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ORIGINAL RESEARCH ARTICLE

Defoliation at berry set alters grape composition and gene expression during berry development in two Greek *Vitis vinifera* L. cultivars

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ABSTRACT

The quality traits of a given grapevine cultivar are determined by the combinatorial action of the genotype, and environmental factors such as soil, landscape, and climate. Nonetheless, another critical factor is vineyard management. The forthcoming effects of climate change on viticulture represent a challenge for the application of sustainable practices. The impact of leaf removal at berry set stage on the Greek *Vitis vinifera* L. cultivars 'Agiorgitiko' and 'Assyrtiko' (a red- and white-berry cultivar, respectively) was investigated during the 2019 and 2020 vintages. Vine physiology measurements and berry sampling were performed at three phenological stages: green berry, veraison and harvest. Grape berry composition and the expression level of specific genes were analysed during ripening. Defoliation decreased berry weight in Assyrtiko, while no significant effect was observed in Agiorgitiko. The treatment resulted in a significant increase in total soluble solids and reduced acidity compared to the controls in both cultivars, while the effect on total phenols mainly depended on climate conditions. The expression profile of the genes examined was also altered during ripening, whereas the response to defoliation mainly depended on the vintage and the cultivar. Taken together, the results suggest that defoliation is a useful technique for maintaining grape and wine quality.

KEYWORDS: Agiorgitiko, Assyrtiko, defoliation, phenols, phenylpropanoid and terpenoid pathway

INTRODUCTION

Viticulture and winemaking have been an indispensable part of the Greek national culture since antiquity. Consisting mainly of small, traditional, family-owned plots, the Greek vineyard is defined by a varied topography and climate (i.e., mountainous and arid regions). Greece is one of the major wine producer countries, ranked 10th in Europe and 18th worldwide (OIV, 2019). A total area of over 60,000 hectares is covered with wine-grapes, which produce approximately 2.2 million hectoliters of wine per year. Domestic cultivars, such as Savvatiano, Roditis, Agiorgitiko, Xinomavro and Assyrtiko, represent more than 90 % of the Greek vineyard. Foreign cultivars, such as Cabernet-Sauvignon, Merlot, Syrah and Sauvignon blanc are cultivated as well (Hellenic Statistical Authority, 2020).

Although grapevine is a crop well-adapted to the Mediterranean climate, the extended summer drought and higher temperatures projected by the climate change scenario for the near future (Intergovernmental Panel on Climate Change, 2013) will indisputably affect grape production and quality. Climate change has already induced noticeable changes in grapevine development and wine composition (Drappier *et al*., 2017). While the predicted 2-4 ^{o}C increase in average temperature in the coming decades is an opportunity for grapevine cultivation in northern countries, it will also threaten the established quality characteristic of traditional cultivars in Mediterranean wine-growing regions (Hannah *et al*., 2013; Drappier *et al*., 2017)). Therefore, the development and application of sustainable practices for maintaining grape quality is of great importance for viticulture, especially in the context of climate change (Keller, 2010; Poni *et al*., 2018; Gutierrez-Gamboa *et al*., 2021).

The improved application of viticultural management practices is essential for mitigating adverse climatic conditions. Basal leaf removal is one of several viticultural practices applied thus far (Palliotti *et al.*, 2011; Poni *et al*., 2018). Selective defoliation increases sunlight exposure, temperature and air circulation in cluster zones, and reduces humidity and the risk of microbial infection (Guidoni *et al*., 2008; Sternad Lemut *et al.*, 2015). Leaf removal applied early in the season (i.e., at pea-size stage) reduces berry size, resulting in higher skin to pulp ratio (Poni *et al*., 2009; Palliotti *et al.*, 2011). Sunlight exposure resulting from leaf removal has been found to increase the concentration of phenolic and aromatic compounds in grape berries (Moreno *et al*., 2015; Stefanovic *et al*., 2021) due to an up-regulation of the corresponding genes (Matus *et al.*, 2009; Pastore *et al.*, 2013; Zenoni *et al.*, 2017). However, the favourable impact of defoliation on grape quality has only been observed in studies conducted in temperate climates. Under dry and hot climate conditions, extensive sunlight exposure could excessively increase cluster temperature, with detrimental effects on phenol and anthocyanin content (Bergqvist *et al.*, 2001; Yamane *et al.*, 2006; Mori *et al*., 2007, Petropoulos *et al*., 2011). Furthermore, several studies have shown that defoliation can have variable effects, depending on cultivar, vintage, time of application and severity (Kotseridis *et al.*, 2012; Acimovic *et al*., 2016; Frioni *et al.*, 2017; Verdenal *et al*., 2018). Therefore, optimising the timing and severity of defoliation represents a challenge in order to ensure the grape and wine quality of a given cultivar (Yue *et al*., 2019; Cataldo *et al*., 2021).

Holistic approaches are essential for determining the impact of environmental conditions on grape quality traits. Grape metabolomics should be supplemented by gene expression studies to elucidate environmental impacts on phenolic and volatile compounds biosynthesis during berry maturation. During the last two decades, the availability of grapevine genome and high-throughput transcriptomic methods has enriched our knowledge about tissue-specific, developmental- or environmental-controlled gene expression patterns (Wang *et al*., 2020). Recent studies have shown that exposure to sunlight alters the expression profile of genes related to aroma and flavour characters in both red- and white-berry cultivars (Young *et al*., 2016; Zenoni *et al.*, 2017; He *et al.*, 2020).

Most studies on abiotic stress and/or viticultural practices conducted under Greek vineyard conditions (Koundouras *et al*., 2006; Koundouras *et al*., 2009; Petropoulos *et al*., 2011; Kotseridis *et al*., 2012; Chorti *et al*., 2016; Theodorou *et al*., 2019; Theocharis *et al*., 2021) have focused almost exclusively on the phenolic composition of grapes. Although phenolic composition is an important quality trait in viticulture and oenology, holistic approaches are still scarce. In a recent study, we combined physiological, chemical and phenolic characters with transcriptomic data and showed that leaf removal and water deficit had diverse significant effects on the gene expression and grape berry composition of the domestic cultivar Xinomavro (Alatzas *et al*., 2023). We have also conducted similar studies on deficit irrigation in two other domestic cultivars, Agiorgitiko and Assyrtiko (Alatzas *et al*., 2021), and on elicitor application in Savvatiano and Mouhtaro (Miliordos *et al*., 2022; Miliordos *et al*., 2023).

In the present study, we applied leaf removal at berry set stage on two emblematic Greek cultivars, the red Agiorgitiko and the white Assyrtiko, in order to examine the effects on grape berry composition and gene expression. Physiological and chemical parameters were measured, and the expression level of genes involved in the initial steps of the phenylpropanoid pathway was evaluated and correlated with grape berry total phenols. Moreover, the expression profile of genes related to the biosynthesis of the volatile organic compounds that contribute to grape aroma and flavour was also determined. The results show that leaf removal can significantly influence both the gene expression pattern and grape berry composition.

