



ORIGINAL RESEARCH ARTICLE

Leaf removal and deficit irrigation have diverse outcomes on composition and gene expression during berry development of *Vitis vinifera* L. cultivar Xinomavro

Anastasios Alatzas^{1,♦}, Serafeim Theocharis^{2,♦}, Dimitrios-Evangelos Miliordos³, Yorgos Kotseridis³, Stefanos Koundouras^{2*} and Polydefkis Hatzopoulos^{1*}

¹ Laboratory of Molecular Biology, Department of Biotechnology, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece

² Laboratory of Viticulture, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

³ Laboratory of Enology and Alcoholic Drinks, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, 75 Iera Odos, 11855 Athens, Greece

♦ These authors contributed equally to this work



*correspondence:
phat@aua.gr
skoundou@agro.auth.gr

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ABSTRACT

The quality characteristics of a certain grapevine cultivar are determined by the combinatorial action of the genotype and environmental factors, such as soil, landscape and climate. Another critical parameter is the viticultural techniques utilised to improve grape berry quality. The influence of two viticultural practices, deficit irrigation and leaf removal, on the Greek *Vitis vinifera* L. cultivar ‘Xinomavro’ was investigated during the 2019 and 2020 vintages. Physiology measurements and berry sampling were performed at three phenological stages (green berry, veraison and harvest). The expression of specific genes known to control grape colour, aroma and flavour was studied in conjunction with berry growth and composition. All the parameters contributing to final yield, such as berry size, cluster weight and yield/vine, were reduced in non-irrigated and defoliated vines compared to their control (irrigated and non-defoliated) vines. Both treatments showed a significant increase in anthocyanin level, whereas total phenols increased in the deficit irrigation treatment only. Water deficit had no effect on total soluble solids, whereas leaf removal resulted in significantly increased levels compared to the controls. The expression profile of the genes examined was altered during ripening. Although both water deficit and defoliation positively influenced gene expression, there were several diverse responses among the phenological stages that depended on the vintage.

KEYWORDS: Xinomavro, water deficit, defoliation, anthocyanin, phenols, phenylpropanoid and terpenoid pathway

INTRODUCTION

Grapevine is an important agricultural crop worldwide, covering an area of 7.4 million hectares and producing 78 million tonnes of grapes. Approximately 57 % of the grapes are used in wine production, with an estimated export value of up to 31 billion EUR (OIV, 2019). The quality of grapes and wine is of great importance for both winemakers and consumers. Therefore, several viticultural practices have been developed in order to improve grape berry quality in terms of flavour, aroma and colour (Poni *et al.*, 2017; Gutiérrez-Gamboa *et al.*, 2021).

Regulated deficit irrigation is considered to be a useful strategy in viticulture and is known to improve the quality of grape berries and, subsequently, wine composition (Roby *et al.*, 2004; Deluc *et al.*, 2009). Reduced vegetative growth leads to improved canopy microclimate (Santos *et al.*, 2007) and the distribution of sugars between the shoots and ripening grape berries (Petrie *et al.*, 2004). Nevertheless, smaller berry size due to transient water deficit results in higher skin to pulp ratio and, consequently, to a higher concentration of skin-located aroma and flavour compounds (Romero *et al.*, 2022). Therefore, applying a water deficit regime results in higher concentration of phenols, anthocyanins and volatile organic compounds (Koundouras *et al.*, 2006; Deluc *et al.*, 2009; Savoi *et al.*, 2016). Deficit irrigation is known to cause changes in gene expression and to modulate metabolic pathways controlling the accumulation of secondary metabolites that influence berry and wine quality traits (Deluc *et al.*, 2009; Savoi *et al.*, 2016). However, numerous studies have shown that the responses of grape berry to water deficit are variable, depending on the cultivar, the weather conditions and the timing and severity of the irrigation regime applied (Gambetta *et al.*, 2020).

Another viticultural technique commonly utilised to improve grape quality is basal leaf removal (Palliotti *et al.*, 2011; Poni *et al.*, 2017). Selective defoliation increases sunlight exposure, temperature and air circulation in cluster zones (Haselgrove *et al.*, 2000), reducing humidity and thereby the risk of fungal or bacterial infection (Lemut *et al.*, 2015). In a similar way to deficit irrigation, early leaf removal (*i.e.*, at pea-size stage) affects berry size, resulting in higher skin to pulp ratio (Palliotti *et al.*, 2011). Sunlight exposure due to leaf removal has been found to increase the concentration of anthocyanins and phenolic compounds in grape berries (Intrieri *et al.*, 2008; Stefanovic *et al.*, 2021) by regulating the expression of the phenylpropanoid pathway genes (Matus *et al.*, 2009; Pastore *et al.*, 2013; Zenoni *et al.*, 2017). Nevertheless, extensive sunlight exposure could increase cluster temperature, especially under hot climate conditions, with detrimental effects on anthocyanin content (Yamane *et al.*, 2006; Mori *et al.*, 2007). Furthermore, several studies have shown defoliation to have variable effects, depending on cultivar, time of application and vintage (Frioni *et al.*, 2017; Poni *et al.*, 2017; Cincotta *et al.*, 2021).

Considering that grape berry development and metabolite biosynthesis are determined by complex gene expression

networks, holistic approaches are fundamental to understanding the effect of viticultural practices on grape quality. Over the last two decades, the availability of grapevine genome and the high-throughput transcriptomic methods have enriched our knowledge of development- or environment-controlled gene expression patterns. Recent studies have shown that viticultural practices, such as deficit irrigation and leaf removal, alter the expression profiles of genes involved in aroma and flavour compound biosynthesis in both red and white cultivars (Pastore *et al.*, 2013; Savoi *et al.*, 2016; Zenoni *et al.*, 2017; He *et al.*, 2020).

Greece is one of the world's major wine producing countries with over 60,000 ha covered with wine-grapes. Domestic cultivars, like Agiorgitiko and Xinomavro (red-coloured), Savatiano and Assyrtiko (white-coloured), represent more than 90 % of the Greek vineyard area. Xinomavro is a leading wine-grape cultivar, covering an area of 2,150 ha. Remarkably, it is cultivated almost exclusively in Northern Greece, representing 23 % of the local vineyard area (Hellenic Statistical Authority; <http://www.statistics.gr/en/home>, accessed on 26 January 2022). Grape berries of Xinomavro are generally poor in anthocyanins and rich in tannins, compared to other domestic cultivars (Kallithraka *et al.*, 2006). This characteristic phenolic composition, though being a challenge for winemakers, leads to the production of distinguished light-coloured, dry and astringent wines. Additionally, Xinomavro has been recently used for the production of single-varietal spirits.

Most of the studies on viticultural practices conducted in Greek vineyard conditions, including those that carry out deficit irrigation (Koundouras *et al.*, 2006; Koundouras *et al.*, 2009; Kyraleou *et al.*, 2016; Theodorou *et al.*, 2019) or leaf removal (Petropoulos *et al.*, 2011; Kotseridis *et al.*, 2012; Chorti *et al.*, 2016), have focused almost exclusively on the phenolic composition of grapes. Despite the fact that berry quality traits are of great importance in viticulture and oenology, holistic approaches in response to the various viticulture practices although challenging, are still scarce. In a recent study, we combined physiological, chemical and phenolic characteristics with molecular data and showed that water deficit treatment had a strong impact on gene expression and grape berry composition in two domestic cultivars, the red-coloured Agiorgitiko and the white-coloured Assyrtiko (Alatzas *et al.*, 2021).

In the present study, we applied two viticultural techniques, namely water deficit and leaf removal, to improve the quality of the grape and wine. We investigated their effects on gene expression, and correlated gene expression level to the grape berry quality traits (such as phenols and anthocyanins) of the leading Greek red-coloured cultivar Xinomavro. Furthermore, we evaluated the expression profile of the genes related to volatile organic compounds that contribute to grape aroma and flavour. The results obtained showed that both practices can significantly influence gene expression pattern and grape berry composition.

MATERIALS AND METHODS

1. Vineyard site and experimental design

All the experiments were conducted throughout the 2019 and 2020 growing seasons in a commercial vineyard located in Kavala (40°48'43" N, 23°59'25" E, at an altitude of 231 m) in Northern Greece planted with the (*Vitis vinifera* L.) cv. Xinomavro variety. The vineyard was 12 years old, located on a sandy-loam soil (50 % sand, 30 % silt and 20 % clay) and grafted on 1103 Paulsen (*V. rupestris* × *V. berlandieri*) at a density of 3333 vines/ha (2.5 m between rows × 1.2 m within rows). Vines were trained on a vertical trellis with three fixed wires and spur-pruned on a bilateral cordon system to a standard 16 nodes per vine.

