Free and glycosylated green leaf volatiles, lipoxygenase and alcohol dehydrogenase in defoliated Nebbiolo grapes during postharvest dehydration

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Abstract

Background and Aims: Nebbiolo grapes are used to produce Sfursat wine, following partial dehydration. This research aimed to clarify the influence of fruit exposure to light and postharvest water loss on the concentration of green leaf volatiles (GLVs) and lipoxygenase (LOX) and alcohol dehydrogenase (ADH) activity of grapes.

Methods and Results: Nebbiolo grapes from Control vines (no defoliation) (ND) and from vines defoliated at fruitset (DFS) or defoliated post-veraison (DPV) were harvested at about 23°Brix and dehydrated at 10 and 20°C, 60% RH and air flow of 1.5 m/s. Berries were sampled at 10 and 20% mass loss (ML). Significant differences in crop yield, bunch mass and berry mass were observed. As expected, the higher the dehydration temperature, the faster the dehydration process: 20% ML at 20°C occurred between 18 and 25 days, the shortest time corresponding to ND and the longest to DFS; at 10°C, the dehydration lasted between 27 and 32 days. At 10°C, the ADH activity was almost double that at 20°C, and in DFS was much higher than in other samples. At harvest, LOX did not show any difference among the samples, while at 10°C and 10% ML, the enzyme activity increased significantly and then declined at 20% ML, especially in defoliated samples. At harvest, the total free GLVs associated with the metabolism of lipid oxidation were 9434, 7212 and 11 656 μg/kg dry weight (DW) in ND, DFS and DPV samples, respectively; the total bound GLVs lipid-derived were 7599, 18 486 and 15 409 μg/kg DW in ND, DFS and DPV samples, respectively. During dehydration at 10°C, the ML induced ADH + LOX activity, especially in defoliated samples, but the bound GLVs, produced by defoliation, greatly decreased.

Conclusions: Defoliation affected the response of Nebbiolo grapes to dehydration temperature: postharvest cold stress (10°C) and ML induced glycosylation of GLVs, alcohol formation (via ADH) and membrane oxidation (via LOX); a further stress effect was observed with leaf removal, regardless of the time of application.

Significance of the Study: The timing of defoliation and postharvest dehydration temperature are significant factors to mitigate the postharvest stress response of Nebbiolo grapes.

Keywords: *alcohol dehydrogenase (ADH), defoliation, dehydration, glycosylated green leaf volatiles (GLVs), grape, lipoxygenase (LOX)*

Introduction

Postharvest dehydration, drying or withering is employed to remove water from grape berries in the production of sweet or dry Passito wines. Postharvest water loss from winegrapes is known to induce significant metabolic changes (Zenoni et al. 2016), depending on the technique and the degree and rate of water loss (Mencarelli and Bellincontro 2013). Cell wall enzymes are the first enzymes expressed in response to water stress and at a low level of water loss (Hsiao 1973). Previous research has shown that genes of cell wall enzymes are activated during winegrape dehydration (Zenoni et al. 2016), which, in turn, are stimulated by postharvest treatment with ethylene (Botondi et al. 2011). Costantini et al. (2006) showed that in Malvasia winegrapes, postharvest dehydration under controlled conditions resulted in a two-step metabolic change commencing at around 10 and 20% mass loss (ML): first, lipoxygenase (LOX) was activated and then alcohol dehydrogenase (ADH).

Glycosylated green leaf volatiles (GLVs) are short-chain acyclic aldehydes, alcohols and esters that form as a result of catalysis by LOX, hydroperoxide lyases (HPLs) and ADH, and they are the main source of green aroma in grapes (Ameye et al. 2018). Glycosylated green leaf volatiles are

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responsible for the aroma imparted by crushed or injured leaves, and plants produce GLVs after damage and biotic or abiotic stress (Hatanaka 1993, Matsui 2006). Lipoxygenase is the first enzyme involved in the production of fatty acid-derived volatiles, via deoxygenation of unsaturated fatty acids (e.g. linoleic and α-linolenic acids) and the production of oxygen free radical molecules; the HPL enzyme cleaves the LOX products, initially producing hexanal and (*Z*)-3-hexenal, from linoleic and α-linolenic acids, respectively. The aldehydes are subsequently converted to their corresponding alcohols, 1-hexanol and (*Z*)-3-hexenol, by ADH. These alcohols can be metabolised by alcohol acyltransferase to produce hexyl, (2,*E*)-hexenyl and (3,*Z*)-hexenyl esters (Vick 1993).

Defoliation is a common vineyard practice used to modify bunch shape or to enhance grape secondary metabolites (Pastore et al. 2013). The rate of postharvest dehydration of grapes is lower in defoliated vines, while defoliation also resulted in changes in the concentration and composition of phenolic compounds (Nicoletti et al. 2013). Moreover, the temperature at which grapes were dehydrated affected the rate of increase of phenolic compounds: at 10°C, the flavonol concentration increased. Most of the phenolic compounds analysed were glycosylated and it is known that glycosylation is strongly influenced by oxidative stress, which is the most common stress (Behr et al. 2020).

In this study, we have hypothesised that defoliation could affect the behaviour of enzymes involved in cell membrane oxidation (LOX and ADH) and their volatile metabolites, and therefore, the GLV aroma of wine. Experiments were therefore established to investigate the effect of defoliation, applied at fruitset and post-veraison in two growing seasons, on the LOX and ADH activity, and on the concentration of lipid-derived volatile organic compounds, in free and glycoconjugate (bound) forms, of Nebbiolo grapes.

Materials and methods

Defoliation and sampling protocol

The experiment was conducted on *Vitis vinifera* L. cv. Nebbiolo grapevines, grown on steep slopes in the Valtellina area, at between 600 and 700 masl by the Cooperativa Cantina di Villa (Sondrio, Italy). Failla et al. (2004) provided a detailed description of the viticultural region. Briefly, Valtellina is a valley running east–west in the central Alps (46°10′N). Vineyards are traditionally grown on the southern slopes between 300 and 700 masl on small artificial terraces (the inclination of the mountain slope ranges between 27 and 70%). Vines are trained to *Archetto Valtellinese* (a modified Guyot system) and planted at a density of about 5880 plants/ha (i.e. row and vine spacing of approximately 1.7 m × 1.0 m), with rows aligned north–south following the maximum slope lines.

The climate of Valtellina is classified as endo-alpine, with a rainfall of 800–1200 mm/year, relatively lower than that with other Alps districts, due to Föhn (also known as Stau) effects (https://www.britannica.com/science/foehn). Rainfall distribution is maximum in the summer semester (from April to September) and minimum in the winter semester. The mean annual temperature in the valley is 11–12°C due to the cold air masses that descend the slopes at night (cold-lake effect), thus increasing the risk of frost. These conditions make the mountain slopes the most favoured thermally for viticulture. The annual growing degree days (GDD) index ranges from 1100 to 1800 GDD units, with a gradient declining with site elevation. Photosynthetically active radiation ranges between 1800 and 3200 MJ/m^2 per annum, with most vineyards within 2700 and 3200 MJ/m^2 on the top of the slopes.

This experiment ran over two consecutive seasons. Meteorological data were recorded at a nearby weather station located in Tirano (Sondrio, Italy) (Figure 1). The two vintages differed mainly in their rainfall (1038 mm vs 637 mm), while the average summer temperature in each season, respectively, was as follows: 20.9 versus 19.6°C for June, 24.5 versus 20.5°C for July, 21.4 versus 22.6°C for August, and 16.7 versus 19.5°C for September.