MATERIALS AND METHODS

1. Vineyard Site and Experimental Design

The trial was conducted during the 2019 and 2020 growing seasons in two 12-year-old commercial vineyards located

in Drama (41°12′03″N, 23°57′10″E, 211 m) and Kavala (40°48′43″N, 23°59′25″E, 231 m) in Northern Greece. The vineyards were planted with the varieties (*Vitis vinifera* L.) cv. Assyrtiko and Agiorgitiko respectively, which were grafted on 1103 Paulsen (*V. rupestris* × *V. berlandieri*) at a density of 3,333 vines/ha (2.5 m between rows×1.2 m within rows). The vines were trained on a vertical trellis with three fixed wires and pruned on a Guyot system with two canes of 6-7 nodes each. The water was applied via a drip irrigation system equipped with 4 L/h drip emitters. Pest and canopy management, irrigation and fertilisation were applied according to standard local viticultural practices. The leaf removal treatment consisted of full manual removal of the total leaf area (main leaves and lateral shoots) of the first six basal nodes (Suppl. Figure S1). The control comprised non-defoliated vines. Defoliation was carried out at berry set of each year (E-L 27; Coombe 1995). A certain phenological stage was considered to have been reached when 50 % of the grapes were at the same phenological stage. Three blocks of 10 consecutive vines in two adjacent rows were randomly chosen for treatment.

2. Vine Parameters

Stem water potential (Ψstem) measurements were conducted with the use of a pressure chamber according to Choné *et al.* (2001). In each measurement set, three mature leaves of the inside 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 Ψs. Measurement of Ψs were performed at midday (12:30 to 13:30), on three cloudless days per season, corresponding to the growth stages of bunch closure (i.e., green berry, veraison and harvest). Only the four central vines of each replication were used for measurements. Net assimilation rate (A), stomatal conductance (gs) and transpiration (E) were recorded at midday simultaneously with Ψs measurements, using the LCi portable gas exchange system (ADC BioScientific Ltd., Hoddesdon, UK). Measurements were taken on three fully expanded, recently matured and sun-lit leaves per plot (photosynthetic photon flux density > 1200 μmol/m^2/s), which were adjacent to those used for Ψs determination.

3. Berry Sampling and Must Analysis

The samplings were conducted at three phenological stages throughout berry maturation (green berry, E-L 33; veraison, E-L 36; harvest, E-L 38, according to Coombe, 1995). Three samplings were performed in 2019 on Day Of Year (DOY) 217, 231, 242 for Assyrtiko and 217, 231, 260 for Agiorgitiko and in 2020 on DOY 216, 234, 246 for Assyrtiko and 216, 234, 259 for Agiorgitiko. Samples of 200 berries were collected randomly from four marked vines (i.e., 50 berries per vine selected randomly from the upper, medium and lower parts of the clusters) in each plot per sampling date. Berry fresh weight was then determined. Afterwards, 50 berries collected per plot were pressed and the must was analysed for soluble solids (°Brix) by refractometry, titratable acidity (g/L tartaric acid) and pH, according to the methods of OIV (2018). The grapes were harvested on the last sampling date (i.e., on 30 August 2019 and 2 September 2020 for Assyrtiko, and on 17 September 2019 and 15 September 2020 for Agiorgitiko), when total yield per plant (kg/vine) and average cluster weight (g) were also estimated.

4. Phenolic Content of Whole Berries

The remaining berries from each sampling date (approximately 150 berries per replicate) were homogenised using an Ultra Turrax T25 (IKA-Werke, Staufen, Germany) at 24,000 rpm for 1 min. Total phenol content was measured according to Iland *et al*. (2004). Briefly, 1 g of the homogenate was transferred to 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

Three samplings and vine physiology measurements were carried out simultaneously on the following DOY: 204 and 220 for both varieties, 242 for Assyrtiko and 260 for Agiorgitiko in 2019; 196 and 224 for both varieties, 246 for Assyrtiko and 259 for Agiorgitiko in 2020. Skins of 10 berries per plot were removed by means of a razor from the grapes, covered with aluminium foil and placed on dry ice. Tissue samples were ground to powder with liquid nitrogen and RNA was extracted by the method of Reid *et al*. (2006). 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. 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 times, and the PCR conditions were optimised for primers corresponding to selected genes (Suppl. Table S1). The samples were further diluted, and quantitative PCR reactions were performed on 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 cycler conditions: 2 min at 50 °C, 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 a melting curve analysis was performed at the end of each reaction to confirm primer specificity. 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 the differences and similarities among samples, as well as to confirm robustness and analytical variability. PCA and three-way analysis of variance (ANOVA) were performed on chemical and phenolic data of grape berries using the XLSTAT software (Addinsoft, New York, USA). All data were subjected to one-way ANOVA, and Student's

t-test was used to assess the statistically significant differences between the mean values.

RESULTS

1. Climatic conditions and vine water status

The experimental vineyards are located in neighbouring narrow valleys, both at an elevation of approximately 200 m, in northern Greece close to the Aegean Sea (Suppl. Figure S2). The Mediterranean climate of the region is generally characterised as having mild winters and temperate summers. During the ripening period of 2019 (i.e., July and August), the total rainfall in both vineyards was lower than that in 2020 (76 mm versus 111 mm and 47 mm versus 62 mm in the Assyrtiko and Agiorgitiko vineyards respectively). However, total rainfall in June was higher in 2019 than in 2020 in both vineyards (Suppl. Table S2). It is worth mentioning that no precipitation occurred in September prior to harvest in both vintages (Figure 1). In the Assyrtiko vineyard, the average daily maximum temperatures during the season from berry set to harvest was 33.9 °C in 2019 and 31.9 °C in 2020. Furthermore, a higher number of days with maximum temperature above 35 °C were recorded in July and August of 2019 than the same months of 2020 (25 versus 4 days respectively) (Figure 1A; https://meteosearch.meteo.gr/index.cfm). The accumulated heat during these months, expressed in growing degree days (GDD, calculated from daily mean temperatures, base 10 °C) was higher in 2019 than in 2020 (958 versus 893 respectively) (Suppl. Table S2). Meanwhile, the temperature differences between the two vintages were almost negligible in the Agiorgitiko vineyard. For instance, the average daily maximum temperatures in July and August were 31.6 °C and 31.9 °C in 2019 and 2020 respectively, and GDD were 983 in both vintages (Figure 1B, Suppl. Table S2).

Water status is known to be an important factor in grapevine development, influencing both yield and berry quality (Chone *et al*., 2001, van Leeuwen *et al*., 2009). We initially examined stem water potential (Ψs), a physiological parameter known to indicate plant water status (Chone *et al*., 2001). Defoliation had no effect on the midday stem water potential of both cultivar vines (Suppl. Figure S3A). The Agiorgitiko vines constantly exhibited Ψs values of between -1.0 and -1.2 MPa, while the Ψs values in Assyrtiko were between -0.9 and -1.2 MPa during maturation in both vintages. Since water deficit is considered weak when Ψs values are higher than -0.9 MPa and severe when they are lower than -1.4 MPa (van Leeuwen *et al.*, 2009), both defoliated and control vines were subjected to moderate drought stress at the time of measurement. In agreement with the water potential profile, other physiological parameters, such as net assimilation rate (A), stomatal conductance (gs) and transpiration (E) were similar in both defoliated and control plants (Suppl. Figure S3B, C and D). In particular, no