Starting at berry set (E-L 27) through harvest, two water regimes were applied on a weekly basis: 1) irrigation at 70 % of crop evapotranspiration ET_c (IR), and 2) non-irrigated (NIR). Each treatment was replicated in three randomised blocks containing 10 consecutive plants each and separated by three buffer rows to avoid any border effects. Quantities of water were applied by a drip irrigation system equipped with 4 L/h drip emitters. ET_c was estimated from potential evapotranspiration data (calculated following the Penman-Monteith method) obtained from an on-site automatic weather station (iMETOS, Pessl Instruments GmbH, Weiz, Austria). Water was supplied on either side of the trunk, through holes positioned at 50 cm intervals along the pipe. For IR treatments total water supply was 413 mm in 2019 and 438 mm in 2020. Pest and canopy management and fertilisation were applied according to standard local viticultural practices.

Leaf removal treatments consisted of 1) non-defoliated vines (CO), and 2) full manual removal of the total leaf area (main leaves and lateral shoots) of the first six basal nodes (LR). Defoliation was carried out at berry set of each year (E-L 27). Three blocks of 10 consecutive vines were randomly assigned to the treatments in two adjacent rows. Pest and canopy management, fertilisation and irrigation were applied according to standard local viticultural practices.

2. Vine parameters

Stem water potential (Ψ_{stem}) measurements were conducted with the use of a pressure chamber according to Choné *et al.* (2001). In each set of measurements, three mature leaves taken from the inner part of the canopy were enclosed in plastic bags and covered with aluminium foil for at least 90 min before measurement to allow equilibration of Ψ_s . The Ψ_s measurements were performed at midday (between 12:30 and 13:30) on three cloudless days per season that corresponded to the following growth stages: bunch closure (E-L 33, approximately 20 days after the beginning of irrigation), middle ripening (E-L 36) and harvest (E-L 38). Only the central 4 vines of each replication were used for measurements.

Net assimilation rate (A), stomatal conductance (gs) and evaporation (E) were recorded at midday at the same time as the Ψ_s measurements using the LCi portable gas

exchange system (ADC BioScientific Ltd., Hoddesdon, UK). Measurements were made on three fully expanded and recently matured, sunlit leaves per plot (photosynthetic photon flux density > 1200 $\mu\text{mol}/\text{m}^2/\text{s}$) that were adjacent to those used for Ψ_s determination.

3. Berry sampling and must analysis

The grapes were harvested on 17 September 2019 and 15 September 2020 from the four chosen vines in each plot. Total yield per plant (kg/vine) and average cluster weight (g) were estimated. Sampling took place three times after veraison in 2019 (DOY (Day Of Year) 217, 231, 260) and in 2020 (DOY 216, 234, 259). Samples of 200 berries were collected randomly from each plot per sampling date and the fresh weight of the berry was determined. The remaining berries per plot were pressed and the must was analysed for soluble solids (oBrix) by refractometry and by measuring titratable acidity (g/L tartaric acid) and pH.

4. Phenolic content of whole berries

Berries (about 150) from each replicate were homogenised using an Ultra Turrax T25 (IKA-Werke, Staufen, Germany) at 24,000 rpm for 1 min. Total phenol and anthocyanin content was measured according to Iland *et al.* (1996). Briefly, 1 g of the homogenate was transferred into a centrifuge tube and mixed with 10 mL 50 % v/v aqueous ethanol (pH 2.0) for 1 h. After centrifugation at 3500 rpm for 10 min, 0.5 mL of the supernatant was added to 10 mL 1N HCl and mixed thoroughly for 3 h, then absorbance at 520 nm and 280 nm was recorded. All analyses were performed in triplicate.

5. RNA extraction and gene expression analysis by RT-qPCR

Sampling was carried out at the same time as the vine physiology measurements: three times in 2019 (DOY 204, 220, 260) and in 2020 (DOY 196, 224, 259). The skins of 10 berries per plot were removed by hand from the grapes, covered with aluminium foil and placed on dry ice. The tissue samples were ground to powder with liquid nitrogen and RNA was extracted using the method of Reid *et al.* (2006). Briefly, approximately 1 g of ground tissue (berry skin) was extracted with buffer containing 300 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2 % (w/v) CTAB, 2 % (w/v) PVPP, 0.05 % (w/v) spermine at 65 °C for 15 min, mixed thoroughly with equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged. The chloroform:isoamyl alcohol step was repeated and the aqueous phase was collected. The RNA was precipitated with 0.6 volumes isopropanol and 0.1 volumes 3 M sodium acetate at -20 °C overnight, centrifuged and finally dissolved in 100 μL ddH₂O. The RNA samples were treated with DNase I (Takara Bio, Shiga, Japan) and further purified using phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation. RNA quantity and quality were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and verified by 0.8 % agarose gel electrophoresis respectively. Reverse transcription was performed with 2 μg RNA using SMART MMLV-Reverse Transcriptase (Takara Bio) and oligo (dT) primer (Eurofins Genomics, Ebersberg, Germany).

The synthesised cDNA was diluted five-fold and PCR conditions were optimised for primers corresponding to selected genes (listed in Supplementary Table S1). The samples were further diluted and quantitative PCR reactions were performed in the PikoReal Real-Time PCR System (Thermo Fisher Scientific, Vantaa, Finland) using KAPA SYBR FAST Master Mix Universal (Kapa Biosystems, Cape Town, South Africa) and applying the following cyclers conditions: 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 62 °C, 30 s at 72 °C. All quantitative PCR reactions were performed in triplicate and melting curve analysis was performed at the end of each reaction to confirm primer specificity. The quantification of gene expression was performed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and elongation factor 1a (*VviEF1a*) was used as the reference gene for data normalisation.

6. Statistical analysis

Principal component analysis (PCA) was used in order to visualise differences and similarities among samples as well as to confirm robustness and analytical variability. PCA was performed on the chemical and phenolic data of the grape berries using the Umetrics SIMCA® 14.1 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden). Data were subjected to one-way analysis of variance (ANOVA) and Student's *t*-test was used to assess the statistically significant differences among the mean values.

RESULTS

1. Climatic conditions and vine water status

The experimental vineyard was located in a narrow valley at approximately 200 m altitude in Northern Greece, close to the Aegean Sea (Supplementary Figure S1). The Mediterranean climate of the region is generally characterised by mild winters and temperate summers. The temperature differences between the two seasons were almost negligible (Figure 1A, 1B), resulting in approximately equal ripening periods. During June and July of 2019, the total amount of rainfall was higher than in 2020 (133mm versus 93mm; Figure 1A). However, total rainfall in August was lower in 2019 than in 2020 (3mm versus 17.5mm). It is worth noting that no precipitation occurred in September prior to the harvest date in both vintages (<http://meteosearch.meteo.gr/stationInfo.asp>, accessed on 26 January 2022).

An important factor in grapevine development that influences both yield and berry quality is the vine water status (Chone *et al.*, 2001; van Leeuwen *et al.*, 2009). Midday stem water potential (Ψ_s) - a physiological parameter regularly utilised to indicate water deficit (Chone *et al.*, 2001) - was clearly affected by the irrigation regime (Figure 1C) during the two vintages. Non-irrigated vines constantly exhibited lower Ψ_s values (i.e., between -1.3 and -1.5 MPa) during maturation, in contrast to irrigated vines (-0.7 and -1 MPa). Drought stress is considered weak when the minimum Ψ_s values are higher than -0.9 MPa and severe when they are below -1.4 MPa (van Leeuwen *et al.*, 2009); therefore, the

non-irrigated vines were under severe drought stress and the irrigated vines were under mild drought stress. In agreement with the stem water potential profile, other physiological parameters, such as stomatal conductance (gs), net assimilation rate (A) and evaporation (E), decreased in non-irrigated vines compared to irrigated ones (Supplementary Figures S2A, S2B and S2C). Leaf removal had no effect on stem water potential in defoliated versus non-defoliated vines during the two vintages (Figure 1C). The Ψ_s values observed in these vines (i.e., between -1 and -1.3 MPa) indicate that both defoliated and non-defoliated vines exhibited the same water status; i.e., they were both under moderate drought stress. Consistently, no significant differences in terms of the other physiological parameters (i.e., stomatal conductance, net assimilation rate and evaporation) were observed among defoliated and non-defoliated vines (Supplementary Figure S2A, S2B and S2C).