Two defoliation treatments were evaluated: an early defoliation applied at fruitset (BBCH 71) (Meier et al. 2009) (DFS) on 11 June, and a late defoliation applied post-veraison (BBCH 81) (DPV) on 14 July. In both treatments, only the leaves shading bunches were removed, increasing bunch exposure to sunlight, without excessive limitation of photosynthate availability. To achieve this goal, at least 1 m^2 of leaf area per kg of grape production was guaranteed in all the treatments (Table 1). Vines that were not defoliated (ND, Control) were also included to complete the experimental design. Vine blocks were distributed in the upper, middle and lower parts of the vineyard. For the ND, DFS and DPV treatments, 583, 583 and 579 vines, respectively, were selected in a completely randomised block design (comprising 9, 8 and 8 blocks for ND, DFS and DPV, respectively). In each of the upper, middle and lower parts of the vineyard, eight rows (one row per block, 80 vines per treatment) were designated for measurement of crop yield (the number of bunches) per vine and the average mass of bunches and berries, the characteristics that were of interest for monitoring the dehydration rate. Canopy assessment to determine the proportion of gaps, leaf layer number and proportion of interior leaves and fruit was achieved via the 'point quadrant' method (Smart and Smith 1988) adapted to grapevines. The sugar concentration of berries was similar among treatments in both years, thus the harvest of all treatments occurred within 2 days, 15–16 October (BBCH 89), when berries reached a TSS of 23 ± 1°Brix.

In the second year, the postharvest dehydration experiment was executed: grape bunches were shipped to the laboratory of the Department for Innovation in Biological, Agro-Food and Forest Systems for dehydration tests. After 6 h of night transport in a closed van, the bunches (arrival temperature at the laboratory was 23°C) were placed in perforated boxes (60 × 40 × 15 cm) in a single layer. For each test, two perforated boxes were used, each one containing 6 kg of fruit, one on top of the other, were placed in a small metallic tunnel (45 × 45 × 100 cm) fitted with an exhaust fan with regulated airflow. The small tunnels were placed in two thermohygrometric controlled rooms (12 m^3) at either 10 ± 1°C or 20 ± 1°C: RH was set at 60 ± 5% and airflow at 1.5 ± 0.3 m/s. The duration of the experiment was the time taken to achieve 20% ML in the bunches. Bunches were sampled prior to dehydration and then after a ML of 10 ± 2% and 20 ± 4%. Bunch mass (using the same ten bunches) was determined using a technical balance (Adam Equipment, Milton Keynes, England). During dehydration, thermohygrometric conditions were monitored with a HYGROclip model probe (Rotronic, Bassersdorf, Switzerland) connected to HYGROwin software to record the data. Air speed was measured by means of a Terman hotwire anemometer (LSI, Milan, Italy).

Sample preparation and analyses

In addition to berries sampled from different bunches at time 0, all berries from ten bunches (about 600 berries) in

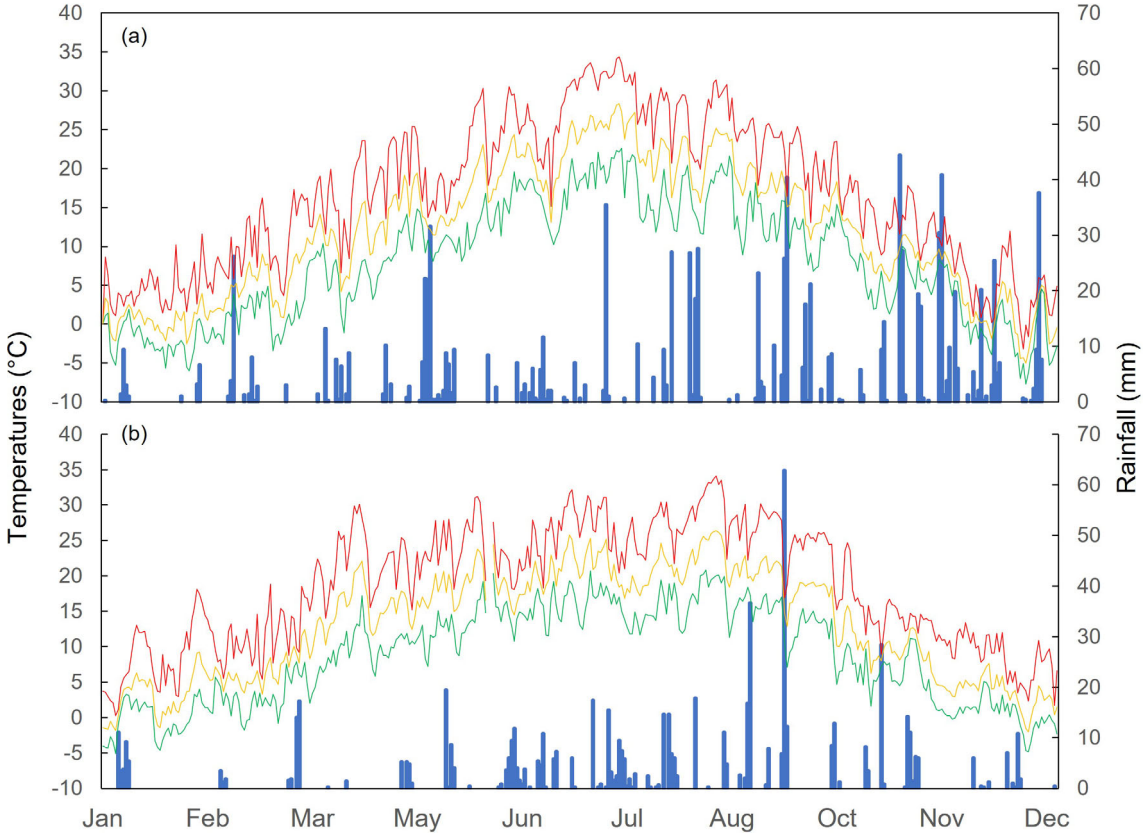

Figure 1. Maximum (——), minimum (——) and average (——) temperature and rainfall (■) recorded during the (a) first year and the (b) second year of the trial.

Table 1. Total leaf area, exposed leaf area and proportion of exposed bunches in two seasons corresponding to Nebbiolo grapevines subjected to no defoliation, defoliation at fruitset and defoliation post-veraison.

	Total leaf area (m^2/vine)		Exposed leaf area (m^2/vine)		Exposed bunches (%)	
Treatment	**First year**	**Second year**	**First year**	**Second year**	**First year**	**Second year**
Not defoliated	3.79 ± 0.21 a	3.84 ± 0.2 a	1.52 ± 0.04 a	1.54 ± 0.06 a	7.9 ± 0.4 b	6.4 ± 0.2 c
Defoliated at fruitset	2.93 ± 0.18 b	3.00 ± 0.07 b	1.46 ± 0.05 b	1.50 ± 0.05 ab	90.7 ± 1.9 a	92.8 ± 3.6 a
Defoliated post-veraison	2.94 ± 0.18 b	3.00 ± 0.11 b	1.47 ± 0.05 b	1.50 ± 0.04 ab	88.8 ± 4.1 a	90.6 ± 4.1 a

Data are means from 80 vines per treatment (±SD) in each season; values followed by different letters (within columns) are significantly different ($P < 0.05$).

each box per treatment were sampled at the sampling times of 10 and 20% ML. Thirty berries were squeezed separately and the juice used for TSS measurement with an Abbe RL-2 table refractometer, calibrated at 20°C (Officine Galileo, Florence, Italy). Part of the remaining berries was frozen in liquid N_2 after seed removal and stored at −75°C for enzymatic assays of LOX and ADH, and the rest were stored at −20°C until needed for GC analysis of GLVs.