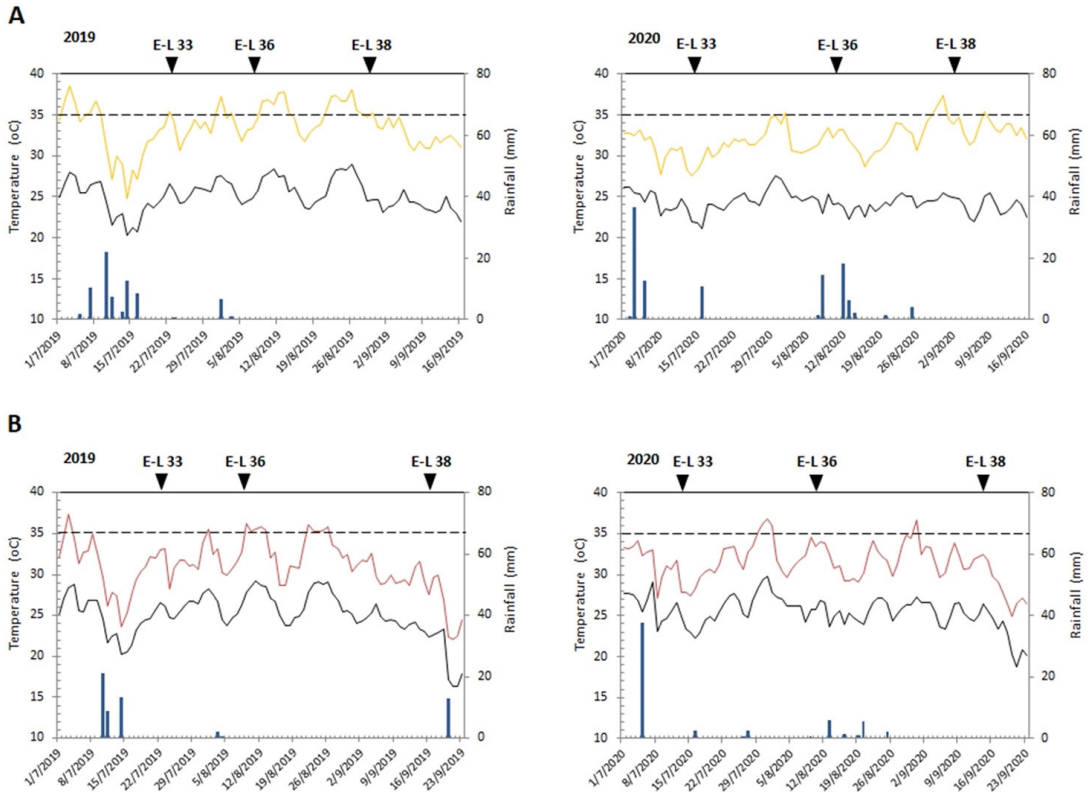


FIGURE 1. Weather conditions at the experimental vineyards of Assyrtiko (A) and Agiorgitiko (B) during the ripening periods of 2019 and 2020. Evolution of daily mean (black lines) and maximum temperature at the vineyards of Assyrtiko (yellow lines) and Agiorgitiko (red lines). Daily rainfall is shown by blue columns and black arrows indicate the days of sampling.

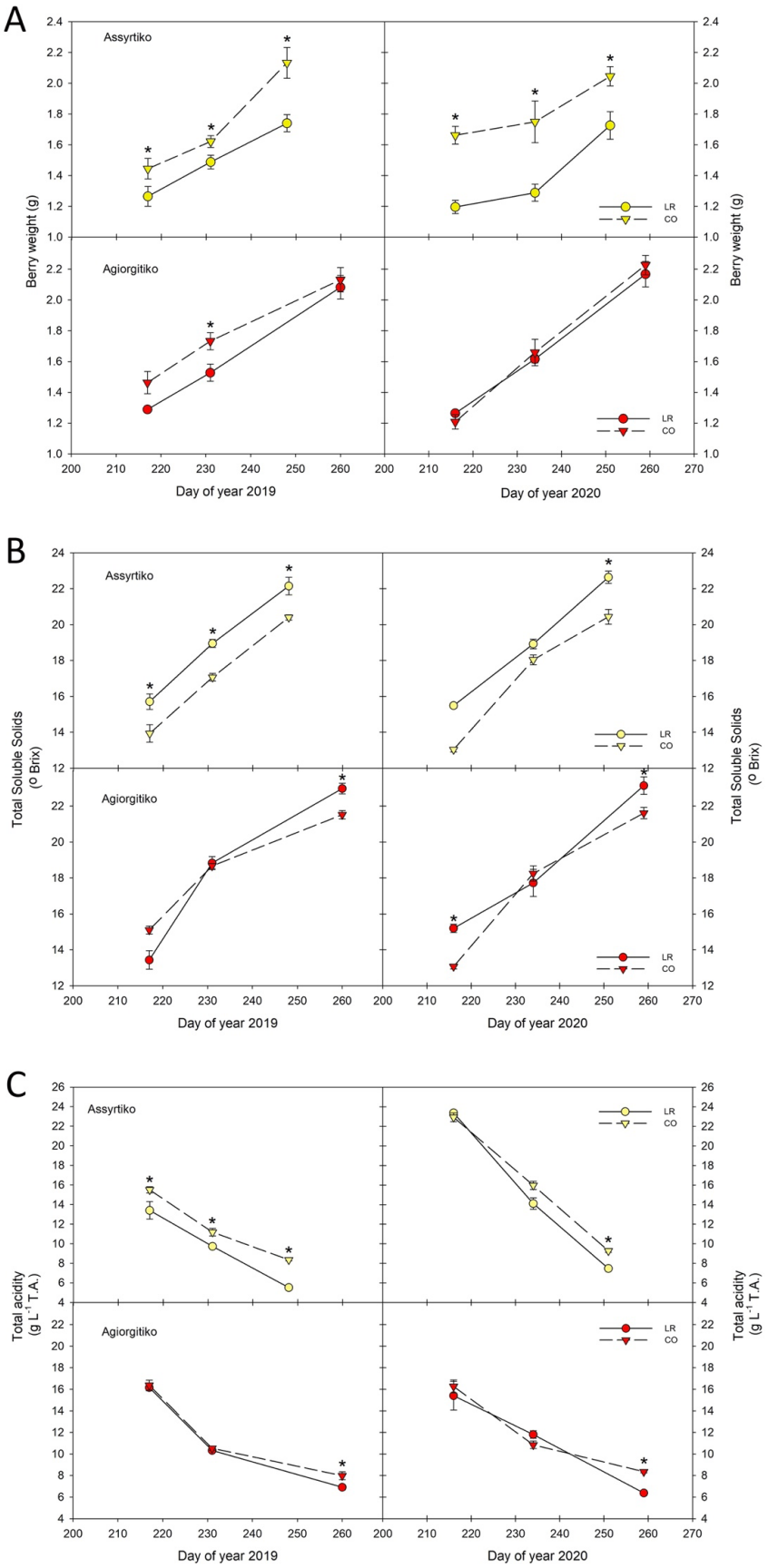


FIGURE 2. The effect of leaf removal on grape berry weight (A), total soluble solids (B) and titratable acidity (C) in Assyrtiko (yellow lines) and Agiorgitiko (red lines) during ripening. Vertical bars indicate the standard deviation of mean values (n = 9) and asterisks indicate statistically significant differences (Student's t-test, p value < 0.05). LR, leaf removal; CO, control.

stomatal conductance values were recorded below 0.05 mol $H_2O/m^2/s$ (Suppl. Figure S3C), which is considered as the threshold of severe water deficit (Cifre *et al*., 2005). Taken together, the results show that defoliation did not affect the water status of the vines.

2. Yield parameters and berry composition

In order to evaluate the effect of defoliation on grape berry quality traits, grape samples were collected during both vintages and a number of parameters (i.e., size, yield, total soluble solids, acidity and total phenols) were measured.

