | ns | *** | ns |
|            | LD              | 2.69ª  | 7.70ª  | 5.20ª  | ns | *** | ns |
|            | EED             | 2.61ª  | 5.39ª  | 4.00ª  | ns | *** | ns |
| tR         | Control         | 0.62ª  | 0.26ª  | 0.44ª  | ns | *** | ns |
|            | ED              | 0.76ª  | 0.74ª  | 0.75ª  | ns | *** | ns |
|            | LD              | 0.84ª  | 1.03ª  | 0.94ª  | ns | *** | ns |
|            | EED             | 0.52ª  | 0.44ª  | 0.48ª  | ns | *** | ns |

Data are average values (n = 32). Within each column: different letters indicate significant difference among treatments after Tukey test (p<0.05). 1 CF: caffeic acid. c and t CFT: cis and trans caftaric acid. COU: coumaric acid. c and t COUT: cis and trans coutaric acid. FE: ferulic acid. c and t FET: cis and trans caftaric acid. tR: trans resveratrol. 2 Treatments: see Table 1. 3 *, **, ***: statistical differences at p<0.05, 0.01, 0.001 respectively; ns: not significant.
generated by early defoliation. Previous studies stated that the increase of anthocyanin content under manual defoliation may be due to improved cluster microclimate (Kliewer & Smart, 1989). Many investigations confirmed that the cluster light exposure increases total anthocyanins and phenols (Kliewer & Antcliff, 1970; Dokoozlian & Kliewer, 1996; Chorti et al., 2010). The increased temperature accelerates the rate of metabolic processes in the plant, with subsequent acceleration in the development and metabolite accumulation (Downey et al., 2006). In the present trial, light exposure in the fruit zone was at the maximum in the LD treatments, whereas the ED and EED treatments had similar fruit exposure (Risco et al., 2014). Besides, the berries from defoliated vines had 1-2 ºC and 0.5-1 ºC lower night-time temperature than that of control berries and the berry temperature difference among the different defoliation treatments was not as clear (results presented in Risco et al., 2014). Shortly after fruit-set, the ED had a berry temperature slightly higher than that of the LD and EED treatments (Risco et al., 2014). In cool climates, improved cluster exposure can enhance berry color (Dokoozlian & Kliewer, 1996), although other works conducted under warm air temperature conditions has warned about the negative effects on berry pigmentation due to over-exposure of the fruiting zone (Bergqvist et al., 2001), that may decrease anthocyanin accumulation (Smart & Robinson, 1991). This is the reason behind testing the EED approach, where only leaves from the west-oriented canopy were removed in order to protect berries from the afternoon sunlight. However, in our case, defoliation carried out early in the season resulted in some vegetative re-growth, which prevented berry over-exposure. Similar results were observed by Poni et al. (2009) in ‘Barbera’ vines grown in Piacenza, Italy.

Our study was particularly focused on profiling the different phenolic families. This is particularly important, as not all phenolic compounds have the same importance for the intensity, tone and color stability of wines (Boulton, 2001). The anthocyanin profile was similar to that observed in other studies carried out on different clones of ‘Tempranillo’ in other terroirs (Santesteban et al., 2011; García Estevez et al., 2015; Gamero et al., 2018). Despite a trend favoring the highest anthocyanin accumulation in defoliation treatments, no differences were observed in total anthocyanins among the treatments. The effects of defoliation on anthocyanin compounds were not always consistent, but were dependent on cultivar grapevine genotype: increases were found in ‘Pinot Noir’ (Verdenal et al., 2017), ‘Merlot’, and ‘Cabernet Sauvignon’ (Kotseridis et al., 2012) but no effects were reported in ‘Cabernet Sauvignon’ and ‘Vranac’ (Bogicevic et al., 2015), or in ‘Tempranillo’ grown in the semiarid terroir of western Spain (Moreno et al., 2015). Similar to previous works (Diago et al., 2010; Moreno et al., 2015), our results showed the relevance of the timing and season of the defoliation period on the effect on these polyphenol families on the ‘Tempranillo’ cultivar. VanderWeide et al. (2018) also found no difference in the total anthocyanin content of ‘Merlot’ when early defoliations treatments were applied manually and mechanically and no alterations occurred in terms of monoglycoside, total acylated and p-coumarylated. According to these authors, the environmental conditions in the cluster zone were enhanced in all treatments, each relative to the time of application and leaf removed (VanderWeide et al., 2018). On the other hand, the trend to increase of the acetylated forms is an interesting result because acetylation can be related to an increase of the anthocyanidin stability against light, temperature or pH changes (He et al., 2012).