2. Grape berry growth and composition

A number of grape berry quality parameters, such as size, yield, total soluble solids, acidity, phenols and anthocyanins, were measured in order to evaluate the effects of the two viticultural practices on berry composition. The measurements were conducted at three phenological stages throughout berry maturation (green berry, E-L 33; veraison, E-L 36; harvest, E-L 38).

2.1. Grape berry size and yield

Berry size was decreased significantly in non-irrigated vines compared to the controls in both vintages (Figure 2A); similar results were obtained for defoliated vines, with the exception of harvest stage of 2020 (Figure 2A). The grape berries of the control (irrigated and non-defoliated) plants reached a similar maximum size during the 2019 and 2020 vintages (approximately 2.1 g and 2.2 g respectively). On the other hand, the grape berries of both non-irrigated and defoliated plants exhibited a smaller size in the 2019 vintage, especially at the harvest stage (1.7 g and 1.8 g respectively), compared to those of the 2020 vintage (1.9 g and 2.1 g respectively) (Figure 2A); this is most likely due to higher precipitation in August 2020 (Figure 1A). Remarkably, no significant differences in the final yield of the control (i.e., irrigated and non-defoliated) vines between the two vintages were observed at harvest, as indicated by yield/vine and cluster weight (Supplementary Figure S3). However, both parameters were significantly higher ($p < 0.05$) in the control vines compared to non-irrigated and to defoliated (Supplementary Figure S3). Interestingly, among the non-irrigated plants, cluster weight was significantly higher ($p < 0.05$) in the 2019 vintage (193 g compared to 167 g in 2020), and a similar tendency was observed in defoliated plants (198 g compared to 177 g in 2020) (Supplementary Figure S3). In conclusion, our data indicate that both of the applied viticultural practices reduced berry size and yield parameters.

2.2. Total Soluble Solids and Titratable Acidity

Total soluble solids (TSS; expressed in °Brix) and titratable acidity (TA; expressed in tartaric acid g/L) are among the most important berry quality traits that determine grape

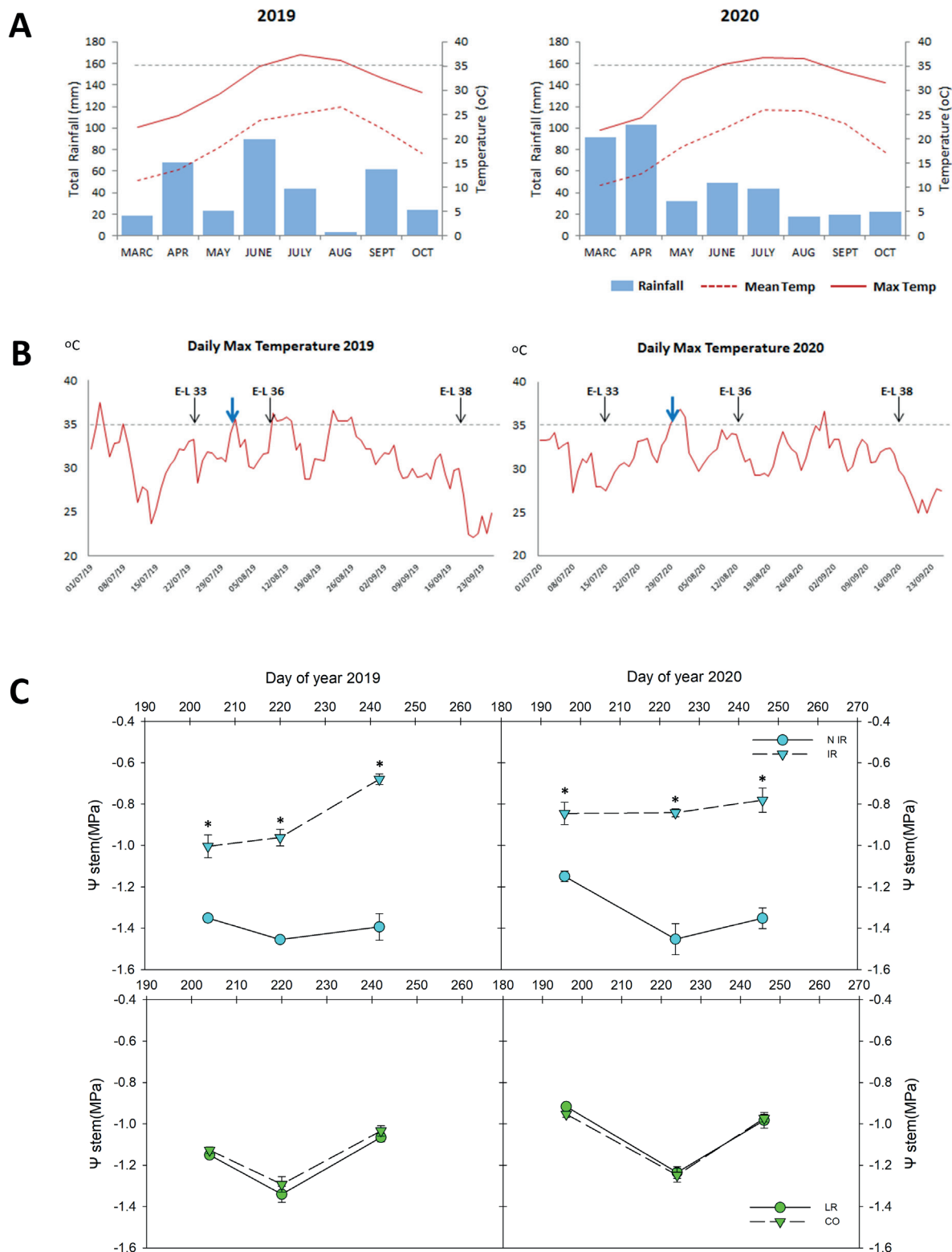


FIGURE 1. Weather conditions at the experimental vineyard. (A) Evolution of monthly mean and maximum temperature and total rainfall from March to October during the 2019 (left) and 2020 (right) seasons. (B) Evolution of daily maximum temperature during the ripening period of 2019 (left) and 2020 (right). Black arrows indicate the days of sampling and blue arrows the period of veraison. (C) The effect of water deficit and leaf removal on stem water potential (Ψ_{stem}) during ripening.

Vertical bars indicate the standard deviation of mean values and asterisks indicate the statistically significant differences (Student's *t* test, *p* value < 0.05) between control and treated plants of the same sampling period. NIR = non-irrigated; IR = irrigated; LR = leaf removal; CO = control