2.1. Grape berry size and yield

Although berry size decreased more in defoliated vines than in the controls of both cultivars, the decrease was statistically significant ($p < 0.05$) in Assyrtiko only (Figure 2A). Moreover, the grape berries of the Assyrtiko control vines were larger at the harvest stage of the 2019 vintage than in 2020, while no significant differences between the two vintages were observed in both defoliated and control Agiorgitiko vines (Figure 2A). Remarkably, no significant differences between the two vintages were observed in the final yield (based on yield/vine and cluster weight) of the control vines at harvest (Suppl. Figure S4A and B). However, yield/vine was lower in the defoliated Assyrtiko plants in the 2020 vintage (4.4 kg/vine compared to 4.8 kg/vine in the control plants), and it was similar in the defoliated and control plants (i.e., 4.9 kg/vine) in 2019. Furthermore, yield/vine in defoliated Assyrtiko plants was significantly lower in the 2020 vintage than in the 2019 vintage (4.4 kg/vine versus 4.9 kg/vine) (Suppl. Figure S4A). A similar tendency was also observed in defoliated Agiorgitiko plants (4.1 kg/vine in 2020 compared to 4.4 kg/vine in 2019), but the difference was not statistically significant (Suppl. Figure S4A). Taken together, the results suggest that defoliation had a stronger impact on yield parameters in Assyrtiko than in Agiorgitiko.

2.2. Total Soluble Solids and Titratable Acidity

One of the most important berry quality traits is grape juice composition in terms of total soluble solids (TSS; expressed in ◦Brix) and titratable acidity (TA; expressed in tartaric acid g/L). In the present study, the grape berries exhibited a constant increase in total soluble solids during maturation, and higher concentrations of TSS were detected in the defoliated vines of both cultivars at harvest stage compared to the controls in both vintages (Figure 2B). In contrast to total soluble solids, titratable acidity was found to decrease during maturation (Figure 2C) and was significantly lower in defoliated Assyrtiko vines at all sampling stages in 2019 and at the harvest stage of 2020 (Figure 2C). In Agiorgitiko, a significant decrease in TA levels was observed only at the harvest stage (Figure 2C). Therefore, leaf removal positively influenced TSS concentrations and negatively influenced TA at harvest, regardless of the cultivar and the vintage.

2.3. Total phenols in grape berries

The phenolic content of grapes is the most important determinant of grape and wine quality, because a number of sensory characteristics of wine are directly associated with phenolic compounds (Hufnagel and Hofmann, 2008). Total phenols level in Assyrtiko was significantly higher in defoliated vines at the harvest stage of 2019 (2.1 versus

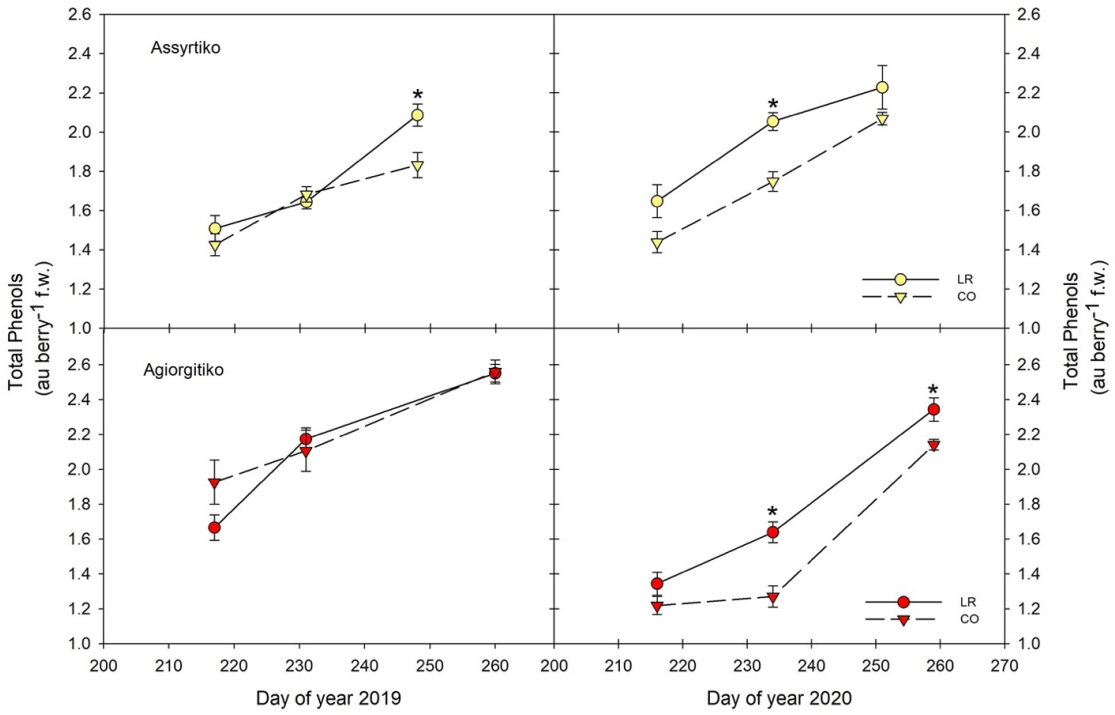


FIGURE 3. The effect of leaf removal on total phenols in Assyrtiko (yellow lines) and Agiorgitiko (red lines) during ripening. Vertical bars indicate the standard deviation of mean values (n=9) and asterisks indicate statistically significant differences (Student's t-test, p value < 0.05). LR, leaf removal; CO, control.

1.8 au/berry f.w.) and at veraison of 2020 (2.1 versus 1.7 au/berry^{-} f.w.) (Figure 3). The increase in total phenols concentration was found to be independent of berry size, thus excluding any possible concentration/dilution effect (Suppl. Figure S5). Interestingly, the total phenols level in the control Assyrtiko vines was significantly higher at the harvest stage of 2020 (2.1 au/berry^{-} f.w.) than at that of 2019 (1.8 au/berry f.w.), suggesting that the high maximum temperatures in the Assyrtiko vineyard during the summer of 2019 (Figure 1A) may have had a negative impact on total phenolic content. In contrast to the controls, the difference in total phenols between the two vintages in the defoliated vines was insignificant (2.1 versus 2.2 au/berry f.w in 2019 and 2020 respectively), indicating that the defoliation impact on phenolic content was independent of climate conditions (Figure 3). Total phenolic content in Agiorgitiko was also significantly higher ($p < 0.05$) in the defoliated vines than in the controls at veraison and the harvest stage of 2020 (1.6 versus 1.3 au/berry f.w. and 2.3 versus 2.1 au/berry f.w respectively). Interestingly, no differences were observed between the defoliated and control vines in the 2019 vintage (Figure 3). Total phenols level was significantly higher in both the defoliated and control plants of the 2019 vintage than that of 2020 at all sampling stages (e.g., 2.6 versus 2.3 au/berry f.w in defoliated and 2.6 versus 2.1 au/berry f.w in the control vines at the harvest stage) (Figure 3), indicating that the lower amount of rainfall during the ripening period of 2019 (Figure 1 and Suppl. Table S2) was associated with total phenols accumulation. Taken together, the results indicate that the environmental conditions may be determinants of phenolic content.