With respect to the different anthocyanin compounds, our results indicate that the effect of defoliation was dependent on the anthocyanin considered, and the anthocyanin profile can be altered by the defoliation treatment. Other authors reported similar results on cultivars sensitive to defoliation (Moreno et al., 2015; Verdenal et al., 2017). In this sense, in agreement with VanderWeide’s et al. (2018), the forms considered more stable following methylation or esterification (trihydroxylated, OCH3-substituted, acylated, and coumarylated), mainly related to the Mv derivatives, were not significantly modified by the early leaf removal treatments. These results suggest a different sensitivity to environmental conditions induced by early leaf removal on the flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase enzymes, whose products are precursors for Cy-based anthocyanins and Dp-based anthocyanins. One possible explanation would be that unlike F3’5’H enzymes, F3’H enzymes, which control the biosynthesis of disubstituted anthocyanins and flavonols, are modulated early in development, providing a longer time period for their accumulation under enhanced microclimate conditions (Falginella et al., 2010).

The increases in catechin and epicatechin concentrations in grape berry skins found in the 2009 results support the general knowledge that cluster exposure stimulated catechin synthesis by increasing leucoanthocyanidin reductase (LAR) activity, the enzyme responsible for the catalyzed reduction of 2R, 3S, 4S-flavan-3,4-diols to the corresponding 2R, 3S-flavan-3-ols i.e. (+)-catechin (Torres et al., 2017). The profiles detected in control skins from both seasons were similar to the existing literature for this cultivar (Torres et al., 2017), where berry skin flavonols were dominated by myricetin and quercetin compounds. Greater synthesis of flavonols in grapes, as a result of increased sun exposure caused by defoliation, has widely been reported by several authors (Spayd et al., 2002; Giovanelli & Brenna, 2007). In fact, flavonol occurrence can be considered as a biomarker for a sun exposure regime achieved in a bunch area within the canopies following microclimate manipulation management. Thus, as expected, the content of flavonols was largely affected...
in berry skins under the LD and ED treatments in both years. By examining the occurrence of individual flavonols, it is important to note that, like anthocyanins, the magnitude of the effect depends on the substance under consideration (increases on Qc and Kp derivatives were much higher than My and Il), timing (LD was the most effective treatment), and intensity (no significant effect of EED was observed in any case). Similar responses were observed in ‘Pinot Noir’ grown in Switzerland (Lemut et al., 2013b) and in ‘Tempranillo’ in La Rioja and Extremadura (Spain) (Diago et al., 2010; Moreno et al., 2015).

These results suggest a similar response of these compounds in the different varieties and terroirs.

The positive effect of early defoliation on the hydroxy-cinnamic acid content has been attributed to an improvement in the microclimate of the bunch by other authors (Tardáguila et al., 2010; Lemut et al., 2013a; Intrigliolo et al., 2014). At the level of individual compounds, these researchers coincided in pointing out increases in COU when leaf pulling was performed, but there was no consistent pattern for the rest of the substances in this group, i.e. an increase of PE found by those studies was not reported. The increases in certain substances belonging to the flavanol, flavonol and hydroxycinnamic acid families found in the skins from defoliated vines are very significant for wine making purposes, as these compounds are mainly involved in copigmentation processes with anthocyanins, which affect the color of red wines. According to González-Manzano et al. (2008), catechin or epicatechin are recognized as powerful cofactors, which can form colored complexes most easily and intensely, and oligomeric flavan-3-ols (B1, B2 and B3) have stronger copigmentation effects than the monomers, and even the ethyl-bridged flavan-3-ols could act as strong cofactors (González-Manzano et al., 2008). Moreover, the most stable anthocyanin-copigmentation bonds take place between the first wine anthocyanins, such as malvidine-3-glucoside, quercetin flavonols and quercetin-O-glucoside (Boulton, 2001). Lastly, hydroxycinnamic acids react with monomeric anthocyanins to form pyranoanthocyanins, which are more stable and thus stabilize wine color (Schwarz et al., 2003) and among these phenolic acids, ferulic acid exhibits a strong color enhancement (Markovic, 2000).

In summary, in a temperate-warm terroir, early defoliation is a useful strategy for improving the phenolic profile of grapes, with the effect more dependent on the intensity of leaf removal than the timing of defoliation. In any case, defoliation at fruit set by removing all leaves from the first six nodes was the most effective treatment for modifying skin phenolic content, mainly flavonol, and hydroxycinnamic acid concentrations. These results indicated that this agronomic technique could increase the concentration of compounds (catechin, quercetin and coumaric derivatives) involved in copigmentation reactions, which are crucial for red wine color stability. Therefore, early defoliation may be suggested as a field practice to improve and increase phenolic composition and grape potential for high-quality red wine production and aging. The slight detrimental effects reported in a previous study by Risco et al. (2014) should be considered and therefore the application of the early defoliation technique for more premium wine production is suggested.

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