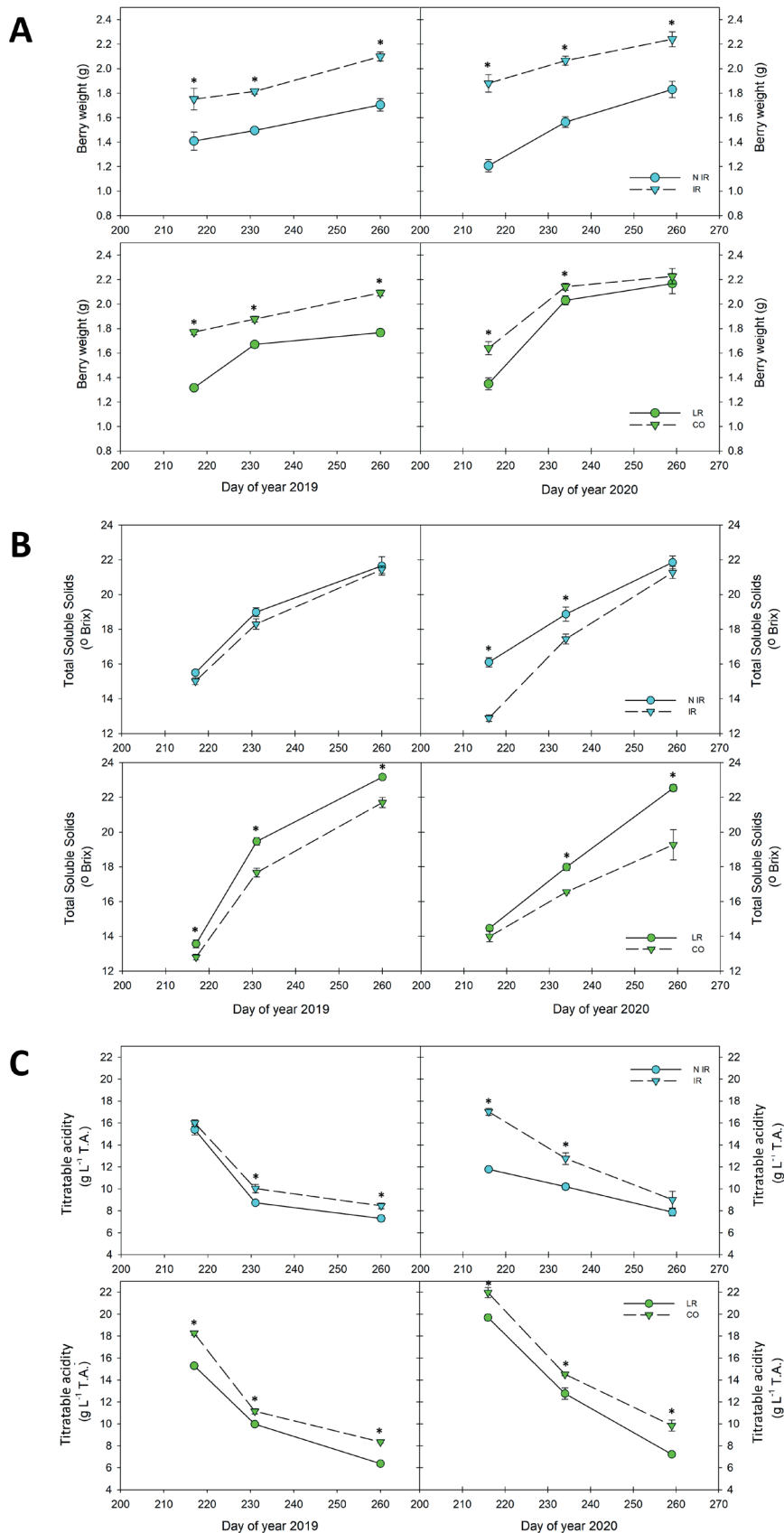


FIGURE 2. The effect of water deficit and leaf removal on grape berry traits during ripening. (A) Berry weight, (B) Total soluble solids (°Brix), and (C) Berry titratable acidity.

Vertical bars indicate the standard deviation of mean values and asterisks indicate the statistically significant differences (Student's *t*-test, *p* value < 0.05) between control and treated plants of the same sampling period. NIR = non-irrigated; IR = irrigated; LR = leaf removal; CO = control.

juice composition. The grape berries in both of the vintages exhibited a constant increase in TSS during maturation (Figure 2B). Water deficit had no effect on TSS in the 2019 vintage, while higher concentrations of TSS were detected at the green berry and veraison stages of the 2020 vintage in the non-irrigated vines (Figure 2B). On the other hand, defoliation resulted in significantly ($p < 0.05$) increased TSS levels at veraison and the harvest stage of both vintages (Figure 2B). In contrast to TSS, TA was found to decrease constantly during maturation (Figure 2C). Defoliation resulted in significantly lower ($p < 0.05$) TA levels compared to the control vines at all the studied stages in both vintages. Similarly, the non-irrigated vines only exhibited lower TA

levels at veraison of both the vintages and harvest of 2019 (Figure 2C). Cumulatively, the results suggest that leaf removal could have a greater impact on the final grape juice composition than water deficit.

2.3. Total anthocyanins and phenols in grape berries

Anthocyanin content is a major quality characteristic of grape berries, as it determines the red colour of grapes and wine. In the present study, anthocyanin concentration was increased during maturation and was higher in both the non-irrigated and defoliated vines compared to their controls (Figure 3A). Anthocyanin level in the non-irrigated vines was increased rapidly after veraison and remained constant

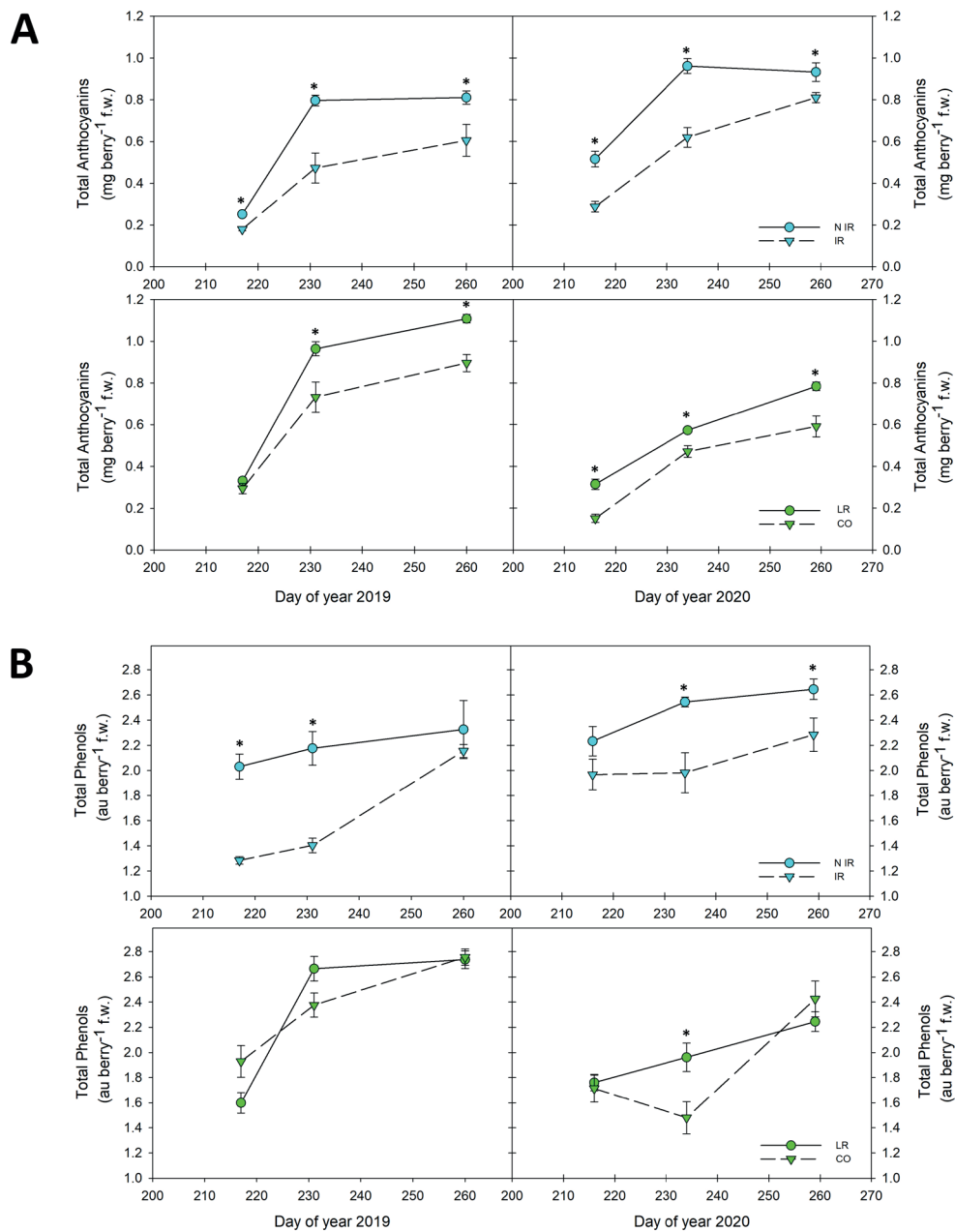


FIGURE 3. The effect of water deficit and leaf removal on anthocyanins (A) and total phenols (B) during ripening.

Vertical bars indicate the standard deviation of mean values and asterisks indicate the statistically significant differences (Student's *t*-test, p value < 0.05) between control and treated plants of the same sampling period. NIR = non-irrigated; IR = irrigated; LR = leaf removal; CO = control.

