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Grape berry position affects the diurnal dynamics of its metabolic profile

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Abstract

Solar irradiance and air temperature are characterized by dramatic circadian fluctuations and are known to significantly modulate fruit composition. To date, it remains unclear whether the abrupt, yet predictive, diurnal changes in radiation and temperature prompt direct metabolic turn-over in the fruit. We assessed the role of fruit insolation, air temperature, and source-tissue CO_2 assimilation in the diurnal compositional changes in ripening grape berries. This was performed by comparing the diurnal changes in metabolite profiles of berries positioned such that they experienced (a) contrasting diurnal solar irradiance patterns, and (b) similar irradiance but contrasting diurnal CO_2 assimilation patterns of adjacent leaves. Grape carbon levels increased during the morning and decreased thereafter. Sucrose levels decreased throughout the day and were correlated with air temperature, but not with the diurnal pattern of leaf CO_2 assimilation. Tight correlation between sucrose and glucose-6-phosphate indicated the involvement of photorespiration/glycolysis in sucrose depletion. Amino acids, polyamines, and phenylpropanoids fluctuated diurnally, and were highly responsive to the diurnal insolation pattern of the fruit. Our results fill the knowledge gap regarding the circadian pattern of source-sink assimilate-translocation in grapevine. In addition, they suggest that short-term direct solar exposure of the fruit impacts both its diurnal and nocturnal metabolism.

KEYWORDS

circadian cycle, diurnal metabolism, fruit metabolic profiling, fruit microclimate, fruit sunlight exposure, source/sink translocation

Abbreviations: Cyan-3-acet, Cyanidin-3-O-(6″-acetyl-glucoside); Cyan-3-coum, Cyanidin-3-O-(6″-p-coumaroyl-glucoside); Cyan-3-glu, Cyanidin-3-O-glucoside; Delph-3-acet, Delphinidin-3-O-(6″-acetyl-glucoside); Delph-3-coum, Delphinidin-3-O-(6″-p-coumaroyl-glucoside); Delph-3-glu, Delphindin-3-O-glucoside; Hex., Hexoside; Kaemp-3-glr, Kaempferol-3-O-glucuronide; Kaemp-3-glu, Kaempferol-3-O-glucoside; Mal-3-acet, Malvindin-3-O-(6″-acetyl-glucoside); Mal-3-caffe, Malvidin-3-O-(6″-caffeoyl-glucoside); Mal-3-coum, Malvidin-3-O-(6″-p-coumaroyl-glucoside); Mal-3-glu, Malvidin-3-O-glucoside; Myr-3-glr, Myricetin-3-O-glucuronide; Myr-3-glu, Myricetin-3-O-glucoside; Narin-chalc-glu, Naringenin-chalcone-4-O-glucoside; Peo-3-acet, Peonidin-3-O-(6″-acetyl-glucoside); Peo-3-coum, Peonidin-3-O-(6″-p-coumaroyl-glucoside); Peo-3-glu, Peonidin-3-O- glucoside; Pet-3-acet, Petundin-3-O-(6″-acetyl- glucoside); Pet-3-coum, Petunidin-3-O-(6″-p-coumaroyl-glucoside); Pet-3-glu, Petunidin-3-O-glucoside; Quer-3-glr, Quercetin-3-O-glucuronide; Quer-3-glu, Quercetin-3-O-glucoside; Rutin, Quercetin-3-O-rutinoside