2.4. Multivariate statistics of chemical and phenolic parameters

To improve the visualisation of the results and to examine whether the measured variables could distinguish defoliated and control plants, a principal components analysis (PCA) was carried out. The previously described berry quality data (i.e., berry weight, total soluble solids, titratable acidity, pH and total phenols) were analysed using the harvest stage samples of both vintages. The first two components of PCA score plots explained 79.64 % of the variation in Assyrtiko (Figure 4A) and 68.58 % of the variation in Agiorgitiko (Figure 4B). The samples clearly fell into two groups (i.e., defoliated and control) for Assyrtiko in both vintages (Figure 4A) and Agiorgitiko in the 2020 vintage (Figure 4B), indicating a difference in grape berry composition depending on the treatment. On the other hand, the 2019 and 2020 samples of each cultivar were not clearly discriminated, indicating that the vintage effect was not significant. Further, the berry quality parameters total phenols, total soluble solids and pH were positively correlated with the defoliated vines, and berry weight and acidity with the control vines. A factorial analysis of variance (three-way ANOVA) was conducted to compare the effects of cultivar, vintage and treatment on the measured grape berry quality traits. The analysis revealed that the treatment significantly affected all of the dependent variables (Suppl. Table 3). The cultivar effect on all quality traits (except titratable acidity) was also evident, while vintage affected total phenols and pH only (Suppl. Table S3). Regarding each cultivar, the results of the analysis showed that the treatment had a significant effect on all of the measured traits in Assyrtiko, but affected only TSS and TA in Agiorgitiko. Meanwhile, the vintage effect was only observed on berry weight and total phenols in Assyrtiko and on pH in both cultivars (Suppl. Table S3).

3. Gene expression during grape berry maturation

Recent transcriptomic studies have demonstrated that exposure to sunlight due to leaf removal causes alterations in grape berry transcriptome (Young *et al*., 2016; Zenoni *et al.*, 2017; He *et al.*, 2020). In the present study, the effect of defoliation on gene expression was examined by targeted RT-qPCR analyses of the initial genes of the phenylpropanoid pathway and of the biosynthetic pathways of volatile organic compounds, such as monoterpenes, norisoprenoids and alcohols. Similar to grape composition analysis, berry samples were collected at three phenological stages (green berry, veraison and harvest) during the 2019 and 2020 vintages and, under the assumption that most of these genes exhibit skin-specific or skin-preferential expression (Grimplet *et al.*, 2007), skin tissue samples were used for RNA extraction.

3.1. Genes of the phenylpropanoid pathway

We initially investigated the expression profile of the genes that encode for enzymes of the first steps of the phenylpropanoid pathway leading to the biosynthesis of phenolic compounds (Grimplet *et al.*, 2007). The expression level of phenylalanine ammonia lyase (*VviPAL*; VIT_13s0019g04460) gene in the Agiorgitiko control vines significantly increased after veraison in both vintages, and then it slightly decreased at the harvest stage of the 2019 vintage while remaining constant in the 2020 vintage. Despite being clearly lower than in Agiorgitiko, the *VviPAL* expression level in Assyrtiko increased gradually towards harvest in both vintages (Figure 5A). The expression of the cinnamate 4-hydroxylase (*VviC4H*; VIT_06s0004g08150) gene exhibited a similar pattern to *VviPAL* during the 2019 vintage in Agiorgitiko, while it gradually increased towards harvest in 2020. Meanwhile, it remained almost constant in Assyrtiko during maturation in both vintages (Figure 4B). Leaf removal resulted in the up-regulation of *VviPAL* at almost all phenological stages of both vintages in Agiorgitiko (except at veraison in 2019). The positive effect of defoliation on *VviPAL* expression in Assyrtiko was also observed at the green berry and veraison stages in both vintages (Figure 5A). Defoliation had no effect on *VviC4H* expression level during the first vintage in Assyrtiko, while a down-regulation was observed at the veraison stage in Agiorgitiko. However, increased transcript accumulation was observed at the two first phenological stages of 2020 in both cultivars, suggesting that the response was dependent on the vintage (Figure 5B). Taken together, the results suggest that the response to leaf removal in many of the cases was associated with the environmental conditions.

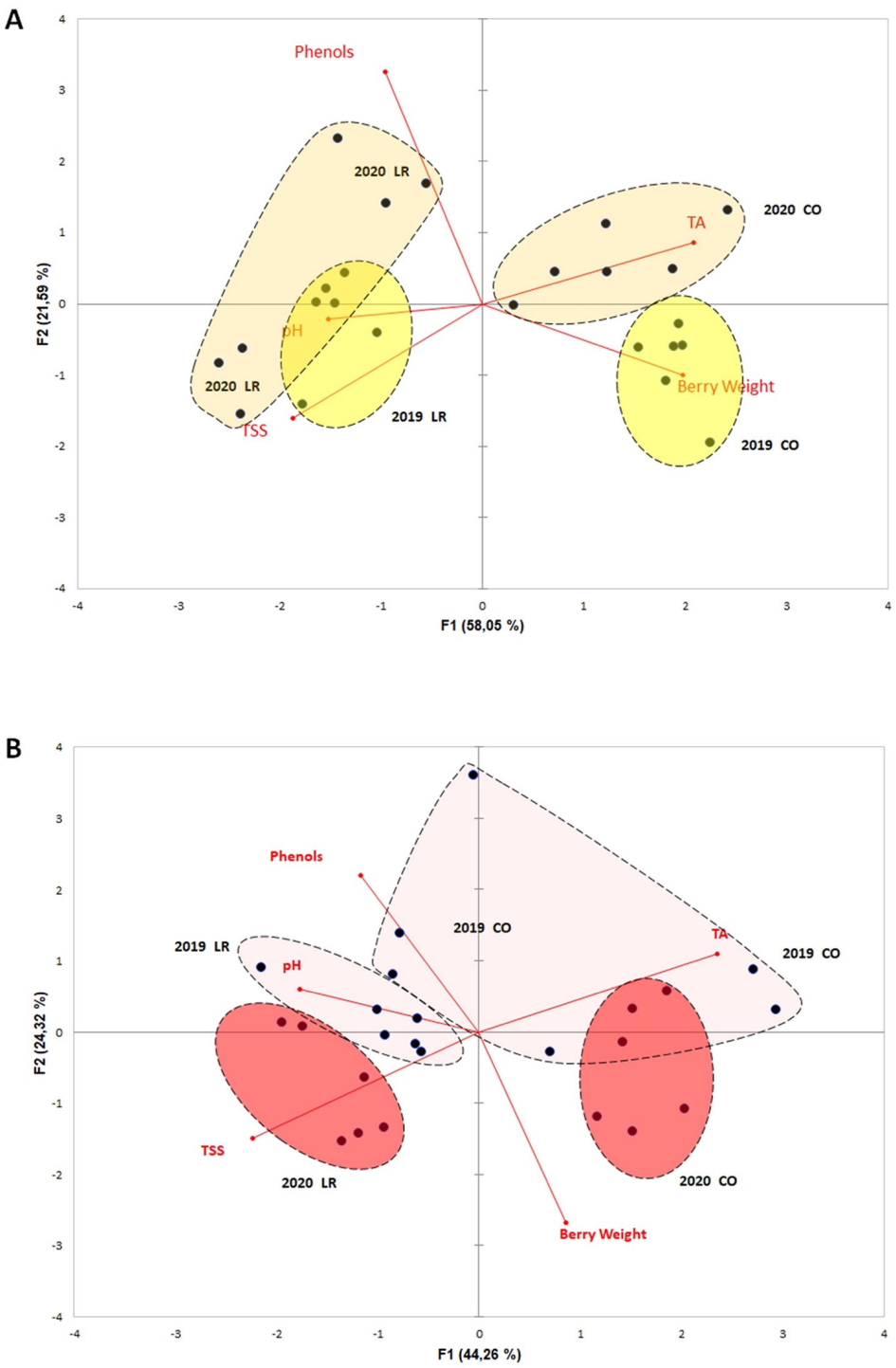


FIGURE 4. Classification using PCA bi-plot of grape berry quality characteristics data (i.e., berry weight, total soluble solids, titratable acidity, pH and total phenols) from defoliated (LR) and control (CO) grape berries at harvest stage of 2019 and 2020 vintages in Assyrtiko (A) and Agiorgitiko (B).