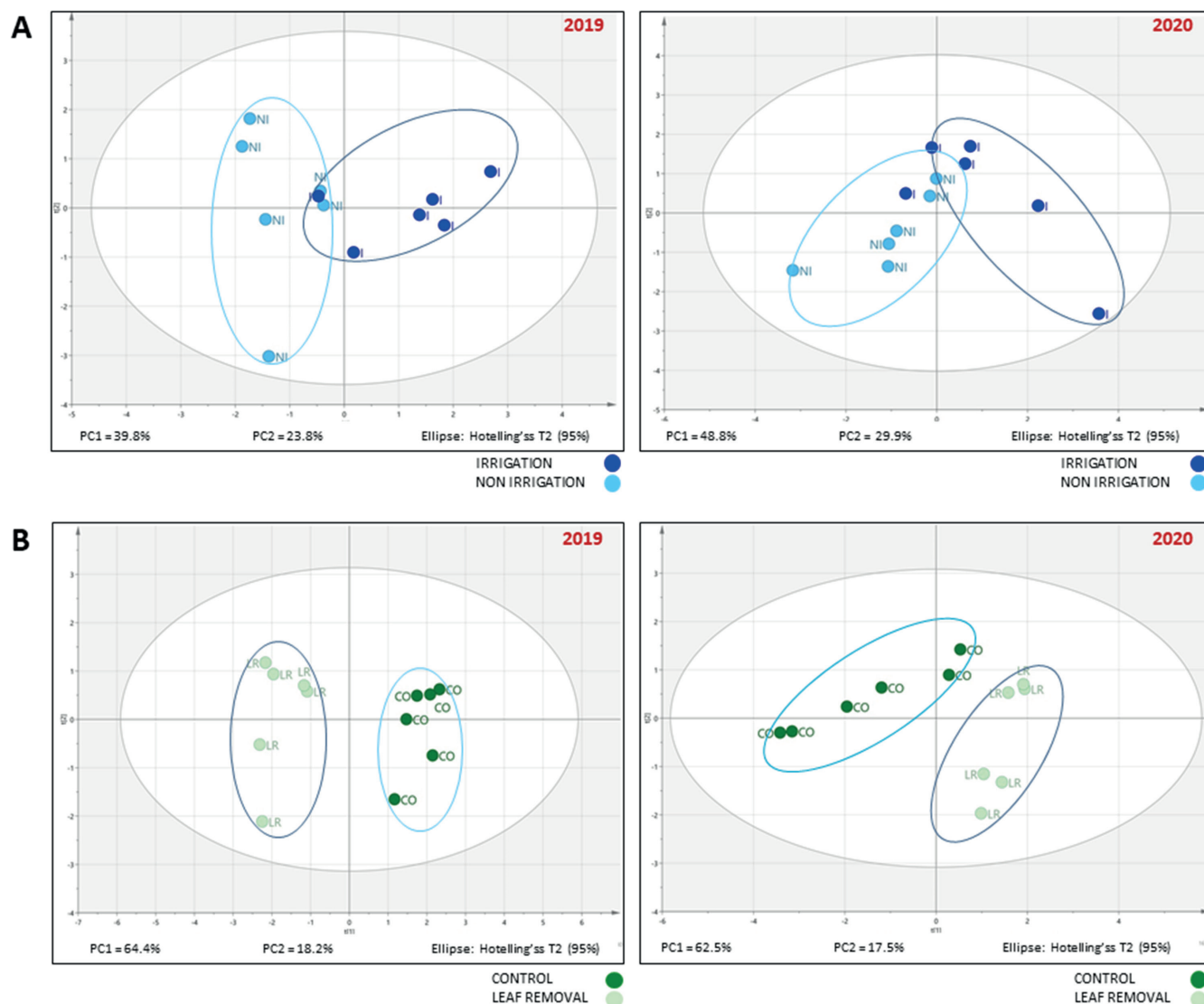


FIGURE 4. Classification of grape berry quality characteristics (chemical and anthocyanins/total phenols) data from grape berries in water deficit (A) and defoliation (B) experiments at the harvest stage of 2019 and 2020 vintages using PCA plots with “treatment” as the dependent variable. Colour of the variables in the score plots correspond to the treatment.

until the harvest stage in both vintages. A similar trend was also observed in the defoliated vines during the 2019 vintage, while the increase in anthocyanin concentration was gradual throughout maturation in the second vintage (Figure 3A). The anthocyanin content in the non-irrigated and defoliated vines was significantly higher ($p < 0.05$) than their controls at all phenological stages (Figure 3A). Remarkably, similar anthocyanin level was reached in the non-irrigated vines at the harvest stage of both vintages (Figure 3A), while the defoliated vines exhibited significantly higher anthocyanin level at the harvest stage of 2019 compared to 2020 (Figure 3A). The two treatments affected total phenol concentration in different ways. The water deficit resulted in significantly increased ($p < 0.05$) total phenol level at the veraison stage of both vintages and at the harvest stage of the 2020 vintage (2.7 au berry⁻¹ f.w. versus 2.3 au berry⁻¹ f.w.). Interestingly, significantly higher total phenolic content in the defoliated vines compared to non-defoliated vines was observed only at

the veraison stage of 2020 (Figure 3B). Taken together, our data indicate that both water deficit and defoliation modulate anthocyanin level and as a consequence, the colour and the characteristics of the final product. However, only the altered irrigation regime has an evident impact on the final total phenols level.

2.4. Multivariate statistics of chemical and phenolic parameters

To improve the visualisation and interpretation of the results, and to examine whether the measured variables can distinguish between pre-established groups (i.e., water deficit and leaf removal), a principal components analysis (PCA) was performed. The previously described berry data were analysed at the three phenological stages (green berry, veraison and harvest), because changes in chemical and phenolic content of the two treatments were observed during all of the stages examined.

The first two components of the PCA score plots explain 83.1 % of the variation in the water deficit experiment and 91.2 % of the variation in the defoliation experiment (Supplementary Figure S4). Remarkably, the unsupervised PCA score plots of the two first components readily discriminated the three stages of berry development (Supplementary Figure S4B). Overall, the PCA showed that the treatment effect was stronger than the vintage effect, whereas they could not be clearly discriminated in the defoliation experiment (Supplementary Figure S4A).

Another PCA was performed as an unsupervised analysis to show the differences in the chemical and phenolic composition of the grape berries at the harvest stage of each vintage. The PCA score plot of the two first components explained approximately 63.6 % and 78.7 % of the variation in the water deficit experiment in 2019 and 2020 respectively (Figure 4A). A similar trend was also observed in the defoliation experiment, with the two first components explaining approximately 82.6 % and 80 % of the variation in 2019 and 2020 respectively (Figure 4B). The fact that two groups of samples could be discriminated in each experiment (i.e., non-irrigated versus irrigated and defoliated versus non-defoliated) at the harvest stage implied a change in grape berry chemical and phenolic composition due to the treatment.

3. Gene expression during grape berry maturation

Recent results have highlighted that viticultural practices, such as regulated deficit irrigation and leaf removal, cause alterations in grape berry transcriptome (Savoi *et al.*, 2016; Zenoni *et al.*, 2017; He *et al.*, 2020). In the present study, the effects of water deficit and defoliation on gene expression were examined by targeted RT-qPCR analysis of the key genes of metabolic pathways known to contribute to grape colour, aroma and flavour (Suppl. Figure S5). The berry samples were collected at three phenological stages (green berry, veraison and harvest) during the 2019 and 2020 vintages, and based on the assumption that most of these genes exhibit skin-specific or skin-preferential expression (Grimplet *et al.*, 2007), skin tissue samples were used to isolate RNA.

3.1. Genes of the phenylpropanoid pathway

We initially investigated the expression profile of genes encoding enzymes of the first steps of the phenylpropanoid pathway that leads to the biosynthesis of phenolic compounds and anthocyanins (Grimplet *et al.*, 2007). The expression analysis of the phenylalanine ammonia lyase (*VviPAL*; VIT_13s0019g04460) gene in the control vines showed a significant up-regulation after veraison followed by a slight decrease at the harvest stage in the 2019 vintage. However, the transcript level remained almost constant throughout berry maturation in 2020 (Figure 5A). Considering the lower rainfall in August of 2019 (Figure 1A), it seems that the environmental conditions had a great impact on *VviPAL* expression. On the other hand, the expression of the cinnamate 4-hydroxylase (*VviC4H*; VIT_06s0004g08150)

gene exhibited a gradual decrease during berry maturation in both vintages (Figure 5B). The water deficit resulted in increased level of *VviPAL* and *VviC4H* expression compared to the controls at all the phenological stages of the 2019 vintage. A similar effect on *VviPAL* expression was also observed in the 2020 vintage, while *VviC4H* expression was increased only at the green berry stage (Figures 5A and 5B). Leaf removal resulted in the up-regulation of *VviPAL* and *VviC4H* genes at veraison stage of 2019, but no effects on their transcript levels during the second vintage were observed (Figure 5A, B).

A different trend was recorded for the UDP-glucose-flavonoid 3-O-glycosyltransferase (*VviUGT*; VIT_04s0023g01290) gene, encoding for the critical step in anthocyanin biosynthesis (Boss *et al.*, 1996). The gene exhibited a significant up-regulation at the veraison stage followed by a decrease towards harvest in both vintages. Moreover, *VviUGT* transcript accumulation during berry maturation was higher in 2020 compared to 2019 (Figure 5C). Similarly to *VviPAL* and *VviC4H* responses, the *VviUGT* expression in non-irrigated vines was increased compared to the controls at all phenological stages of 2019 vintage. However, no differences were observed in 2020 (Figure 5C). On the other hand, defoliation resulted in the up-regulation of the *VviUGT* gene at the veraison stage of both vintages (Figure 5C). Taken together, the results concerning the phenylpropanoid pathway genes suggest that although both viticultural practices can increase transcript accumulation, the environmental conditions are crucial determinants of gene expression.