1 | INTRODUCTION

Fruits exposed to excessive solar radiation and elevated air temperatures face the risk of oxidative damage, sunburn, and pericarp dehydration (Bondada & Keller, 2012; Greer & Weedon, 2013; Racsko & Schrader, 2012; Torres, Andrews, & Davies, 2006) with potential negative implications on seed dispersal (Allen & Lee, 1992; Garcia, Zamora, Gomez, & Hodar, 1999; Martin, 1985). To counter these degrading processes, fruits have complex protection mechanisms, which include the accumulation of compounds conferring potent reactive oxygen species (ROS) scavenging activity, osmotic adjustment, and ultraviolet B (UV-B) radiation screening. These protective functions are largely attributed to ascorbate and glutathione, sugars, amino acids, ions, polyols and polyamines, and carotenoids and phenylpropanoids (Agati et al., 2013; Conde et al., 2015; Foyer & Noctor, 2011; Handa, Bressan, Handa, Carpita, & Hasegawa, 1983; Lev-Yadun & Gould, 2009; Liu, Wang, Wu, Gong, & Moriguchi, 2015; Loreto & Schnitzler, 2010; Winkel-Shirley, 2002). In wine grapes, several protective-metabolites have a well-established role in the flavor and color of the grape and wine, as in the case of the phenylpropanoids (Peters & Constabel, 2002; Steyn, 2009; Winkel-Shirley, 2001), thus affecting the fruit commercial value. This fact, along with the increasingly unpredictable climate, and the expansion of viticulture to warmer regions, prompted studies of the effect of micrometeorological conditions on the metabolism and chemical composition of the grape.

The effect of solar irradiance and air temperature on the accumulated levels and composition of numerous primary and secondary metabolites were extensively described (Cohen, Tarara, Gambetta, Matthews, & Kennedy, 2012; Haselgrove et al., 2000; Pereira et al., 2006; Reshef, Agam, & Fait, 2018; Reshef, Walbaum, Agam, & Fait, 2017; Spayd, Tarara, Mee, & Ferguson, 2002; Young et al., 2016). Nevertheless, daily changes in the levels of fruit primary and secondary metabolites and their underlying causes received little attention. Can the abrupt, yet oscillatory, changes in solar irradiance and air temperature lead to instantaneous metabolic responses in the fruit, and eventually modulate the overall metabolic development of the berry? Is this a direct response to changes in fruit conditions or one that is mediated by changes in whole-plant physiology, for example, photosynthetic activity?

Specific metabolite groups are known to exhibit diurnal changes in grapes. These include 3-meracptohexan-1-ol precursors and amino acids (Kobayashi et al., 2012; Krueger & Kliewer, 1995; L. Wang et al., 2014). The latter exhibited temporal trends that were less pronounced during cloudy days and, in the absence of relevant data, were attributed to differences in leaf photosynthesis. In addition, grape transcriptome was recently found to differ between day and night (Rienth et al., 2014), and exhibit circadian patterns (Carbonell-Bejerano et al., 2014). That said, although season-long trials show a strong association between grape gene expression patterns and metabolite accumulation (Degu et al., 2014; Deluc et al., 2007; Matus et al., 2009), in a short-term diurnal trial in potato, transcripts and the metabolite profile of leaves were poorly correlated (Urbanczyk-Wochniak et al., 2005). This stresses the importance of metabolomic evidence to verify the phenotypic relevance of such findings.

Here, we used a mass spectrometry (MS)-based approach to unravel diurnal dynamics in grape metabolism under field conditions. Berry-level micrometeorology and leaf CO_2 assimilation were measured to assess their role as mediating factors. To separate these factors, we created two systems in which we compared the diurnal metabolism between (a) berries experiencing contrasting diurnal solar irradiance patterns, and (b) berries experiencing contrasting diurnal CO_2 assimilation patterns of their adjacent leaves. We discuss the daily changes in grape metabolic profile and relate them to quantitative micrometeorological and physiological measurements.

2 | MATERIALS AND METHODS

2.1 | Experimental layout

The experiment was conducted in a vineyard located in the Negev desert highlands, Israel (30°36′55.22″N, 34°45′12.00″E, 800 m altitude). The vineyard was planted in 2007 with *Vitis vinifera L*. Cabernet Sauvignon grafted on Ruggeri 140 rootstock. Rows were orientated north–south with a 30° angle to the north–east/south–west and were trained in a vertical shoot positioning. Three vine rows, separated by border rows, were used for the experiment. In each experimental row, two groups of three adjacent vines were marked as field repetitions and the basal leaves in the vicinity of the clusters (up to 30 cm above the cordon) were completely removed at the onset of veraison, creating full exposure of the clusters to sunlight (Figure 1a). More details on the experimental design are provided in Reshef et al. (2017).