The activity of ADH (EC 1.1.1.1) was measured by the method of OuYang et al. (2015), with some modifications. Powder (5 g) obtained from grinding frozen grape berries with an Ultraturrax (IKA-Werke, Staufen im Breisgau, Germany) kept in ice bath was suspended in 10 mL of 100 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) containing 5 mmol/L dithiothreitol (DTT), 1 mmol/L EDTA and 1% mass per volume (m/v) polyvinylpyrrolidone (PVP, Polyclar-AT). The homogenate was centrifuged at 31 000 *g* for 10 min at 4°C and the supernatant was used for the assay. The assay mixture consisted of 0.05 mL of 1.6 mmol/L NADH, 0.05 mL of 80 mmol/L acetaldehyde and 0.1 mL of crude extract in 0.8 mL of 100 mmol/L MES buffer (pH 6.5). Enzyme activity was measured at 20°C by the increase in absorbance at 340 nm for 15 s. Results were expressed as (μmol/NADH consumed per min)/g dry weight (DW).

The activity of LOX (EC 1.13.11.12) was measured according to a previously published method (Bellincontro et al. 2017). Powdered frozen berries (10 g) were suspended in an extraction buffer consisting of 10 mL (1:1) of 0.5 mol/L Tris–HCl buffer (pH 8.0) containing 1% m/v ascorbic acid, 1% m/v EDTA and 1% m/v polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 3500 *g* for 15 min at 4°C. Five mL of 1 mol/L aqueous $CaCl_2$ solution (2% v/v) was added to the supernatant and kept for 2 h to induce precipitation of pectic substances. The mixture was centrifuged at 3500 *g* for 20 min, and the supernatant desalted using a PD-10 Sephadex G-25 M column (Pharmacia Biotech, Uppsala, Sweden), previously

equilibrated with extraction buffer described previously. All operations were carried out at 4 ± 1°C. The assay mixture consisted of 100 μL of crude extract in 2.7 mL of 0.2 mol/L phosphate buffer (0.2 mol/L KH_2PO_4 + 0.2 mol/L Na_2HPO_4*12 H_2O), pH 6.5 and 0.3 mL of incubation substrate prepared with 1 mL of 0.1 N NaOH, 5 μL of Tween 20, and 10 μL of linoleic acid; the final volume of 25 mL was reached adding twice distilled water. The reaction mixture was incubated at 37°C in a water bath for 10 min. Enzyme activity was measured at 234 nm, using a Lambda 25 UV/Vis spectrophotometer (Perkin-Elmer Instruments, Seer Green, Beaconsfield, England), reading the change in absorbance after 3 min due to hydroperoxide formation. One unit of enzyme was defined as the change of 0.001 absorbance at 234 nm for 1 min at 20°C and expressed as μmol/g DW.

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich, Milan, Italy.

For analysis of GLVs, 400 g of berries of each treatment (ND, DFS and DPV) was processed and analysed in duplicate by a previously optimised method (Genovese et al. 2013a) applied to both grapes (Genovese et al. 2013b) and wine (Piombino et al. 2010). Peduncles were removed from the berries and the skins were carefully separated from the pulp using tweezers. To avoid oxidation, the skins were immediately immersed into bottles containing 250 mL of a must-like buffer solution for extraction. The buffer solution contained 5 g/L of tartaric acid, 10 g/L of PVPP and 2 g/L of sodium azide and was pH adjusted to 3.2 with 1 N NaOH. The skins were then stirred for 24 h at 20°C without light, after which they were centrifuged at 10 000 *g* for 20 min at 20°C. After separation of skins, deseeded pulp was homogenised with a CJ60 laboratory homogeniser (Black & Decker, Towson, MD, USA) for 2 min following the addition of 2 g/L of sodium azide and then centrifuged at 10 000 *g* for 10 min at 10°C in an ALC 4239R centrifuge (Daihan Scientific, Wonju, South Korea). Liquids were filtered with cellulose paper to obtain sample solutions that were stored at −20°C until analysis (two replicates). For each grape sample, 50 mL of the sample solution obtained was spiked with 250 μL of 2-octanol (200 mg/L in methanol) and passed through a C18 reversed-phase solid-phase extraction (SPE) (1-g C18 cartridge (Phenomenex, Torrance, CA, USA). The column was rinsed with Milli-Q water and the adsorbed volatiles were eluted with dichloromethane; bound volatiles were then eluted with methanol. The methanol was evaporated under reduced pressure at 37°C, and the residue dissolved in 5 mL of citrate–phosphate buffer (pH 5.0) containing 80 mg of Rapidase AR 2000 pectolytic enzyme with secondary glycosidase activities (DSM, Delft, Holland), before incubation for 16 h at 40°C. The volatiles released by enzymatic hydrolysis were eluted with dichloromethane on preconditioned C18 cartridges after addition of 250 μL of 2-octanol as an internal standard. The extracts were dried over Na_2SO_4 and finally concentrated to 50 μL under a N_2 stream. Free and bound GLVs were analysed by GC/MS and GC/flame ionisation detector (GC/FID), according to Genovese et al. (2005). For each sample, the SPE procedure was performed in duplicate. The GC/MS analysis was performed using a Shimadzu GC/MS-QP2010 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a split/splitless injector and a DB-WAX column (60 m × 0.250 i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The temperature program was 40°C for 5 min, increased to 220°C at 2°C/min, and then held at 220°C for 20 min. The carrier gas (He) flow was 1.02 mL/min. Samples of approximately 1.2 μL were injected in splitless mode, while the injector port and ion source were maintained at 250°C and 230°C, respectively. Positive electron impact spectra were recorded at 70 eV in the range m/z 33–350. The identification of compounds was confirmed by injection of pure standards and comparison of retention times and MS spectra with those reported in the NIST 2.0 library. *cis*-2-Hexen-1-ol was not available as a pure reference standard; therefore, it was tentatively identified based on retention time and MS spectrum and then semi-quantified (against 2-octanol). An Agilent 7890 A chromatograph equipped with a split/splitless injector (Agilent Technologies, Palo Alto, CA, USA) and a J&W DB Wax column (60 m length × 0.32 i.d. × 0.5 film thickness) (J&W Scientific) was used for GC/FID analysis. The same temperature program used for GC/MS analysis was employed. The carrier gas (He) flow was 2.20 mL/min. Samples (1.2 μL) were injected in splitless mode, and the temperature of both the detector and injector was 250°C. Volatile compounds were quantified with calibration curves. The peak area of each compound was normalised with respect to the area of the internal standard peak and interpolated using the calibration curve. Calibration graphs were built by analysing a blank solution as reported by Genovese et al. (2013a) and spiked with known amounts of each analyte and internal standard. The solution was then diluted to obtain five to seven calibration points for each analyte. The concentration range considered for the calibration curve of each molecule was within the values typically found in grapes of Italian grape cultivars (Genovese et al. 2013b). The linear regression coefficient (r^2) for each of the volatile compounds studied was ≥0.9918, in agreement with the previously cited literature.

Dry mass (DM) instead of fresh mass was used to avoid concentration effects during computation, that is, due to water loss from partial dehydration.

For both enzymatic assays and GC analysis of GLVs, the DM was calculated after measuring the moisture content loss using an oven-dry method (AOAC International 2005).