3.2. Genes related to volatile organic compounds

We further examined the expression patterns of genes belonging to the biosynthetic pathways of volatile compounds that contribute to the aroma potential of grape berries. The expression of the 1-deoxy-D-xylulose-5-phosphate synthase (*VviDXS*; VIT_05s0020g02130) gene that encodes for the enzyme that catalyses the initial step in terpenoid biosynthesis (Battilana *et al.*, 2009) showed a declining trend during berry maturation (Figure 6A). The water deficit led to increased *VviDXS* expression levels at the green berry stage of both vintages (Figure 6A). In the defoliated vines, *VviDXS* expression was also increased at the green berry stage in 2020 (Figure 6A). The carotenoid cleavage dioxygenase 1 (*VviCCDI*; VIT_13s0064g00840) gene, which encodes for the key enzyme in the norisoprenoid pathway (Young *et al.*, 2012), exhibited a different profile with increased expression after veraison (Figure 6B). Water deficit resulted in the up-regulation of the *VviCCDI* gene at veraison in 2019, but no differences in the controls was observed in the following vintage (Figure 6B). Leaf removal resulted in an increased *VviCCDI* transcript level at veraison in both vintages, and at harvest and the green berry stages of 2019 and 2020 respectively (Figure 6B). Taken together, our findings indicate that the two viticultural practices influenced the responses of the genes controlling terpene and norisoprenoid biosynthesis positively.

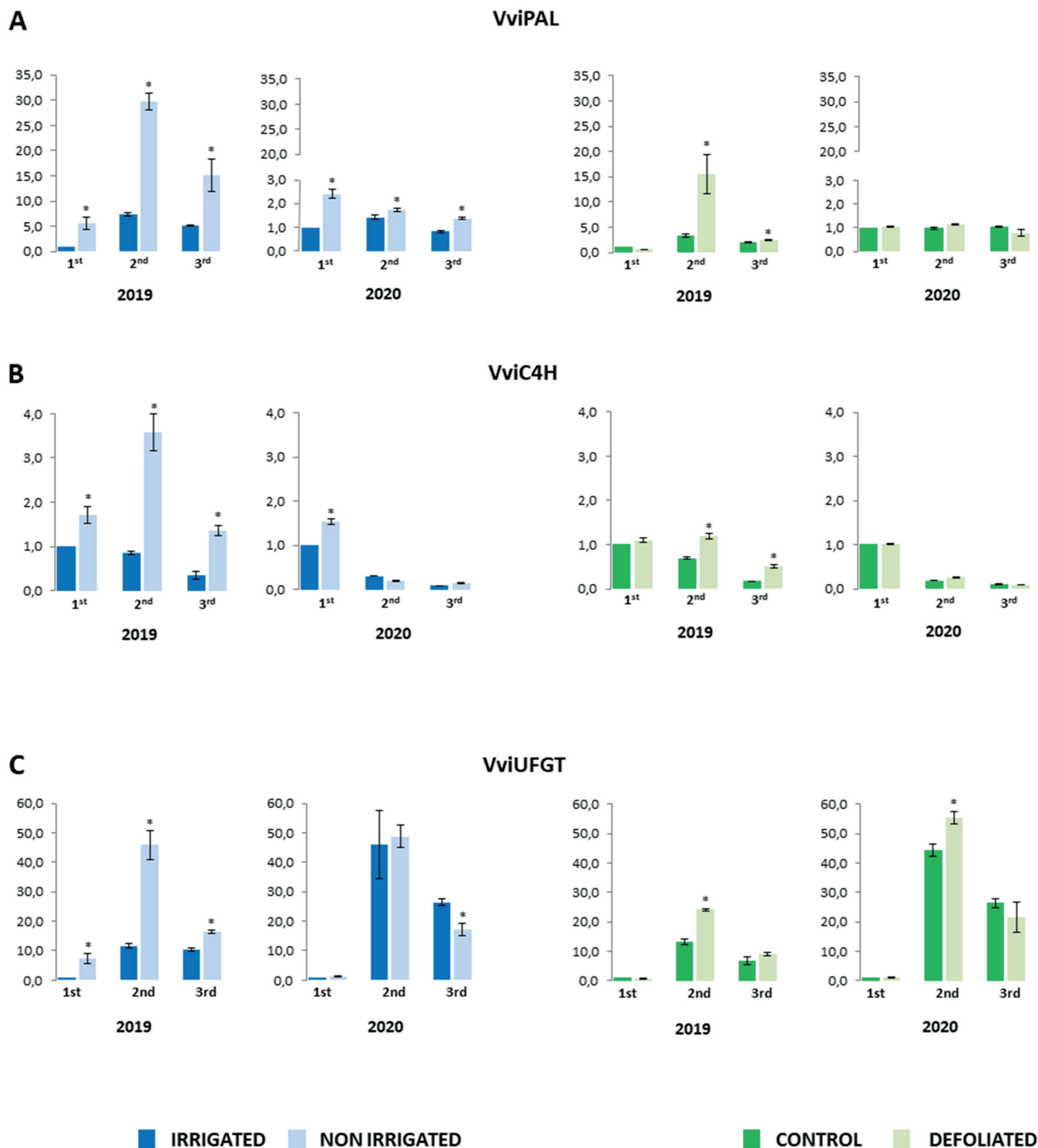


FIGURE 5. Expression level of genes involved in the phenylpropanoid pathway (*VviPAL* (A), *VviC4H* (B) and *VviUFGT* (C)) during the two vintages (2019 and 2020).

The expression level in the water deficit experiment is shown in dark blue (irrigated) and light blue (non-irrigated). Similarly, the expression level in the defoliation experiment is shown in dark green (control) and light green (defoliated). Vertical bars represent the standard deviation of mean values and asterisks indicate the statistically significant differences (Student's *T* test, *p* value < 0.05) between control and treated plants of the same sampling period. The three times of sampling are indicated with numbers under each pair of graphs (green berry, 1st; middle veraison, 2nd and harvest, 3rd).