A high-resolution diurnal trial was performed on 19 August 2016, 26 days after veraison, to represent the mid-stage of the ripening process, spanning approximately over 52 days from veraison to technical maturity (i.e., 24°Brix) (Reshef et al., 2018). The experiment was undertaken from dawn to dusk (6:00 a.m. to 8:00 p.m.) and involved the sampling of berries for metabolite profiling and the acquisition of micrometeorological and physiological data.

2.2 | Berry sampling

On each vine, four exterior-located clusters (furthest from the trunk towards the inter-row) were marked on both sides of the vine canopy, representing different cluster positions. From each of the six field repetitions, positionally-labeled berries were sampled: three pooled berry samples (each containing 12 berries) were obtained, two from clusters positioned on the east side of the vine row (a and b), and one from clusters positioned on the west side of the vine row (c). From the clusters positioned on the east side of the vine row we sampled: (a) berries orientated towards the inter-row (i.e., facing east) labelled as "E," and (b) berries orientated towards the vine row (i.e., facing west) labelled as "EW." From the clusters positioned on the west side of the vine row we sampled (c) berries orientated towards the inter-row (i.e., facing west) labelled as "W" (Figure 1c). Berries were sampled every 2 hr,

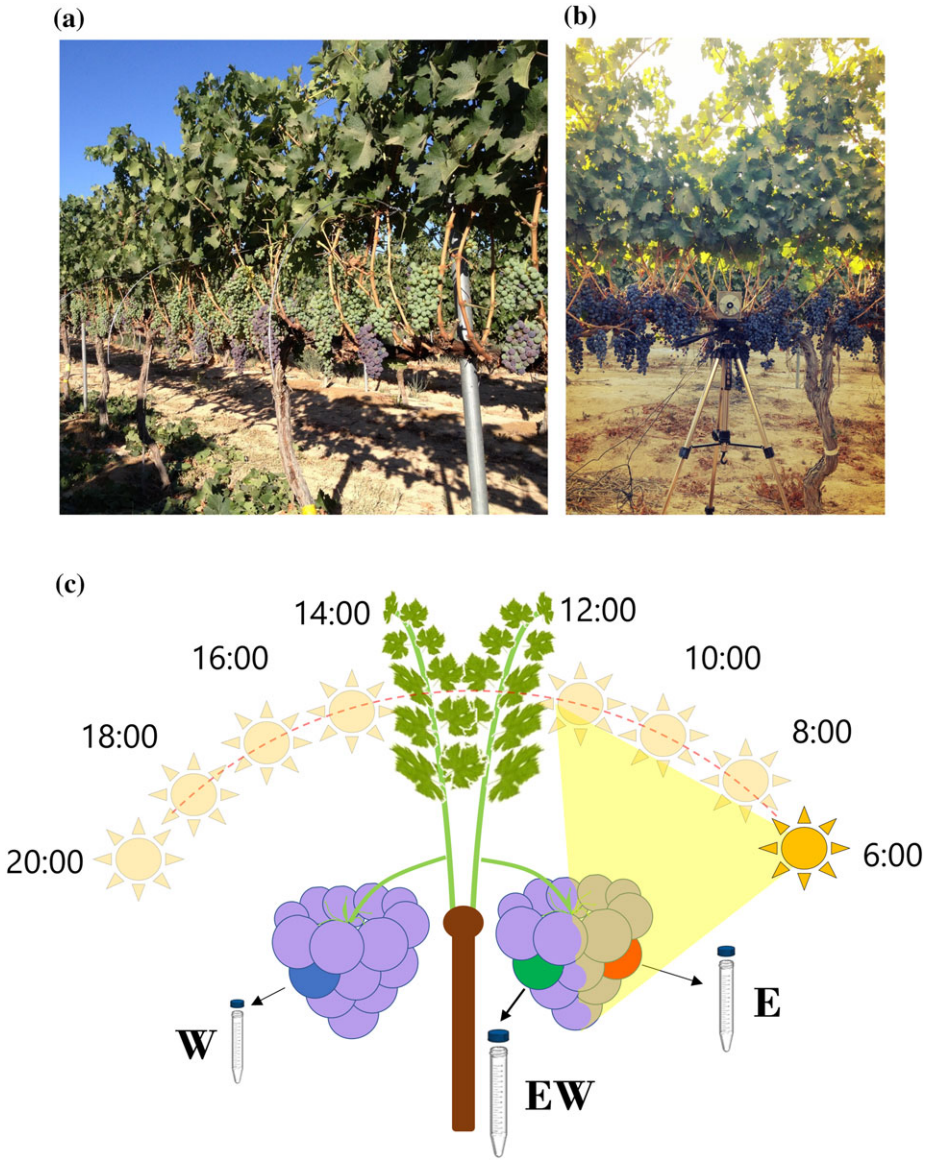

FIGURE 1 (a) Defoliation of basal leaves at the onset of veraison to create full exposure of clusters to sunlight, (b) a two-pyranometer system positioned within the cluster-zone to measure the incoming shortwave radiation reaching the sampled cluster orientations, and (c) an illustration of the diurnal sampling scheme. Sampled berry position color corresponds with the color scheme of the figures throughout the manuscript [Colour figure can be viewed at wileyonlinelibrary.com]

from the middle height of the corresponding face of the cluster, to avoid the effect of the vertical location on fruit composition. Samples were snap-frozen in liquid nitrogen in the vineyard and stored at −80°C until further processing. In the lab, berries were weighed and separated to skin, pulp, and seeds, while kept frozen on dry ice.

2.3 | Meteorological and cluster micrometeorological measurements