3.2. Genes related to volatile organic compounds

We further examined the expression patterns of genes belonging to the biosynthetic pathways of the volatile organic 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, which encodes for the enzyme that catalyses the initial step in monoterpene biosynthesis (Battilana *et al.*, 2009), showed a declining trend during berry maturation in both cultivars (Figure 5A). In the defoliated Agiorgitiko vines, *VviDXS* expression was lower after veraison in both vintages. By contrast, defoliation had a positive impact on Assyrtiko, leading to the up-regulation of *VviDXS* at the harvest

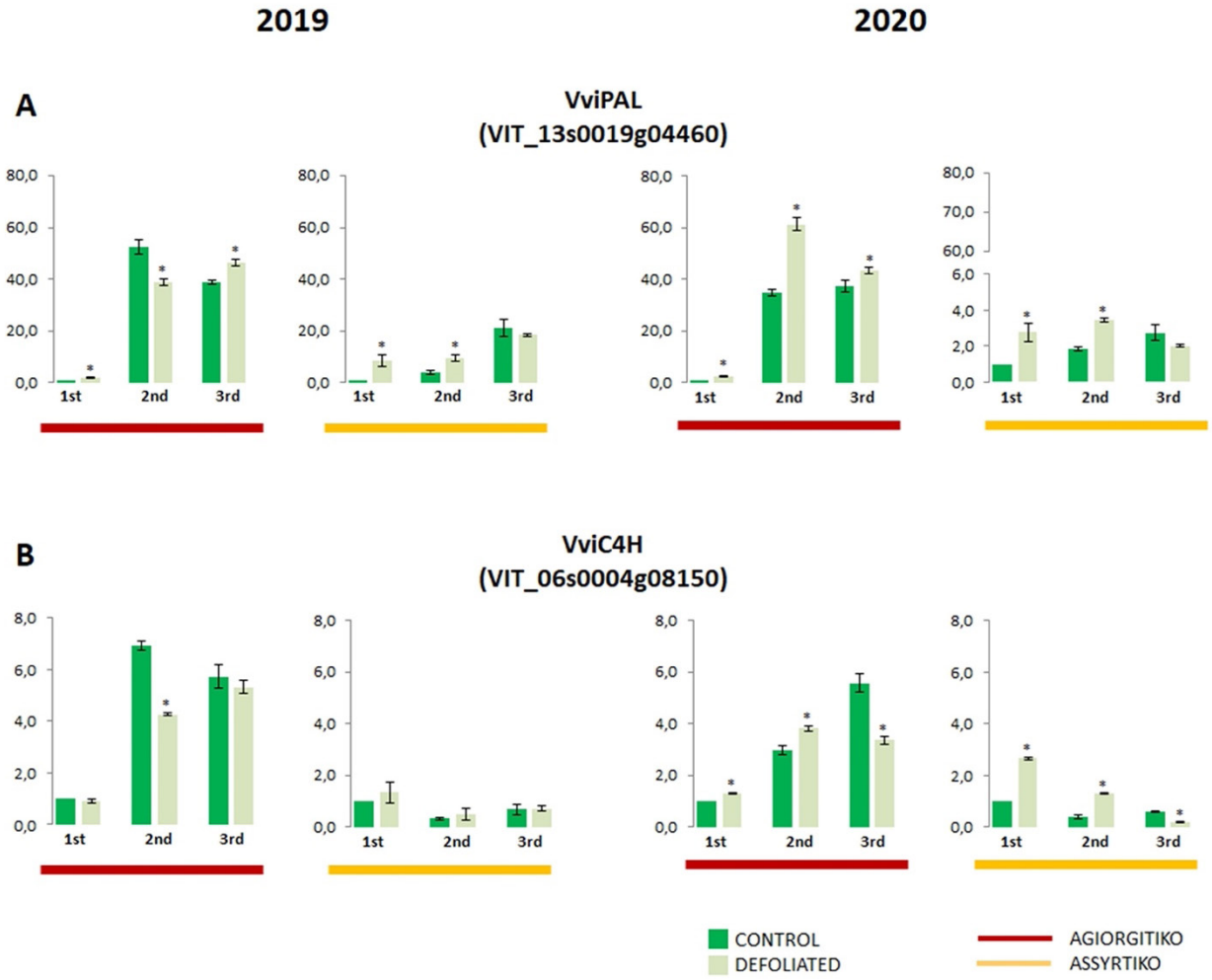


FIGURE 5. Expression level of genes involved in phenylpropanoid pathway (*VviPAL* (A) and *VviC4H* (B)) during the two vintages (2019 and 2020) in Agiorgitiko (red bars) and Assyrtiko (yellow bars). The expression level is shown in dark green (control) and light green (defoliated). Vertical bars represent the standard deviation of mean values (n = 9) and asterisks indicate the statistically significant differences (Student's t-test, p value < 0.05) between control and treated plants of the same sampling date. The three samplings are indicated with numbers under each pair of graphs (green berry, 1st; veraison, 2nd and harvest, 3rd).

stage of 2019 and the green berry and veraison stages of 2020 (Figure 6A). The carotenoid cleavage dioxygenase 1 (*VviCCD1*; VIT_13s0064g00840) gene, which encodes for the key enzyme in the norisoprenoid pathway (Young *et al.*, 2012), exhibited differing profiles depending on the cultivar and vintage (Figure 6B). Leaf removal resulted in a decrease in *VviCCD1* transcript level at the harvest stage of both vintages in Agiorgitiko, while no differences in the Assyrtiko control vines were observed (Figure 6B). Taken together, our findings indicate that the responses to defoliation of the genes controlling terpene and norisoprenoid biosynthesis mainly depended on the cultivar. 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 a similar expression profile in both cultivars, with an up-regulation after veraison followed by a decrease at the harvest stage. This profile coincides with the decrease in vegetal flavours towards harvest and points to a developmental regulation of *VviLOXA* (Figure 6C). In defoliated plants, the *VviLOXA* gene in Agiorgitiko was down-regulated after veraison during both vintages, and the same effect was observed at all phenological stages of the 2019 vintage in Assyrtiko. Conversely, a significant increase in *VviLOXA* transcript accumulation was observed in Assyrtiko with defoliation at veraison and the harvest stage of 2020 (Figure 6C), suggesting that the response was associated with the vintage.