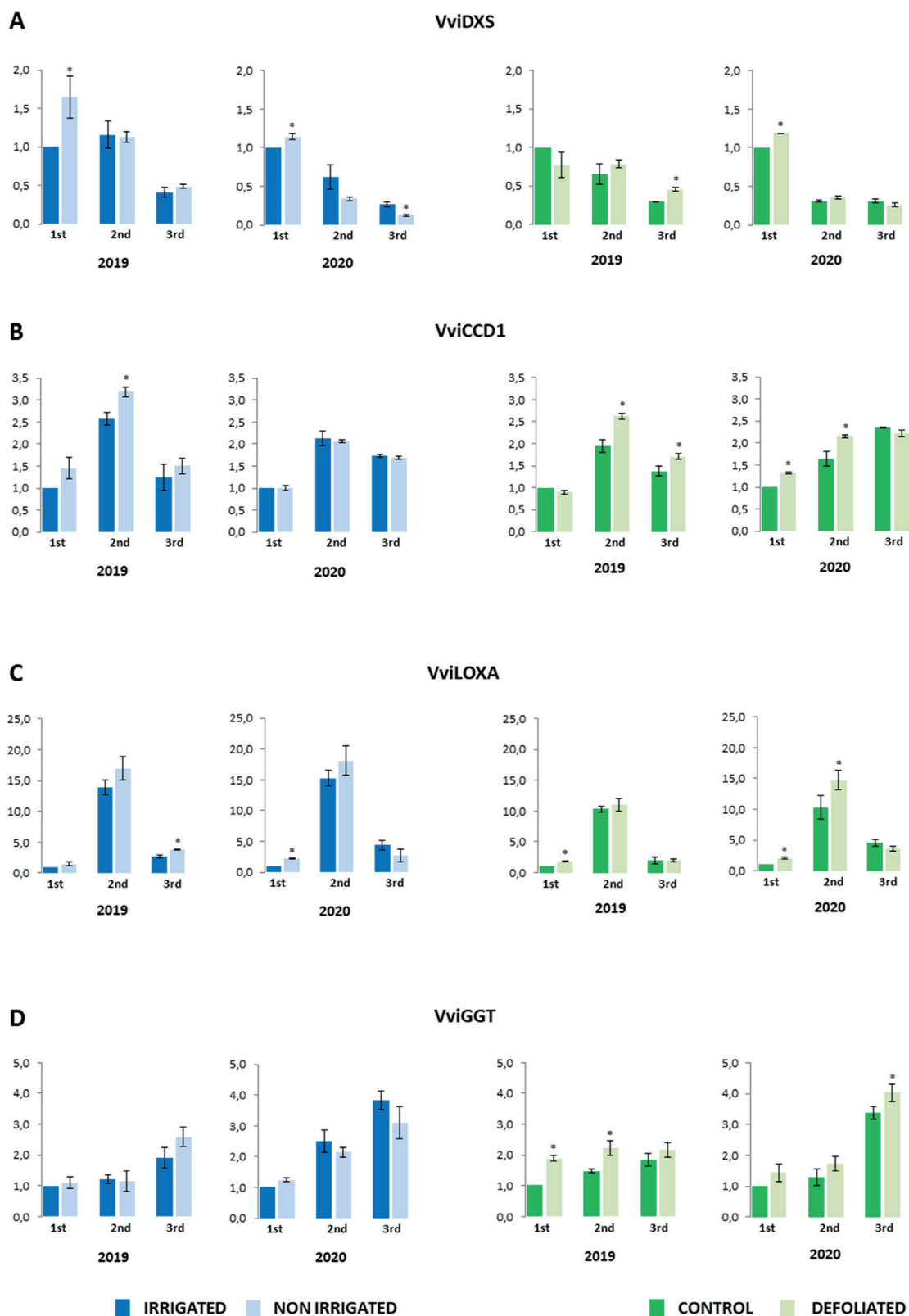


FIGURE 6. Expression level of genes involved in the biosynthesis of various aroma compounds (*VviDXS* (A), *VviCCD1* (B), *VviLOXA* (C) and *VviGGT* (D)) during the two vintages (2019 and 2020).

The expression level in the water deficit experiment is shown in dark blue (irrigated) and light blue (non-irrigated). Similarly, the expression level in the defoliation experiment is shown in dark green (control) and light green (defoliated). Vertical bars represent the standard deviation of mean values and asterisks indicate the statistically significant differences (Student's *t*-test, *p* value < 0.05) between control and treated plants of the same sampling period. The three times of sampling are indicated with numbers under each pair of graphs (green berry, 1st; veraison, 2nd and harvest, 3rd).

The lipoxygenase A gene (*VviLOXA*; VIT_06s0004g01450), which is involved in the biosynthesis of volatile compounds via fatty acid metabolism (Podolyan *et al.*, 2010), exhibited an almost similar expression profile in both vintages, with an up-regulation after veraison followed by a decrease at the harvest stage, which coincided with the decrease in vegetal flavours approaching harvest (Figure 6C). The water deficit resulted in higher *VviLOXA* expression level at the harvest stage of 2019 and at the green berry stage of 2020 (Figure 6C). In defoliated plants, the *VviLOXA* gene was up-regulated at the green berry stage of both vintages, as well as at veraison in 2020 (Figure 6C). Finally, the expression analysis of the γ -glutamyl-transpeptidase gene (*VviGGT*; VIT_11s0016g02830), which is related to volatile sulphur compounds biosynthesis (Kobayashi *et al.*, 2011), exhibited an up-regulation during the progression of berry maturation in both vintages, suggesting developmental regulation (Figure 6D). The water deficit had no effect on *VviGGT* gene expression, while leaf removal resulted in an up-regulation during the two first phenological stages of 2019 and the harvest stage of 2020 (Figure 6D).

DISCUSSION

The productivity and distinguishing grape quality characteristics of a certain cultivar are determined by the combinatorial action of the genotype and environmental factors, such as soil, landscape and climate. Another critical parameter is human intervention via the application of viticultural techniques which aim to cause beneficial changes to the maturation process.

In our study, all the parameters contributing to final yield, such as berry size, cluster weight and yield/vine, were reduced in both non-irrigated and defoliated vines compared to the control (i.e., irrigated and non-defoliated) vines. Although water deficit is known to reduce yield components (Romero *et al.*, 2022), leaf removal studies have resulted in diverse outcomes. For instance, decreased berry size has been observed in Cabernet-Sauvignon grapes, but not in Merlot and Sangiovese grapes, suggesting that the response to defoliation is cultivar-dependent (Kotseridis *et al.*, 2012). Pre-bloom leaf removal can lead to low yield due to reduced fertilisation and berry set (Hickey and Wolf, 2018). However, according to Intrieri *et al.* (2008), the same result can also be observed when defoliation occurs in the early stages of berry growth (i.e., less than 5 mm in diameter) due to reduced carbohydrate availability; this is probably a better explanation for the results of the present study. Additionally, early leaf removal can lead to smaller berries with increased skin thickness, skin-to-pulp ratio and concentration of phenolic (Stefanovic *et al.*, 2021) and aromatic compounds (Moreno *et al.*, 2015). This is probably associated with the reduced intensity of cell divisions and the degree of cell enlargement during the first weeks after berry set due to changes in the availability and distribution of synthesised carbohydrates (Caccavello *et al.*, 2017). The number and size of berry cells is also likely to be affected by increased temperature as a result of the direct exposure of the grapes to

light. According to van Leeuwen *et al.* (2017), when smaller berry size is linked to an increased percentage of skins and seeds, it is not due to the reduced weight per se but to the factor that caused it - which in most cases is water deficit and light exposure. Smaller berries is a desirable characteristic in red wine-making cultivars such as Xinomavro due to the increased contribution of the solid components of the skin and seeds, and thus to a higher concentration of their chemical components in the produced wine (Ivanišević *et al.*, 2020).

In contrast to the yield components, the two treatments had different effects on grape juice composition. The water deficit did not affect TSS level, whereas leaf removal resulted in significantly increased level compared to the controls in both vintages. Although both treatments led to reduced titratable acidity, grape berries from the defoliated vines exhibited lower TA level compared to those from non-irrigated vines in the 2020 vintage. Water deficit is known to improve grape juice composition parameters (Santos *et al.*, 2007; Romero *et al.*, 2013). However, reduced water availability during the post-veraison period can negatively affect the sugar accumulation in berries due to a reduced rate of assimilation (Intrigliolo *et al.*, 2012). An intense water deficit during the ripening period has been shown to lead to a reduction in sugar accumulation in Tempranillo grapes due to a lack of photosynthetic function (Intrigliolo and Castel, 2010). On the other hand, the effect of leaf removal depends mainly on climate conditions, the cultivar and the timing of the treatment (Petropoulos *et al.*, 2011; Kotseridis *et al.*, 2012; Frioni *et al.*, 2017; Cincotta *et al.*, 2021). Previous studies (Tardáguila *et al.*, 2008) have reported that despite transiently reducing total photosynthesis, the removal of photosynthetic leaves at berry set or earlier, stimulates the growth of lateral shoots and secondary leaf area. As the leaves of the secondary leaf area have a better photosynthetic efficiency than those of the main leaf area, early defoliation may lead to a better photosynthesis during ripening, especially in late-ripening cultivars such as Xinomavro. Lukić *et al.* (2017) reported that leaf removal after berry set exposes grapes to increased solar radiation and temperature, resulting in reduced acidity and increased total soluble solids in the must. However, other researchers have reported increased acidity in the must of grapes exposed to light compared to shaded ones (Koundouras *et al.*, 2009); this may be due to the increased synthesis of ascorbic acid, which is a precursor of tartaric acid (Melino *et al.*, 2011). Although leaf area was not estimated in the present work, the increased concentration of sugars in grapes from the defoliated vines could be explained by the possibly greater participation of secondary leaf area in the defoliated vines, and thus the more favourable ratio of total leaf area/yield.