Global incoming shortwave radiation (R_G), air temperature (T_{air}), relative humidity (RH%), and wind speed and direction were continuously monitored 1 m above the canopy. Measurements were made at 10 s intervals using a multisensor (WS501-UMB, Lufft, Fellbach, Germany), and 15 min averages were logged by a data logger (CR200, Campbell Scientific, Utah, USA). Cluster-zone air temperature and RH% were measured at a shaded spot in the immediate vicinity of the clusters, by placing sensors equipped with an internal data logger (Hobo ProV2, Onset, MA, USA). Measurements were performed at 1 min intervals, and 15 min averages were recorded. The shortwave radiation flux reaching the east and west faces of representative clusters from both canopy sides (R_C) were measured by four pyranometers (LI200R, Li-Core, NE, USA) connected to a data logger (21X, Campbell Scientific, UT, USA). Each two pyranometers were set on a tripod and placed within the cluster-zone, facing vertically (i.e., looking sideways in a 90° angle relative to the sky) and directed away from the sampled orientations of representative clusters (Figure 1b). Berry surface temperature (BST) was measured hourly using an infrared camera (T640, FLIR

systems, OR, USA). Images were taken from two representative clusters from both sides of the canopy. The images captured the portion of the clusters corresponding to the sampling locations and pyranometer measurements. Data analysis of the acquired thermal images included a careful delineation of berries located in the middle section of the cluster's vertical axis (i.e., middle height), and the exclusion of pixels representing non-grape background data.

2.4 | Leaf assimilation measurements

Gas exchange and fluorescence measurements were performed every 2 hr using a portable LI-6400 Infrared Gas Analyzer (Li-Cor Biosciences Inc., Nebraska, USA). The system maintained the leaf chamber at the ambient temperature and at a steady CO_2 level of 400 μmol mol^{-1}. The instrument's photosynthetically active radiation flux density was manually adjusted, at every measuring point, in accordance with the ambient values (R_G). All measurements were conducted on young, fully-matured leaves, from both sides of the canopy of a representative vine, at each field replicate.