Statistical analysis

Statistical analysis was evaluated by ANOVA) using $P < 0.05$. Mean values were compared by using Tukey's test ($P < 0.05$) and significant differences were attributed by letters. Calculations were made with Minitab 15 (Minitab, College Station, PA, USA).

Results and discussion

Defoliation modified the grapevine canopy structure (Table 1), increasing the proportion of bunches exposed to direct solar radiation by 7.9, 90.7 and 88.8% in ND, DFS and DPV treatments, respectively. As a result, the latter two treatments significantly reduced the number of leaves/plant; however, at least 1 m^2 of leaf area per kg of grape production was retained in these treatments. Thus, the availability of photosynthate was not considered a limiting factor for berry growth (Howell 2001).

The effects of defoliation on berry and bunch mass are shown in Table 2. Compared with ND, only early defoliation (DFS) was significantly affected (in both harvests); crop yield/vine in the two seasons was 0.66–0.70 and 0.83–0.89 kg for ND and DFS, respectively, while average bunch mass was 104–110 g for ND and 128–136 g DFS. Late defoliation (DPV) did not yield any significant effects. These results confirmed that canopy management did not cause

Table 2. Effect of defoliation at fruitset, defoliation post-veraison and no defoliation of Nebbiolo grape vines on bunches per vine, bunch mass and berry mass in two seasons.

	Bunches/vine (kg)		Bunch mass (g)		Berry mass (g)	
Treatment	**First year**	**Second year**	**First year**	**Second year**	**First year**	**Second year**
Not defoliated	0.66 ± 0.04 b	0.70 ± 0.03 b	104 ± 8 b	110 ± 6 b	2.1 ± 0.1 b	2.1 ± 0.1 b
Defoliated at fruitset	0.83 ± 0.04 a	0.89 ± 0.04 a	128 ± 6 a	136 ± 7 a	2.5 ± 0.1 a	2.6 ± 0.1 a
Defoliated post-veraison	0.60 ± 0.05 b	0.69 ± 0.03 b	100 ± 8 b	110 ± 6 b	2.0 ± 0.1 b	2.1 ± 0.1 b

Data are means from 90 grape bunches chosen from 583, 583 and 579 vines from not defoliated, defoliated at fruitset and defoliated post-veraison treatments, respectively, in each season. Values followed by different letters (within columns) are significantly different ($P < 0.05$).

vegetative and productive imbalance, indicating sufficient photosynthate for berry development, whereas the literature reports that early leaf removal can reduce yield, modifying the source–sink balance (Intrieri et al. 2008, Sabbatini and Howell 2010), in the current study (which did not limit photosynthate availability for berries). DFS had a small stimulating effect on berry growth probably due to increased cell division during phase I of berry development (Dokoozlian and Kliewer 1996).

The carpological differences influenced the duration of dehydration; indeed in DFS samples, which had the highest bunch mass, a longer duration time was needed to achieve 10 and 20% ML (Table 3). A preliminary test carried out in the first season showed the different behaviour of samples in terms of dehydration time (Table S1), behaviour that was confirmed in the following year whose data are presented here. As expected, the higher the dehydration temperature, the faster the dehydration process; to achieve 20% ML, at 20°C, DPV and DFS samples required 18 and 25 days of dehydration, respectively. Less time was needed for ND samples; at 10°C, the dehydration time was between 27 (ND) and 32 (DFS) days, and samples lost mass similarly to those dehydrated at 20°C. After 10% ML, different trends were observed depending on the dehydration temperature: the ND, DFS and DPV samples dehydrated over different times at 10°C, but not at 20°C. To date, few published papers have reported the grape dehydration response following defoliation of grapevines. Constantinou et al. (2019) reported the effect of leaf removal at veraison on grape composition, but for sun-dried grapes, which is a different process, compared to dehydration of grapes under controlled ambient conditions. In the current study, the controlled conditions enabled accurate measurement of the time of dehydration. The DFS sample required a longer dehydration time, attributable to heavier bunches and berries (Table 2); this result was clear after 20% ML at 10 and 20°C. After 10% ML, a difference was notable at 10°C but not at 20°C, likely because, at the higher temperature, the dehydration process is fast and it is known that the rate of ML is higher earlier and then slows down over time (Mencarelli and Bellincontro 2013). Therefore, potential differences were not detectable.

Table 3. Duration of dehydration required to achieve 10 and 20% ML in bunches corresponding to Nebbiolo grapevines subjected to no defoliation, defoliation at fruitset and defoliation post-veraison at 10 and 20°C.

	Duration of dehydration (days ± 1)			
	10% ML		20% ML	
Treatment	**10°C**	**20°C**	**10°C**	**20°C**
Not defoliated	11 h	7 i	27 b	15 fg
Defoliated at fruit set	16 ef	8 i	32 a	25 c
Defoliated post-veraison	14 g	7 i	28 b	18 de

Data are means from ten grape bunches; SD was ±1 day for all measurements; values followed by different letters are significantly different ($P < 0.05$). ML, mass loss.

On completion of dehydration (i.e. at 20% ML), the sugar concentration of ND, DPV and DFS samples was 28, 27 and 31 (±1) °Brix, respectively, for dehydration at 20°C and 27, 28 and 27 (±1) °Brix for dehydration at 10°C.

The highest sugar concentration corresponded to DFS samples dehydrated at 20°C, which was unexpected. The proportion of ML was the same for the other samples, thus this could not be attributed to a concentration effect. The amount of water that evaporates in a given time is directly proportional to the evaporation coefficient, the air speed, the surface area of water (or bunch surface area in the current study) and the difference in absolute humidity at 10 versus 20°C. Given that the air speed and RH were the same at each dehydration temperature, variation in bunch surface area (due to differences in berry mass and number) might explain differences in evaporation rates. Indeed, ND and DPV bunches, comprising smaller bunches and berries (2.1, 2.5–2.6 and 2.0–2.1 g, for ND, DFS and DPV berries, respectively), had a higher surface–mass ratio; thus, a higher volume of water to evaporate with similar heat supply (10 or 20°C), losing water faster, whereas at 20°C DFS samples took longer to achieve 20% ML. Our supposition is that during prolonged dehydration at the higher temperature, cell wall degradation could accelerate, combining water stress and cell senescence effects, permitting an increase in refractometric materials, sugars and mineral ions and, thus, the increased TSS.

The enzymatic activity was also affected by the timing of defoliation and the dehydration temperature (Table 4). At harvest, ND and DPV samples show similar ADH activity, while the DFS sample shows higher activity.

Despite the defoliation treatments employed in this experiment being managed to avoid symptoms of excessive sunburn, we can suppose that the mechanisms involved in tissue adaptation to light are also involved, at least partially. It has been demonstrated that susceptibility to photo-oxidative sunburn decreases during berry growth due to low chlorophyll concentration (Rustioni et al. 2015) and, thus, a higher effect of radiation on early stages of berry growth is consistent with the literature. It has recently been shown that radiation of grape berries can significantly affect ascorbate peroxidase and catalase activity (Rustioni et al. 2020).

The variable ADH activity observed during dehydration at 10 and 20°C (for all samples) contradicted the van't Hoff law, because activity decreased at 20°C. Although ADH

Table 4. Alcohol dehydrogenase and lipoxygenase activity in berries from bunches corresponding to Nebbiolo grapevines subjected to no defoliation, defoliation at fruitset and defoliation post-veraison, at harvest and after 10 and 20% ML due to dehydration at 10 and 20°C.