Although total soluble solids and titratable acidity are important grape berry parameters, appropriate sugar/acid maturity alone is not enough to ensure the good quality of grapes and therefore, the good quality of the wine. The most important factor affecting grape and wine quality is the phenolic content of grapes at harvest, considering that many sensory attributes of wine are directly associated with

phenolic compounds (Hufnagel and Hofmann, 2008). In the present study, the water deficit resulted in a significant increase in total phenol level compared to the controls in both vintages, while the higher phenolic content of the defoliated vines was only observed at the veraison stage of 2019. These findings concur with the response of the phenylpropanoid pathway genes *VviPAL* and *VviC4H* to both treatments. Similarly, anthocyanin content per berry was higher in both non-irrigated and defoliated vines compared to their controls. Although this trend is consistent with the *VviUFGT* up-regulation at the veraison stage observed in defoliation in both vintages, surprisingly, it only concurs with the *VviUFGT* response to water deficit in the first vintage. The beneficial effect of deficit irrigation on phenolic compounds has been reported for several cultivars, including Xinomavro (Theodorou *et al.*, 2019; Theocharis *et al.*, 2021). The favourable effect of a mild water deficit on the accumulation of anthocyanins is usually linked to an increased berry skin-to-pulp ratio due to a reduction in their size (Koundouras *et al.*, 2009), changes in the microclimate of the grapes (increased illumination) or the preferential distribution of the photosynthesis products in the berries during ripening (Romero *et al.*, 2013). However, according to Roby *et al.* (2004), the increased polyphenol content of berries under water deficit conditions occurs independently of any changes to the morphological and anatomical characteristics of berries. On the other hand, a cultivar-dependent response to defoliation (i.e., increased phenols level in Merlot and Cabernet-Sauvignon and no effect in Sangiovese) has been observed in Greek summer conditions (Kotseridis *et al.*, 2012). The effect of exposing grapes to light at specific stages of the vegetative cycle seems to differ between cultivars. Chorti *et al.* (2010) reported that in the Nebbiolo cultivar very low as well as excessive exposure of grapes to light led to a reduced concentration of anthocyanins in the skins. Ristic *et al.* (2007) reported that shading in the early developmental stages of Syrah berries mainly affects the type and concentration of individual anthocyanins and not the final concentration of total anthocyanins, a conclusion also reported by Ivanišević *et al.* (2020) in Cabernet-Sauvignon. However, in dry heat viticultural regions, the subsequent warming of exposed grapes may cause reduced anthocyanin accumulation. For instance, Mori *et al.* (2007) observed a significant decrease in the anthocyanin content of Cabernet-Sauvignon grapes at 35 °C compared to at 25 °C.

Given that the altered phenolic and anthocyanin level of grape berries under different viticultural techniques depend on transcriptional changes, it was challenging to investigate the expression profiles of genes involved in phenolic compounds biosynthesis. Recent transcriptomic studies have shown phenylpropanoid pathway genes to be differentially expressed under various treatments (Pastore *et al.*, 2013; Savoi *et al.*, 2016; He *et al.*, 2020). In the present study, we showed that in Xinomavro control vines the expression of *VviPAL* and *VviUFGT* was increased significantly at veraison and then declined slightly towards harvest, mainly during the first vintage. The same profile has been observed in other red grape cultivars, such as Norton (Ali *et al.*, 2011), Merlot

(Yanli *et al.*, 2019) and the domestic cultivar Agiorgitiko (Alatzas *et al.*, 2021), suggesting that the expression of these genes is developmentally regulated, at least in red grape cultivars. The water deficit resulted in an early up-regulation of *VviPAL* and *VviC4H*, regardless of the vintage, while defoliation led to increased transcript accumulation in the 2019 vintage only. The opposite trend was observed for *VviUFGT*; i.e., while the water deficit increased *VviUFGT* expression level in 2019 only, defoliation increased it at the veraison stage in both vintages. Therefore it seems that the response of these three genes to the treatments was affected by climatic variation in different ways. Transcriptomic analyses of red grape cultivars, such as Cabernet-Sauvignon (Castellarin *et al.*, 2007a; Deluc *et al.*, 2009) and Merlot (Castellarin *et al.*, 2007b), have shown that water deficit increases the expression levels of phenylpropanoid pathway genes. Similar results have also been obtained after leaf removal in Cabernet-Sauvignon (Matus *et al.*, 2009) and Sangiovese vines (Pastore *et al.*, 2013), as well as in sunlight exposure/shading experiments in white Riesling grapes (Friedel *et al.*, 2016).

Apart from phenolic and anthocyanin content, grape and wine quality is affected by volatile organic compounds, such as terpenes, C₁₃ norisoprenoids, aldehydes and thiols. We selected genes that are involved in the initial steps of terpenes and C₁₃ norisoprenoids biosynthesis (i.e., *VviDXS* and *VviCCDI*), the gene that encodes for the enzyme that catalyzes the first step of the lipoxygenase pathway leading to volatile aldehydes synthesis (*VviLOXA*), and a γ -glutamyl-transpeptidase gene (*VviGGT*) related to the subsequent production of sulphur volatile compounds.

The *VviDXS* expression was decreased towards harvest in the control plants, while the expression of *VviCCDI* was increased at the veraison stage. Similar profiles have been reported in previous studies (Young *et al.*, 2016; Chen *et al.*, 2017). The expression profile during maturation suggests that both the genes appear to be developmentally regulated, although in different ways (i.e., down-regulation of *VviDXS* and increased level of *VviCCDI*). In contrast to previous studies that have reported the negative effect of drought on terpenoid pathway genes (Grimplet *et al.*, 2007; Savoi *et al.*, 2016), the water deficit in the present study resulted in an up-regulation of *VviDXS* at the green berry stage of Xinomavro, indicating a cultivar-dependent response. The *VviCCDI* expression level was increased at the veraison stage - a response to water deficit that has been also reported in Cabernet-Sauvignon (Deluc *et al.*, 2009) and Agiorgitiko (Alatzas *et al.*, 2021). The accumulation of norisoprenoids under water deficit conditions reported in previous studies (Koundouras *et al.*, 2006; Bindon *et al.*, 2007) indicates that norisoprenoid biosynthesis is resilient to drought. Leaf removal resulted in an up-regulation of *VviDXS* at the green berry stage of the second vintage and an increased expression level of *VviCCDI* at the veraison stage of both vintages. To date, the positive effect of sunlight exposure on terpenoid and norisoprenoid biosynthesis has only been observed in

white grape cultivars, namely Riesling (Friedel *et al.*, 2016) and Sauvignon blanc (Young *et al.*, 2016).

The expression of the lipoxygenase *VviLOXA* gene was increased after veraison and subsequently decreased at harvest in both vintages. The same profile has been also reported in red grape cultivars Tamina (Qian *et al.*, 2017) and Agiorgitiko (Alatzas *et al.*, 2021), as well as in Sauvignon blanc (Podolyan *et al.*, 2010) and Xiangfei (Qian *et al.*, 2017). On the other hand, the *VviGGT* expression level was constantly increased during maturation, similarly to Agiorgitiko (Alatzas *et al.*, 2021) and Sauvignon blanc (Helwi *et al.*, 2016). The expression patterns of *VviLOXA* and *VviGGT* suggests that they are developmentally regulated. Although lipoxygenase genes have been found to be up-regulated due to drought throughout berry development (Deluc *et al.*, 2009), we found that the *VviLOXA* expression level in non-irrigated vines was affected by the vintage (i.e., it was increased at harvest in 2019 and at the green berry stage in 2020). Remarkably, water deficit had no effect on *VviGGT* expression level, regardless of the vintage. Leaf removal caused early up-regulation of *VviLOXA* in both vintages, while the effect on *VviGGT* expression, although positive, was dependent on the vintage.

CONCLUSIONS

The effect of environmental conditions and viticultural practices on berry development and metabolite profile has been substantially studied with diverse results (reviewed in Poni *et al.*, 2017; Gutiérrez-Gamboa *et al.*, 2021). This variability obviously reflects diverse expression patterns of the corresponding genes. Our results revealed gene profiles that were developmentally regulated during maturation and responses to treatments that occurred regardless of the vintage. Water deficit caused early up-regulation of specific genes at the green berry stage (e.g., *VviPAL*, *VviCH* and *VviDXS*), while it had no effect on others (e.g., *VviGGT*). On the other hand, defoliation resulted in the up-regulation of genes at specific phenological stages (e.g., *VviUFGT* and *VviCCD1* at veraison and *VviLOXA* at the green berry stage). Although both treatments positively affected the expression of the genes examined, there were many diverse responses among the developmental stages and the vintages. All the parameters contributing to the final yield, such as berry size, cluster weight and yield/vine, were reduced in both non-irrigated and defoliated vines compared to their control vines (irrigated and non-defoliated respectively). Both treatments showed a significant increase in anthocyanin level, whereas total phenols only increased in the water deficit treatment. On the other hand, leaf removal resulted in significantly increased level of total soluble solids. Our data suggest that applying a water deficit regime could increase total phenol and anthocyanin content without affecting total soluble solids and acidity. On the other hand, leaf removal could be helpful when a higher level of anthocyanins and total soluble solids is desired. Considering that the outcome of viticultural practices depends on cultivar, climate conditions, timing and severity, more extensive and in-depth studies are necessary

to comprehend grapevine responses. Such knowledge would be useful for determining the optimal viticultural practice to ensure the desired grape and wine quality.

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