2.5 | Berry sample preparation

Grape skin tissues were lyophilized and ground under liquid nitrogen, using a Retsch-mill (Retsch, Haan, Germany) with prechilled holders and grinding beads, and 40 mg of tissue powder were weighed. Grape pulp samples were manually crushed using a mortar and pestle under liquid nitrogen, and 100 mg of tissue powder were weighed.

Metabolites of both tissues were separately extracted, following the protocol for metabolite profiling as described in Weckwerth, Loureiro, Wenzel, and Fiehn (2004), with adaptation to grape skin as described in Degu et al. (2014). The methanol:chloroform:water extraction solution (2.5:1:1 v/v) contained the following internal standards: 0.2 mg ml^{-1} sorbitol $6C^{13}$ in water, 1 mg ml^{-1} ampicillin in water, and 1 mg ml^{-1} corticosterone in methanol.

Skin extracts were filtered (0.22 μm Millipore, MA, USA) and transferred to glass vials for analysis using ultra performance liquid chromatography coupled to a quadrupole time-of-flight mass-spectrometer (UPLC QTOF-MS; Waters, MA, USA).

2.6 | LC/MS conditions

UPLC QTOF-MS conditions were exactly as described previously by Hochberg et al. (2013). Briefly, separation was performed using a C18 column (Waters MS Technology, Manchester, UK) maintained at 40°C. Leucine enkephalin was used for lock mass calibration. The mobile phase transitioned from 95% water, 5% acetonitrile, 0.1% formic acid (phase A) to 0.1% formic acid in acetonitrile (phase B), with gradient transitioning from 100 to 60% phase A (0–8 min), 60–0% phase A (1 min), a gradual return to 100% phase A (3.5 min), and conditioning at 100–60% phase A (2.5 min), with a total run time of 15 min.

2.7 | Liquid chromatography–mass spectrometry (LC/MS) annotation

MassLynxTM software version 4.1 (Waters) was used for system control and data acquisition. Metabolite annotation was validated using the standard libraries described in Arapitsas et al. (2012), based on retention time order (Degu et al., 2014). Metabolites were also annotated based on fragmentation patterns searched against the Chemspider metabolite database (http://www.chemspider.com/), the consistency of their retention times with those of identified metabolites, and comparison with the data in the scientific literature.

2.8 | Gas chromatography–mass spectrometry (GC/MS)

For the grape pulp, 70 μl of the extracts were dried using a Concentrator Plus (Eppendorf, Hamburg, Germany) and derivatized for 120 min at 37°C (40 μl of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine), followed by a 30 min treatment with 70 μl N-methyl-N-(trimethylsilyl) trifluoroacetamide at 37°C. 7 μl of a retention time standard mixture (0.029% v/v n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotracontane, and n-hexatriacontane dissolved in pyridine) was added prior to trimethylsilylation.

2.9 | GC/MS conditions

The gas chromatography–mass spectrometry (GC–MS) system consisted of a 7,693 autosampler, a 7890B GC, and a 5977B single quadrupole mass spectrometer (Agilent, Santa Clara, CA). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC was performed on a 30 m VF-5 ms column with 0.25 mm i.d. and 0.25 m film thickness + 10 m EZ-Guard (Agilent, Santa Clara, CA), using a liner split/splitless with Wool, (Restek, USA). The gradient of injection temperature (PTV) was from 70°C to 300°C in 14.5°C s^{-1}, the Transfer line was 350°C, and the ion source adjusted to 250°C. The carrier gas used was helium set at a constant flow rate of 1.8 ml min^{-1}. The temperature program was 1 min isothermal heating at 70°C, followed by a 1°C min^{-1} oven temperature ramp to 76°C, followed by a 6°C min^{-1} oven temperature ramp to 340°C, and a final 5 min heating at 340°C. Mass spectra were recorded at 1.6 scans per second with a mass-to-charge ratio 70:550 scanning range. Post-column back-flushing was used during the post-run time in every injection to keep detector clean, column flow was reversed for few minutes to redirect high-boiling components to the inlet split vent.