	Alcohol dehydrogenase activity (μmol/g DW)						Lipoxygenase activity (μmol/g DW)					
		10% ML		20% ML				10% ML		20% ML		
Treatment	0% ML	10°C	20°C	10°C	20°C		0% ML	10°C	20°C	10°C	20°C	
No defoliation	85 ± 3 gh	112 ± 4 d	84 ± 4 fgh	159 ± 11 c	76 ± 7 h		36 ± 4 i	67 ± 6 e	37 ± 2 i	73 ± 5 def	63 ± 3 fg	
Defoliated at fruitset	100 ± 6 de	190 ± 11 b	75 ± 4 h	322 ± 17 a	79 ± 7 h		38 ± 4 i	96 ± 8 ab	55 ± 4 gh	76 ± 5 cde	60 ± 6 gh	
Defoliated post-veraison	89 ± 4 fg	110 ± 9 d	95 ± 5 ef	206 ± 10 b	79 ± 6 gh		30 ± 5 i	86 ± 6 bc	53 ± 3 h	63 ± 4 fg	59 ± 3 gh	

Data are the mean (±SD) of three enzymatic analyses per treatment, of berry lots taken from different grape bunches, each treatment; values followed by different letters are significantly different ($P < 0.05$); values were measured on dry weight (DW) basis. ML, mass loss.

activity during dehydration at 20°C approximated ADH activity at harvest, possibly due to temperature similarities (being 23°C for harvested grapes at receival and 20°C for dehydration), at 10°C the ADH activity of the ND samples was almost double that observed after 20% ML at 20°C. In the DFS sample, ADH activity increased by two- and three-fold after 10 and 20% ML at 10°C, but decreased significantly at 20°C. The DPV and DFS samples showed similar trends, but lower activity level. It is known that during grape ripening on the vine, ethanol can form due to over-expression of ADH in berries (Tesniere et al. 2004). Moreover, it has recently been discovered that during ripening, there is almost no oxygen around berry seeds (Xiao et al. 2018b), even under water stress (Xiao et al. 2018a). Alcohol dehydrogenase is a diffuse enzyme in plant cells, active in the formation of alcohol during the lipid oxidation process, but also in the formation of higher alcohols. The increased ADH activity at harvest in the samples of the larger DFS berries could be due to the diminished availability of oxygen inside the berry during ripening. As the surface area : mass ratio for larger spheres (e.g. berries) is lower than that for smaller spheres, the oxygen diffusion inside the berry core, from the outside, is lower; moreover, the oxygen permeability of the air coming from the pedicel and permeating through mesocarp cells is lower, taking into account that flow through the pedicel is similar. It is well known that the concentration of oxygen in fruit pulp decreases from the outside to the inside, and that the larger the fruit is, the lower the oxygen concentration in the seed area (i.e. the core). This is known as a gas gradient and may cause anoxic or hypoxic conditions inside the fruit, eventually triggering internal breakdown, leading to disorders (Nugraha et al. 2020). Indeed, a low effective O_2 diffusivity affects the internal O_2 concentration, and tissue samples with different microstructures have different O_2 diffusivity values. Along the radial direction of pear tissue, the effective O_2 diffusivity was low under the skin, larger in the cortex region, and lower again in the core region (Schotsmans et al. 2003). This diffusivity gradient has never been measured in grape berries, but it is expected to occur as in pear and even worse in grape berry, taking into account the barrier effect of berry pruine (bloom). Xiao et al. (2018b) showed that the oxygen content of the berry peaked at the central vascular bundle region and that the presence of air spaces in the pedicel affected the oxygen concentration, respiration and ethanol formation in the berry. The oxygen gradient was also observed to decrease sharply across the skins, as ripening progressed in all of the cultivars studied, resulting in a lower overall oxygen concentration across the berry. Thus, it can be assumed that in larger berries, relative to smaller ones with similar pedicel diameters, oxygen diffusion is hindered according to the distance between the skin and bundle region. At 10°C, the significant increase in ADH activity detected in all samples could be due to a combination of temperature and water stress, as reported by Cirilli et al. (2012), who showed the relative expression of the *VvAdh2* gene was highest at 10°C and after 10% ML. In the DFS sample, the significant increase compared to other samples is likely attributable to the synergistic effect of ethanol formed during ripening and that produced during postharvest dehydration. The different samples achieved 20% ML after different durations of dehydration: DFS samples required 32 and 25 days, respectively, at 10 and 20°C, while ND and DPV required a similar duration (27–28 and 15–18 days, at the same dehydration temperature) (Table 3).

Thus, in DFS samples, the potential for a senescing process, mimicking that which occurs on the vine (Tesnière and Verriès 2000), should be taken into account. As such, our hypothesis is that the significant increase in ADH activity in DFS samples is due to the synergistic effects of the following: (i) increased ethanol formation prior to harvest due to larger berry size; (ii) ethanol formation due to postharvest water stress; and (iii) berry senescence due to the extended duration of dehydration. Moreover, the temperature shock might activate ADH as previously mentioned.

At 20°C, ADH activity decreased with the increase of ML, irrespective of sample, in agreement with previous research (Cirilli et al. 2012). This emphasises that, at the optimal metabolic temperature, there is no temperature stress and, likely, the ability of berry cells to afford the water stress is higher. The process of postharvest water loss in grape berries involves several steps, including oxidative and osmotic stress response, anaerobic respiration, defence response, cell wall metabolism, and carbohydrate metabolism. Over-expression of lactate dehydrogenase and the down-regulation of ADH have been observed during postharvest dehydration of winegrapes (Zamboni et al. 2008, Rizzini et al. 2009). These results conflict with the increase in ADH activity observed during controlled dehydration of Malvasia and Pecorino grapes (Costantini et al. 2006, Bellincontro et al. 2017), but Cirilli et al. (2012) showed asynchrony among gene expression, enzyme activity and metabolite concentration. Zenoni et al. (2016) stated, however, 'our data were based on berries subjected to slower and more prolonged dehydration compared to these other studies, and inconsistencies in the expression of genes related to anaerobic respiration may therefore reflect such differences'. Chkaiban et al. (2007) and Bellincontro et al. (2017) showed that it is important to define and accurately control the ambient conditions during dehydration in order to study berry metabolism.

No difference in LOX activity was observed among samples at harvest (0% ML) or in ND samples dehydrated to 10% ML at 20°C. In all other samples, the LOX level increased relative to 0% ML samples. At 10°C, enzyme activity increased significantly, by double or more than double after 10% ML, while at 20% ML, only the activity of ND samples continued to increase; in defoliated samples, activity diminished. At 20°C, the LOX activity increased after 20% ML, reaching similar values in all samples (Table 4). The rapid LOX response confirmed observations made by Costantini et al. (2006), and also findings from Chkaiban et al. (2007) and Bellincontro et al. (2017), who showed that LOX increase also depends on the conditions of grape dehydration. It appears that in the defoliated samples, LOX activity responds more rapidly during water stress, regardless of temperature, but mainly at 10°C. Our supposition is that a synergistic stress effect occurs, with defoliation, low temperature and water stress all affecting membrane oxidation. Grapes are not known to be sensitive to chilling, and 10°C is not considered to be a postharvest chilling temperature. Our hypothesis is that the observed effect on LOX activity could be due to the temperature change, that is, from the higher harvest/shipping temperature of 21–23°C down to the dehydration temperature of 10°C. The influence of temperature in postharvest partial dehydration of winegrapes has been studied by Mencarelli and Bellincontro (2013), who showed that carbon dioxide production from berries that were partially dehydrated at 10°C under a higher air flow rate was greater (by approximately 30%) than under lower ventilation, and similar to that of grapes partially dehydrated at 20°C, leading to the hypothesis of increased oxidative stress. The gene expression of phenylalanine ammonia lyase (PAL), stilbene synthase (STS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) was up-regulated in grapes that were partially dehydrated at 10°C, relative to dehydration at 20°C (Mencarelli et al. 2010). It is known that water stress and, to a lesser extent, low-temperature stress can induce the expression of aerobic fermentation genes, mediated by abscisic acid (ABA) under normoxic conditions (De Bruxelles et al. 1996). Thus, its implication in inducing aerobic fermentation when grapes undergo a significant temperature change from harvest to dehydration, where water stress occurs, is plausible. In summary, for ND samples, an increase in LOX and ADH activity was observed at 10°C while, at 20°C, only LOX activity increased significantly after 20% ML. In DFS, both enzymes increased at 10°C, with the increase for ADH more significant than LOX, especially at 10% ML; at 20°C, ADH activity decreased while LOX activity increased. Similar behaviour was exhibited by both enzymes following DPV.