DISCUSSION

Traditionally cultivated in semi-arid regions, grapevine is considered a stress-resilient crop. However, the predicted global warming highlights the need for sustainable techniques for mitigating potential negative impacts on viticulture. Viticultural practices, such as regulated deficit irrigation and/or leaf removal, can bring beneficial changes to the maturation process and ensure grape yield and quality regardless of environmental changes. The present study was

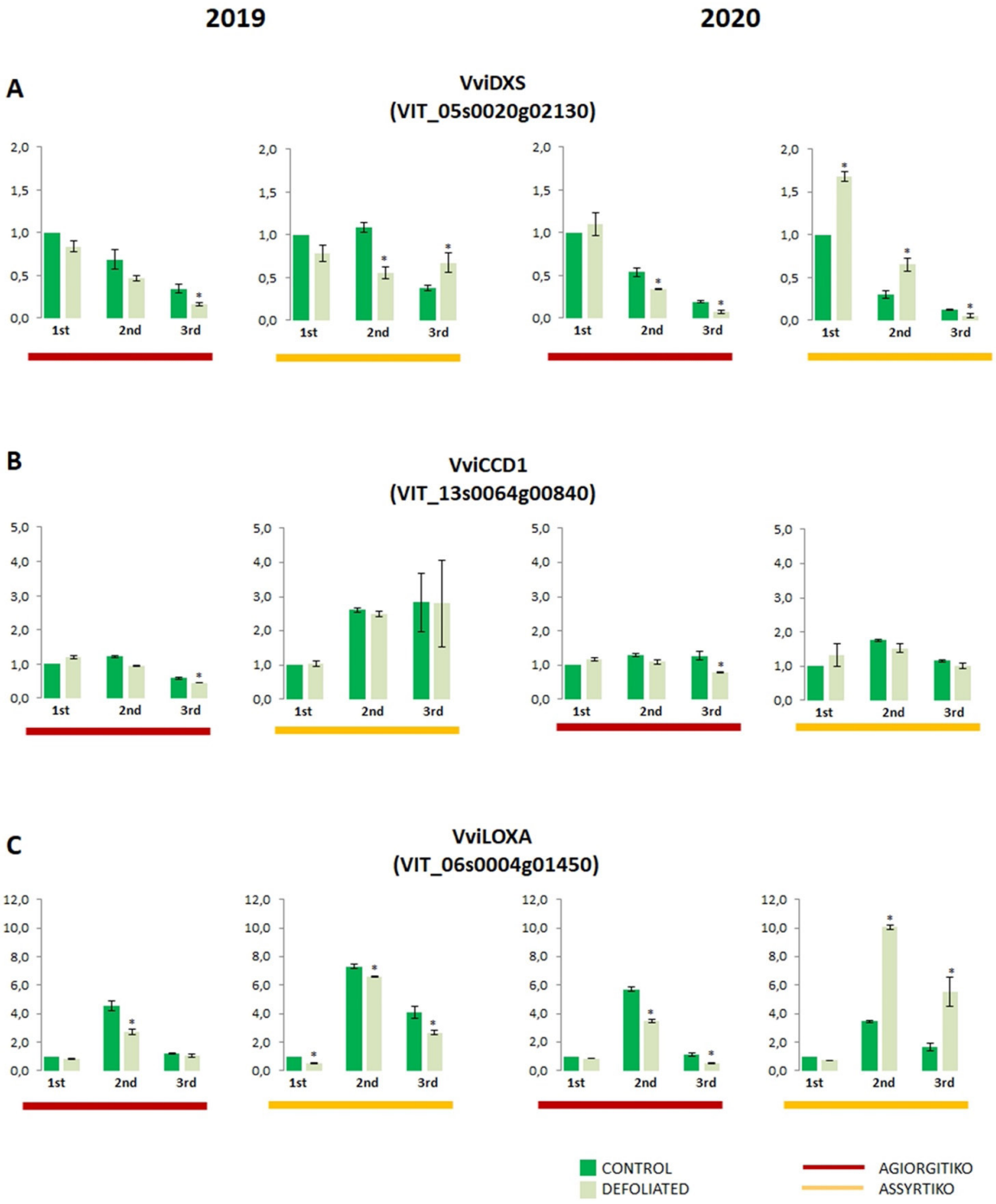


FIGURE 6. Expression level of genes involved in the biosynthesis of various aroma compounds (VviDXS (A), VviCCD1 (B), and VviLOXA (C)) during the two vintages (2019 and 2020) in Agiorgitiko (red bars) and Assyrtiko (yellow bars). The expression level is shown in dark green (control) and light green (defoliated). Vertical bars represent the standard deviation of mean values (n = 9) and asterisks indicate the statistically significant differences (Student's t-test, p value < 0.05) between control and treated plants of the same sampling date. The three samplings are indicated with numbers under each pair of graphs (green berry, 1st; veraison, 2nd and harvest, 3rd).

conducted during two consecutive vintages under different weather conditions. The 2019 vintage was characterised as having reduced rainfall during grape berry maturation (July and August) in both vineyards and higher mean and maximum temperatures (approximately 2 °C higher than the 2020 vintage) in the Assyrtiko vineyard. We were thus able to assess the impact of defoliation on two cultivars under diverse environmental conditions.

The values of the parameters contributing to final yield, such as berry size (both vintages) and yield/vine (second vintage only), were lower in the defoliated Assyrtiko vines

than in the control vines. However, no statistically significant differences were observed in Agiorgitiko. It is known from previous studies that leaf removal has diverse outcomes in terms of grapevine productivity; for instance, a decrease in berry size has been observed in Cabernet-Sauvignon but not in Merlot and Sangiovese grapes, suggesting that the response to defoliation is cultivar-dependent (Kotseridis *et al*., 2012). As well as the cultivar, the severity and timing of removal can significantly affect yield parameters (Acimovic *et al.*, 2016; Verdenal *et al.*, 2018): the removal of 8-10 leaves can dramatically decrease yield/vine, while the removal of 4 leaves has no effect (Acimovic *et al.*, 2016). Pre-bloom leaf removal can lead to low yield due to a decrease in fertilisation and berry set (Hickey and Wolf, 2018). However, the same result can also be observed when defoliation is carried out in the early stages of berry growth (i.e., less than 5 mm in diameter) due to reduced carbohydrate availability (Intrieri *et al*., 2008). Changes in the availability and distribution of synthesised carbohydrates will result in decreased rate of cell divisions and cell enlargement during the first weeks after berry set (Caccavello *et al*., 2017). However, smaller grape berries that have high skin thickness and skin-to-pulp ratio will contain higher concentrations of phenolic (Stefanovic *et al*., 2021) and aromatic compounds (Moreno *et al*., 2015); these are therefore desirable characteristics in wine-making cultivars (Ivanišević *et al*., 2020).

Defoliation resulted in significantly higher levels of total soluble solids and reduced titratable acidity than the controls of both cultivars at the harvest stage. Similar results have also been reported in previous studies (Bubola *et al*., 2017; Cincotta *et al*., 2022; Alatzas *et al*., 2023). The removal of photosynthetic leaves at berry set or earlier, while transiently reducing total photosynthesis, stimulates the growth of lateral shoots and increases the secondary leaf area (Tardaguila *et al*., 2008). Although leaf area was not estimated in the present study, the possible increased photosynthetic activity of the secondary leaf area in the defoliated vines, and thus the more favourable total leaf area/yield ratio, may explain the higher concentrations of sugars in the grapes of defoliated vines. However, contradictory results of numerous studies indicate that the effects of leaf removal on total soluble solids and titratable acidity levels depend on the cultivar, the severity and timing of the treatment, and climate conditions. For instance, intensive defoliation at berry set led to an increase in acidity in Merlot, but had no effect in Cabernet-Sauvignon and Sangiovese (Kotseridis *et al*., 2012); and defoliation at veraison led to reduced soluble solids in the white-coloured cultivar Xynisteri (Constantinou *et al.*, 2019), while the opposite effect was observed when the treatment was applied at berry set stage (Georgiadou *et al.*, 2023).