An additional run with a split ratio of 1:20 was performed on all samples to correctly determine the levels of glucose, fructose, sucrose, malic, and tartaric acids, due to their high abundance in the pulp. Initial oven temperature was set to 70°C, rising in a gradient of 95°C min^{-1} to 165°C and in 6°C min^{-1} to 276°C. Total run time was 19.5 min and post-run of 5 min at 340°C. 1 μl was injected. Gain factor of 15 was applied from 0 to 7 min (augmenting tartaric and malic acid peaks)

and reverted to 1 for the rest of the run. Sucrose, amino acids, and polyamines were quantified using a calibration curve of standards (Sigma-Aldrich, MO, USA).

2.10 | GC/MS annotation

Spectral searching utilized the MassHunter Qualitative and Quantitative analyses (Agilent, Santa Clara, CA) against RI libraries downloadable from the Max-Planck Institute for Plant Physiology in Golm, (http://gmd.mpimp-golm.mpg.de/) and finally normalized by the internal standard sorbitol $6C^{13}$ (Cortecnet Corporation, Mill valley, CA) and tissue fresh weight.

2.11 | Elemental analyzer

Grape pulp samples were lyophilized and ground to powder (Retschmill, Retsch, Haan, Germany). Samples of 6 mg were analyzed by a FlashEA™1112 CHNS-O Analyzer (Thermo Fisher Scientific Inc., UK).

2.12 | Statistical analysis

Statistical analysis was performed using R version 3.3.1 (R Development Core Team, 2017) in RStudio. The significant effect of time, berry orientation, and their interaction on the metabolic composition of the berries was tested using a two-way ANOVA repeated measures model, on each metabolite, using the built-in *aov* function. Metabolite–metabolite correlation matrix was constructed by separately scaling the dataset of each berry orientation to its daily mean, applying a log-transformation, calculating the mean of all biological replicates, combining these data from all orientations, and using the built-in *cor* function with the "Pearson" algorithm. The correlation matrix was visualized using the *corrplot* function in the "corrplot" package (Wei & Simko, 2016). Micrometeorology–metabolite correlation was calculated in the same manner, and a network analysis was conducted using the MetScape application and the NetworkAnalyzer tool, available in Cytoscape version 3.4.0. Correlations were incorporated into the network if they were statistically significant (p value < 0.05) and their correlation coefficient was higher than 0.3 or lower than −0.3. Clustering of metabolites by time-series patterns was calculated by Euclidean distances and the Ward.D2 clustering method in functions *dist* and *hclust*, built-in "stats" package. The number of clusters chosen for each dataset was based on the silhouette method using the *fviz_nbclust* function from the "factoextra" package (Kassambara & Mundt, 2017). Clustered heatmaps were created using *heatmap.2* function from the "gplots" package (Warnes et al., 2016). Multiple regression analysis was performed using the built-in *lm* function using strictly irradiance and air temperature values as explanatory variables. Principle component analyses (PCA) were performed in JMP®, version 13 (SAS Institute Inc., Cary, NC, 1989–2007). Data was scaled, dividing values by the mean of each metabolite and log transformed.