At day 0 (0% ML), the total free GLVs resulting from metabolism of lipids (hexanal, *trans*-2-hexenal, 1-pentanol, 1-hexanol, *cis*-3-hexen-1-ol, *trans*-3-hexen-1-ol, *cis*-2-hexen-1-ol, 1-heptanol and hexanoic acid) were 9434, 7212 (66, 23 and 11%) and 1656 (75, 18 and 7%) μg/kg in ND, DFS and DPV samples, respectively (Figure 2); the total bound GLVs were 7599 (11% aldehydes, 89% alcohols), 18 486 (12 and 88%) and 15 409 (4 and 96%) μg/kg in ND, DFS and DPV samples, respectively (Figure 2). Thus, the concentration of bound compounds was significantly higher than for free forms in the defoliated samples, while in ND samples, bound compounds had slightly lower concentration than free GLVs. The difference was greater when defoliation was applied earlier (i.e. in DFS samples). Considering the effect of temperature during postharvest dehydration, at 10°C and at 10% ML of ND samples, there was a decrease in free compounds and a slight increase at 20% ML. Conversely, the concentration of bound compounds increased three- to fourfold for both 10 and 20% ML. For DFS, free GLVs increased significantly only after 20% ML, while bound GLVs decreased significantly. For DPV, a decrease in free and bound GLVs was observed after both 10 and 20% ML. At 20°C, the ND sample comprised free GLVs at a concentration higher than those observed at 10°C after 10% ML, while values were similar after 20% ML. In contrast, the increase in bound compounds was greater than at 10°C after 10% ML, while bound compounds decreased after 20% ML, achieving a concentration similar to that observed for the samples dehydrated at 10°C. Free GLVs in DFS samples increased after 10% ML, but decreased significantly after 20%, while bound compounds decreased, mainly after 10% ML. In the DPV sample, free GLVs decreased slightly, while bound GLVs declined considerably (Figure 2). It is clear that defoliation enhanced free GLVs in the DPV sample and bound GLVs in both DFS and DPV samples. By summing free and bound compounds in the DFS and DPV samples at day 0 (0% ML), similar values were obtained (about 27 000 and 26 000 μg/kg, for DPV and DFS, respectively), but the ratio of bound: free GLVs was about 2.6 and 1.3 for DFS and DPV, respectively. Therefore, early defoliation significantly increased the bound lipid derived GLVs. To the best of our knowledge, previous studies have not investigated the effect of grapevine defoliation

on glycosylation of volatile organic compounds (VOCs), but in Sauvignon and also in Muscat Blanc à Petits Grains, it has been reported that shading decreased glycosylated monoterpenes and C13 norisoprenoids (Marais et al. 1999, Bureau et al. 2000); similar results have been reported in Pinot Noir (Feng et al. 2015). It is known that plants may glycosylate volatile flavour compounds as a detoxification strategy (Sarry and Günata 2004). It has been proposed that the improved aqueous solubility of small lipophilic compounds arising from glycosylation might prevent their diffusion into the tonoplast, inhibiting their ability to move outside of the vacuole (Wink 2010). Zenoni et al. (2017) reported overexpression of the putative genes involved in flavonoid glycosylation of berries when Sangiovese vines were defoliated at the pre-flowering stage. Thus, our supposition is that the longer the time of berry sun exposure (due to early defoliation, not natural conditions) during berry growth, the higher the stress condition [greater formation of reactive oxygen species (ROS)], especially in membrane lipids, triggering the glycosylation process to protect cells from toxic volatile compounds formed due to stress. Moreover, water stress is effective at activating glycosylation; indeed, during postharvest water loss of ND samples (i.e. without any effect of defoliation), the increase in bound GLV concentration (three- and 1.5-fold increased at 10 and 20% ML, respectively) was noted, while free GLVs decreased (10°C) or were similar (20°C) after 10% ML, or increased slightly after 20% ML. Water stress impacts the biosynthesis of aroma compounds in different ways depending on the molecular family concerned. Previous studies showed that the concentration of bound 3-hydroxy-β-damascenone, a precursor to β-damascenone, increased under water deficit treatments (Peyrot des Gachons et al. 2005, Koundouras et al. 2009). A significant increase in the concentration of hexanal, hexenol and hexenal in grapes subjected to postharvest dehydration has been reported (Costantini et al. 2006), but no discrimination between free and bound volatiles was recorded. A low dehydration temperature (i.e. 10°C) appears to be synergistic to water stress in defoliated samples: after 10% ML, bound volatiles of defoliated samples were two- to threefold higher at 10°C, rather than at 20°C, while at 20% ML this increase was lower and sometimes not significant. Tea plants under cold stress conditions yielded significantly higher sesquiterpene UDP-glucosyltransferases (UGT) in order to protect against ROS forming nerolidol glucoside, with the capacity to scavenge (Zhao et al. 2019). Maintaining tablegrapes at 15°C (compared to 25 or 35°C) stimulated anthocyanin synthesis and the over-expression of related genes, including the flavonoid 3-*O*-glucosyltransferase (Gao-Takai et al. 2019). In this experiment, we argue that the activity of glucosyltransferase is triggered by a temperature shift from 21–23 to 10°C, as mentioned earlier, for LOX activity, and previously reported for phenylpropanoid pathway genes (Mencarelli et al. 2010), thereby forming glycosylated compounds.

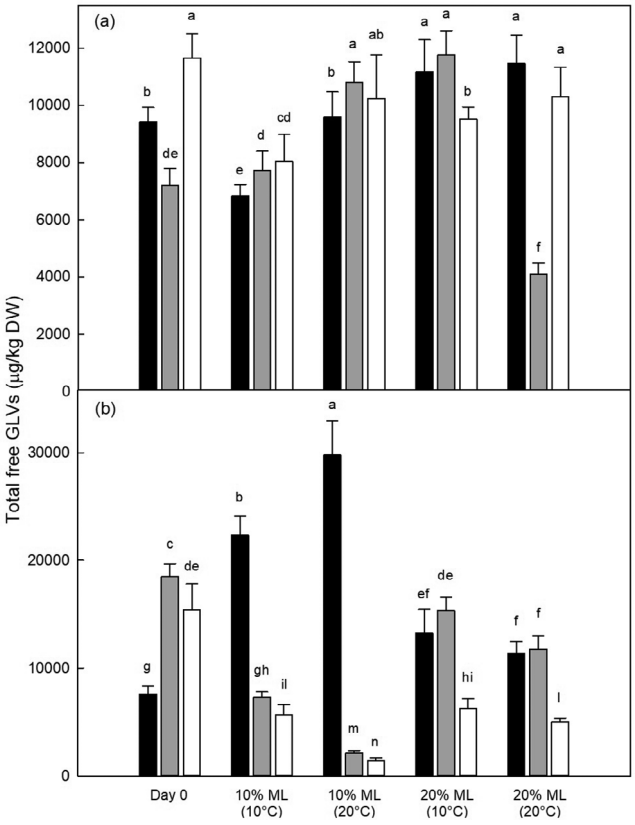

Figure 2. (a) Total free and (b) bound glycosylated green leaf volatiles (GLVs) related to lipid catabolism in berries corresponding to grapevines subjected to no defoliation (■), defoliation at fruitset (▩) and defoliation post-veraison (□), at harvest (day 0) and then after 10 and 20% mass loss (ML) due to dehydration at 10 and 20°C. Data are means ± SD of three GC/MS analyses of berry lots taken from different grape bunches for each treatment. Values with different letters are significantly different ($P < 0.05$). Values were measured on dry weight (DW) basis.