In the present study, an important grape quality trait found to be mainly related to climate conditions was phenolic content. For instance, the lower amount of rainfall during the ripening period of 2019 was associated with higher total phenols concentration than in 2020 in both defoliated and control Agiorgitiko vines. This vintage effect has been previously observed in Xinomavro in a trial conducted in the same vineyard (Alatzas *et al*., 2023). Meanwhile, the total phenols level in the Assyrtiko control plants was lower in the 2019 vintage, probably due to the maximum temperatures recorded during the ripening period being higher than in 2020. Such negative effects of high temperatures on the accumulation and composition of phenolic compounds in grape berries have already been demonstrated in previous studies by Mori *et al*. (2007) and Xu *et al*. (2011); moreover, in a trial conducted during an extremely hot and dry summer (i.e., daily temperatures exceeding 40 ^{o}C and no rainfall), the phenol content of Agiorgitiko wine was found to significantly decrease (Petropoulos *et al*., 2011). In the present study, defoliation resulted in an increase in the phenolic compound levels of both cultivars, which concurs with the *VviPAL* gene response to the treatment. Furthermore, the treatment seemed to moderate the negative effect of high temperatures on Assyrtiko phenol levels in the 2019 vintage. However, a significant difference in the Agiorgitiko controls was observed only during the 2020 vintage. As well as being associated with weather conditions, response to defoliation has also been found to be cultivar-dependent (increased phenol levels in Merlot and Cabernet-Sauvignon and no effect in Sangiovese) under Greek summer conditions in a previous study (Kotseridis *et al*., 2012).

Given that alterations to the phenolic levels of grape berries are linked to transcriptional changes, it was interesting to investigate the profiles of genes involved in the initial steps of phenolic compounds biosynthesis, and to correlate their expression with the total phenols level. Here, we showed that the expression of *VviPAL* in Agiorgitiko control vines significantly increased at veraison and then slightly decreased towards harvest, mainly during the first vintage. The same profile has been reported in other red-berry cultivars (Ali *et al*., 2011; Sun *et al*., 2019; Alatzas *et al*., 2023), suggesting that *VviPAL* expression is developmentally regulated. In contrast to our previous findings in Xinomavro, *VviC4H* expression also increased during maturation in Agiorgitiko, indicating a cultivar-dependent expression profile. The transcript accumulation of both these genes in the white Assyrtiko was clearly lower than in Agiorgitiko. *VviPAL* expression gradually increased during maturation, while *VviC4H* expression remained constant. The positive effect of defoliation on phenylpropanoid pathway genes has also been previously observed in other red- (Matus *et al*., 2009; Pastore *et al*., 2013; Alatzas *et al*., 2023) and white-berry (Friedel *et al*., 2016) cultivars. In our trial, defoliation led to the up-regulation of *VviPAL* in Assyrtiko at the first two phenological stages in both vintages, resulting in higher total phenol levels than in the controls. By contrast, the increase in transcript accumulation in Agiorgtiko at the green berry stage of 2019 was followed by a significant decrease at veraison, which may explain the similar phenol levels in the defoliated and control vines. The response of *VviC4H* to defoliation in Assyrtiko was associated with climate conditions; interestingly, a significant decrease in Agiorgitiko was observed at veraison in 2019. Similar results regarding *VviPAL* and *VviC4H* expression profiles have been previously obtained in Agiorgitiko under water deficit treatment in the

same vineyard (Alatzas *et al*., 2021), indicating a cultivar-dependent response to abiotic stress.

As well as phenolic content, grape and wine quality is affected by volatile organic compounds, such as terpenes, C_{13} norisoprenoids and alcohols. Previous studies have shown that volatile organic compounds contribute to the aroma of both Agiorgitiko (Koundouras *et al.*, 2006) and Assyrtiko wines (Kechagia *et al*., 2008). Therefore, in order to indirectly estimate the impact of defoliation on grape aroma potential, we selected genes involved in the initial steps of monoterpene and C_{13} norisoprenoid biosynthesis (i.e., *VviDXS* and *VviCCD1*), as well as the gene encoding for the enzyme that catalyses the first step of the lipoxygenase pathway, which leads to volatile alcohol synthesis (*VviLOXA*). *VviDXS* expression decreased towards harvest in the control plants of both cultivars, while *VviCCD* expression increased in Assyrtiko control vines. Similar profiles have been reported in previous studies (Young *et al*., 2012; Chen *et al*., 2017; Alatzas *et al*., 2021). Although in a different way (i.e., down-regulation of *VviDXS* and up-regulatiuon of *VviCCD1*), the expression profile during maturation suggests that both genes are developmentally regulated. Leaf removal resulted in the down-regulation of *VviDXS* in Agiorgitiko and an up-regulation in Assyrtiko (mainly in the second vintage), indicating a cultivar-dependent response; meanwhile, defoliation had no effect on *VviCCD1* expression in both cultivars. It is worth mentioning that both these genes were up-regulated with defoliation in Xinomavro in a previous study (Alatzas et al. 2023). Regarding other studies, sunlight exposure has been found to have a positive effect on monoterpene and norisoprenoid pathway genes in the white-berry cultivars Riesling (Friedel *et al*., 2016) and Sauvignon blanc (Sasaki *et al*., 2016; Young *et al*., 2016); this was correlated with the increased photoprotection needs of the exposed berries (Young *et al*., 2016). However, negative effects have been observed in Cabernet-Sauvignon in a trial conducted in a dry and hot climate region (He *et al*., 2020). In the present study, the expression of the lipoxygenase *VviLOXA* gene increased after veraison and subsequently decreased at harvest in both cultivars. The same profile has been reported in red- (Qian *et al*., 2017; Alatzas *et al*., 2023) and white-berry (Podolyan *et al*., 2010; Qian *et al*., 2017) cultivars, suggesting that *VviLOXA* is developmentally regulated. Leaf removal caused down-regulation of *VviLOXA* in Agiorgitiko, while the response in Assyrtiko depended on the vintage. These results, along with the up-regulation of *VviLOXA* with defoliation reported for Xinomavro in a previous study (Alatzas et al., 2023), indicate that the response is associated with both cultivar and vintage.

CONCLUSIONS

To date, the effects of environmental conditions and viticultural practices on berry development and metabolite profiles have been substantially studied, producing diverse results (reviewed in Poni *et al*., 2018; Gutierrez-Gamboa *et al*., 2021). This variability clearly reflects diverse expression patterns of corresponding genes. Our results revealed that the gene expression profiles were developmentally regulated during maturation, and responses to treatment occurred regardless of vintage (e.g., the early up-regulation of *VviPAL*). However, the variability in gene expression profiles clearly depended on the cultivar (e.g., *VviDXS*), vintage (e.g., *VviC4H*), or both (e.g., *VviLOXA*). Remarkably, leaf removal increased total phenol levels, mitigating any negative effects of the high temperatures recorded in the Assyrtiko vineyard. Finally, increased total soluble solids and reduced titratable acidity were observed with defoliation in both cultivars, while yield parameters were only affected in Assyrtiko. Given that the outcome of leaf removal depends on the cultivar, climate conditions, timing and severity, further extensive and in-depth studies are necessary to increase our understanding of grapevine responses. High-throughput transcriptomic studies combined with detailed metabolomics would provide significant data about the responses of a given cultivar to various environmental conditions and viticultural techniques. Organoleptic analyses of the produced wine can complement the holistic approach, from plant to final product. The knowledge gained would contribute to helping viticulturists and winemakers maintain desired grape and wine quality characteristics, particularly in the context of climate change.

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