3 | RESULTS

3.1 | Fruits and leaves on opposing orientations experienced contrasting diurnal pattern of solar irradiance

The diurnal patterns of incoming solar irradiance reaching the cluster zone is a direct result of the diurnal course of the sun, relative to the vine-row orientation. As a result, clusters at the different positions were exposed to distinct diurnal course of radiation (Figure 2a). Berries at the E orientation were exposed to direct sunlight during

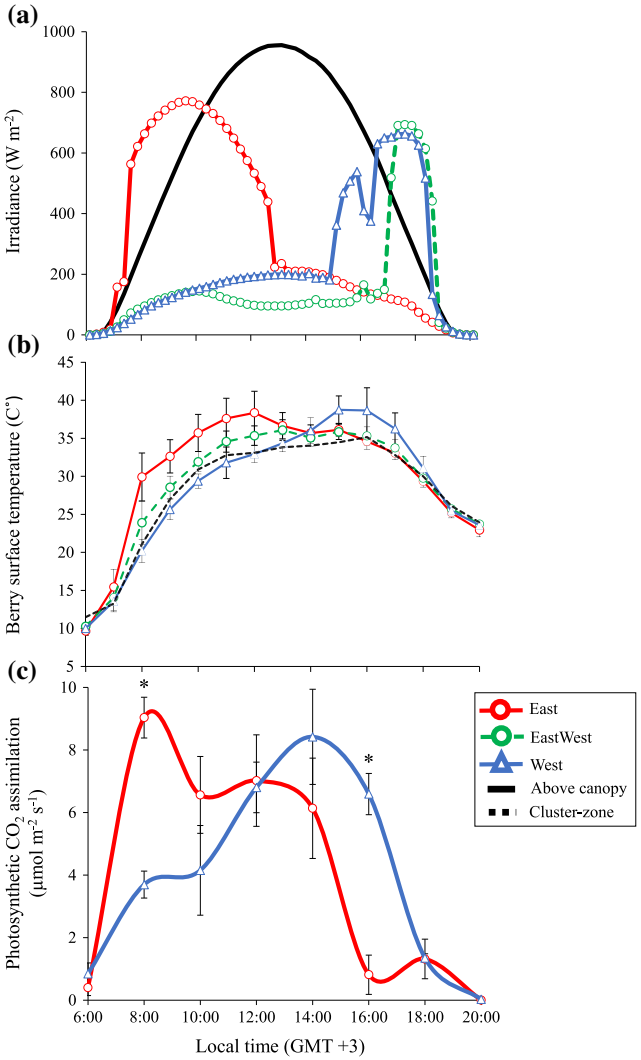

FIGURE 2 Diurnal measurements of intercepted solar irradiance (a) and berry surface temperature (b) of sampled cluster sections, and the photosynthetic CO_2 assimilation of their adjacent leaves (c). Global horizontal irradiance measured above the canopy and cluster-zone air temperature are given in a and b, respectively. Error bars represent standard error (n = 4) in c, and standard deviation in b. Due to the large dataset in b, all differences were statistically significant and, therefore, are not marked. Data points in c marked by * represent significant differences between East and West measurements [Colour figure can be viewed at wileyonlinelibrary.com]

the morning, between 7:30 a.m. and 12:30 p.m., whereas EW and W berries were exposed during the afternoon, between 5:00 p.m. to 6:30 p.m., and 3:15 p.m. to 6:15 p.m., respectively. Thus, E orientated berries experienced a mirror-like diurnal pattern of insolation compared with EW and W orientated berries. Berry surface temperature followed a similar diurnal trend (Figure 2b), yet differences between orientations were less profound. Noteworthy is the decrease in the surface temperature of west-facing berries (EW and W orientations) between 4:00 p.m. to 6:00 p.m., albeit being exposed to high levels of solar irradiance. This is the result of greater sensible heat loss from the fruit, driven by the decrease in air temperature during these hours. Overall, in these conditions, berry orientation (i.e., berry location on the cluster) had a stronger influence than the position of the cluster itself (i.e., canopy side) on the diurnal micrometeorological patterns experienced by the berry. Leaf photosynthetic CO_2 assimilation showed high rates during sunlit hours and lower rates when leaves were shaded. This positive association with the diurnal irradiance pattern resulted in different CO_2 assimilation patterns of opposing canopy sides (Figure 2c).