As LOX and ADH are, respectively, the first and last enzymes involved in the oxidation of membrane lipids, we summed their activity. The results are quite interesting and, overall, the behaviour at 10 and 20°C is completely different. In Figure 3 the patterns of LOX + ADH, and free and bound GLVs in ND samples at 10 and 20°C are reported. At 10°C, a direct relationship was observed between enzyme activity and bound GLVs, with increasing ML; whereas at 20°C, no relationship was observed, and no factors increased with ML. In DFS samples (Figure 4), at 10°C, the enzyme activity increased at a higher rate than for ND samples, while free GLVs increased slightly, giving the same concentration as in ND; bound GLVs decreased significantly. At 20°C, enzyme patterns were similar to the ND sample; while bound GLVs decreased slightly with ML and, free GLVs, initially increased and then decreased, giving values similar to the ND samples. In DPV (Figure 5), at 10°C, enzymes behaved in a manner similar to the other samples, that is, giving a significant increase with ML, but less than for DFS; free and (mostly) bound GLVs, decreased substantially, as for DFS. At 20°C, enzyme activity increased slightly, and free GLVs increased with ML, while bound GLVs decreased significantly.

Thus, it appears that at 10°C, ML induces enzyme activity (mainly ADH) especially in defoliated samples but bound GLVs produced by defoliation greatly diminished during postharvest dehydration, notwithstanding the increase in enzyme activity. No significant change occurred in free GLVs. At 20°C, the enzyme activity was similar among the samples, increasing a little only in dehydrated DPV samples. Free GLVs did not change during ML, except for a slight impact in DFS samples, while bound GLVs were higher in defoliated samples, but declined with ML.

This decoupling of the concentration of GLVs and LOX + ADH activity in the defoliated samples dehydrated at 10°C was unexpected. What is the fate of the GLVs? Cirilli et al. (2012) found asynchrony between *VvAdh2* gene

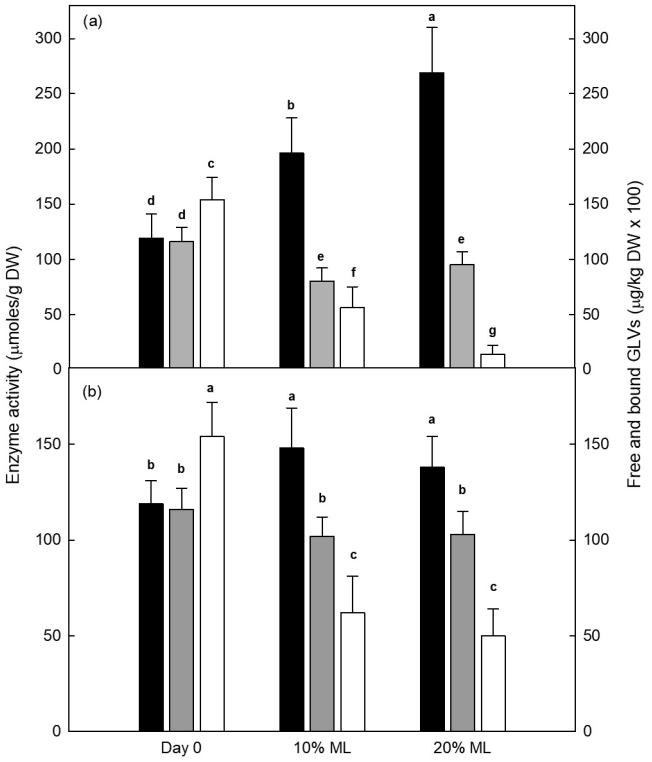

Figure 3. Effect of (a) 10°C and (b) 20°C dehydration temperature and mass loss (ML) on the total activity of alcohol dehydrogenase (ADH) and lipoxygenase (LOX) (■), and on the concentration of free (▨) and bound (□) glycosylated green leaf volatiles (GLVs) in grape berries from bunches corresponding to grapevines subjected to no defoliation, at harvest (i.e. 0% ML), and after 10 and 20% ML. Data are means ± SD of three enzymatic and three GC/MS analyses of berry lots taken from different grape bunches for each treatment. Values with different letters are significantly different ($P < 0.05$). Values were measured on dry weight (DW) basis.

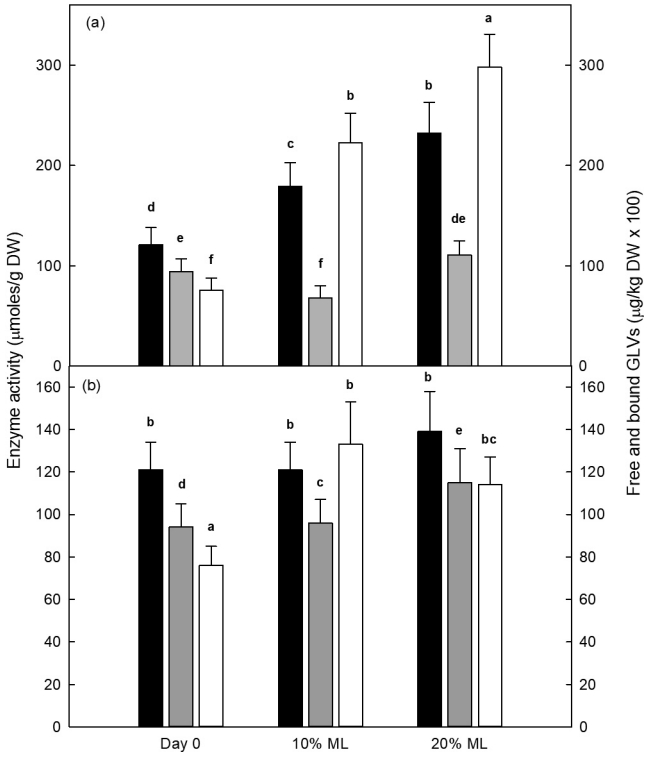

Figure 4. Effect of (a) 10°C and (b) 20°C dehydration temperature and mass loss (ML) on the total activity of alcohol dehydrogenase (ADH) + lipoxygenase (LOX) (■), and on the concentration of free (▨) and bound (□) glycosylated green leaf volatiles (GLVs) in grape berries from bunches corresponding to vines defoliated at fruitset and after 10 and 20% ML. Data are the mean (±SD) of three enzymatic measurements, or three GC/MS analyses from berries of different grape bunches, for each treatment. Values with different letters are significantly different ($P < 0.05$). Values were measured on dry weight (DW) basis.

expression and ADH activity. Gene expression decreased while enzyme activity increased for dehydration at 10°C. Enzymatic activity is a biological event occurring after transcription, so there might be a feedback regulatory mechanism of gene expression. Gao-Takai et al. (2019) found over-expression of the genes involved in anthocyanin biosynthesis, but not of the transcription factors MybAs, when grape berries were kept for a few hours at low temperature (15°C). Zhang et al. (2015) showed that low-temperature storage after harvest greatly enhanced the expression of the five anthocyanin biosynthesis genes, but the concentration of anthocyanins did not increase. In our case, the asynchrony was between enzyme activity and secondary metabolites. The significantly higher gene expression observed at 10°C and 10% ML (than at 20 or 30°C) could reflect tissue reaction to low temperature (Cirilli et al. 2012). Glycosylation appears to be stimulated by defoliation, and leaf removal could be perceived by grapevines as a stress. Plant cells are challenged by a large range of adverse conditions perturbing their redox homeostasis, such as heavy metals, pathogens, drought, salinity, high light intensity and extreme temperature (Schützendübel and Polle 2002, Farooq et al. 2019). Oxidative stress is a cellular consequence of these conditions. The expression of several UGTs is induced during particular abiotic conditions (Rehman et al. 2018) and the down-regulation of two UGTs from tea, UGT78A14 and UGT91Q2, resulted in a lower tolerance to cold stress (Zhao et al. 2019), which also triggers oxidative stress (Behr et al. 2020). During ML, activation of cell wall enzymes, including some glycosidases, has been observed (Botondi et al. 2011) but, in our case, ML in the ND sample increased glycosylation, while in defoliated samples, ML decreased it. As postharvest ML of berries must be considered as a water stress, and thus an oxidative stress, the activation of UGTs is expected, and it has been reported that glycosylation of anthocyanins results in their storage in the vacuole, de-repressing the product feedback inhibition on PAL (Zhang et al. 2015). But PAL is strongly activated by ethylene, which as a stress hormone also triggers the enzymes involved in membrane and cell wall degradation. A core set of genes was consistently modulated in different winegrape cultivars during postharvest dehydration, representing the common features of berries undergoing dehydration and/or commencing senescence, genes controlling ethylene and auxin metabolism, as well as genes involved in oxidative and osmotic stress, defence responses, anaerobic respiration, and cell wall and carbohydrate metabolism (Zenoni et al. 2016). Thus, one hypothesis is that the coupling effect of defoliation and postharvest water stress has induced greater production of ethylene, resulting in higher cell membrane and wall degradation, promoting glycoside hydrolysis (deglycosylation). This was not the case of the ND sample, where only postharvest water stress occurred; therefore, glycosylation acts to confine GLVs in the vacuole to avoid toxicity. In addition, the glycosylation or the deglycosylation came about mainly at 10°C in the ND or defoliated samples, respectively, indicating that cold stress affected all samples, regardless of leaf removal. Our final

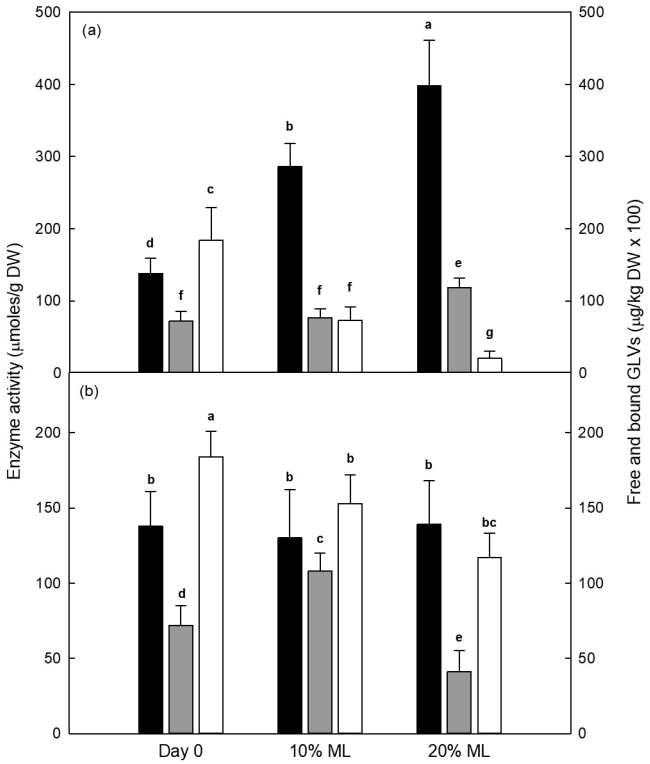

Figure 5. Effect of (a) 10°C and (b) 20°C dehydration temperature and mass loss (ML) on the total activity of alcohol dehydrogenase (ADH) + lipoxygenase (LOX) (■), and on the concentration of free (▨) and bound (□) glycosylated green leaf volatiles (GLVs) in grape berries coming from bunches corresponding to vines defoliated post-veraison and after 10 and 20% ML. Data are the mean (±SD: bars) of three enzymatic measurements or three GC/MS analyses of berry lots taken from different grape bunches for each treatment. Values with different letters are significantly different ($P < 0.05$). Values were measured on dry weight (DW) basis.

hypothesis is that postharvest cold stress (10°C) and water loss induced glycosylation, alcohol formation through ADH and membrane oxidation through LOX, but when grapevines were subjected to leaf removal, irrespective of the timing of defoliation, the postharvest cold and water stresses acted synergistically with field stress (e.g. high temperature, drought, light exposure or simply leaf removal) activating membrane degradation (LOX and ADH) and also overripening (ADH), probably mediated by ethylene, thus deglycosylation prevails. The significantly lower concentration of bound GLVs in DPV samples (compared with DFS samples), at harvest and during dehydration, regardless of temperature, could be due to the highest rate of water loss being expressed by the days of dehydration : 28 versus 32 and 18 versus 25, for DPV and DFS, respectively. These data confirm observations from the preceding year (Table S1), in which DPV samples completed dehydration 3 days before DFS samples. It has been reported that cell death during controlled postharvest water loss begins around 30% ML (Costantini et al. 2006), and Zenoni et al. (2016) emphasised 'Although we did not set up a specific analysis to assess the vitality of berry cells, it could be hypothesized that postharvest stress conditions may have induced this phenomenon to a different extent in the six grape genotypes', and in their study, 30% water loss was targeted. Thus, the acceleration of water loss could favour deglycosylation, in addition to other stress factors mentioned earlier. The accelerated water loss was surely due to berry size being similar to the ND sample, for which the DPV sample had similar dehydration times, but further effects of leaf removal on berry physiology compared to the ND sample affect volatile compound biosynthesis and release.

Conclusions

Defoliation and postharvest dehydration impact not only bunch size but also berry physiology during postharvest water loss. Synergistic effects of stress from the vineyard to postharvest treatment appeared to significantly affect the GLV metabolism, decoupling enzyme activity and volatile compound formation, as well as stimulate glycosylation. In particular, vine defoliation favoured glycosylation of GLVs regardless of the timing of leaf removal, likely affecting not only the physical features (bunch and berry mass) but also bunch and berry physiology, which is well known. Less is known about the effects of leaf removal on postharvest dehydration of grapes, which has been shown here to significantly decrease the overall bound GLVs in DPV samples after 20% ML due to the higher rate of water loss relative to DFS samples.

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Supporting information

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Table S1. Water loss due to dehydration of Nebbiolo grapes that were subjected to no defoliation (ND), defoliation at fruitset (DFS) and defoliation post-veraison (DPV) during two seasons.