3.2 | Berry orientation affected the levels of several metabolites that were stable throughout the day

As shown in a previous study (Reshef et al., 2017), berry orientation has a significant impact on its chemical composition. In support of our previous findings, the level of a large number of metabolites significantly differed between berries positioned at the different orientations, yet most did not change significantly in their levels throughout the day. These included the tricarboxylic acid cycle-related intermediates malate, citrate, and fumarate, other organic acids as erythronate and gluconate, anthocyanin metabolites of malvidin and peonidin, and flavan-3-ols. All were found at higher levels in EW berries that exhibited the lowest daily accumulated radiation. In contrast, several sugars and sugar alcohols including galactose, trehalose, turanose, galactinol, arabitol, and threitol, and flavonol glucosides, were found at higher levels in E and W berries, which, although exposed to different diurnal radiative pattern, received similar daily accumulated radiation.

3.3 | Fruit composition significantly changed during the day

Among 51 primary metabolites, unequivocally annotated in grape pulp, the level of 11 metabolites in E, 12 in EW, and eight in W-orientated berries significantly changed diurnally, for example, amino acids and sugars in E and EW, and sugars and organic acids in W. Metabolites displaying similar diurnal trends were grouped by hierarchical clustering (Figure S1). A prominent group of amino acids, including glycine, leucine, threonine, and valine, clustered together in all three orientations. In addition, serine was clustered with the mentioned group in E and EW, and putrescine in EW and W. Significant diurnal changes in the levels of these amino acids and others, including cysteine, serotonin, pyroglutamate, and GABA, were orientation-specific. The sugars sucrose and glucose-6-phosphate showed significant diurnal changes in all orientations and were grouped together. Fruit organic acids associated with photorespiration (glycolate and glycerate) were clustered together in all berry orientations, whereas those associated with respiration (malate, maleate, and fumarate) were clustered together in E and EW, but not in W. In contrast, isoascorbate and its cleavage product threonate were clustered together only in west-facing berries (EW and W). Although clear diurnal patterns in the levels of organic acids were identified in all berry orientations, statistically significant changes were found only for galactarate and threonate, in E and EW berries, respectively, and for glycolate, isoascorbate, and threonate in W-orientated berries. In addition, no significant diurnal changes were observed in the levels of glucose, fructose, or malate, which are known to dramatically change during fruit maturity.

Contrary to the circadian changes found in the grape transcriptome (Carbonell-Bejerano et al., 2014), a lower number of phenylpropanoid metabolites were found to significantly change diurnally compared with primary metabolites (Figure S2). Compounds showing significant diurnal changes clearly differed between contrasting orientations of the same cluster (E vs. EW). Flavonols were the dominant group in the inter-row-orientated E berries, whereas anthocyanins dominated the change in the vine row-orientated EW berries. The aglycon flavonol quercetin was the only phenylpropanoid metabolite found to significantly fluctuate in all three orientations.

3.4 | Fruit metabolite levels exhibited position-dependent diurnal patterns

An overview of the orientation-specific diurnal changes in grape, primary and secondary metabolism profiles was plotted by PCA (Figure 3). Values for each berry orientation were expressed as fold changes relative to the respective values at the initial time point (6:00 a.m.). In a GC/MS-based PCA (grape pulp, Figure 3a), E and W samples were separated on principle Component 1, explaining 35.1% of the total variance in the dataset. On the basis of the major coefficients comprising this eigenvector (Table S1), different diurnal patterns of change were observed for galactose, its intermediate galactinol, and melibiose; the sugar alcohols erythritol and threitol; and different amino acid-related compounds including GABA, valine, serine, leucine, and putrescine. Principle Component 2 explained 19.6% of the overall variance and separated between E and EW berries sampled from opposite orientations of the same clusters. They differed in the diurnal patterns of several organic acids, including the TCA cycle-related intermediates pyruvate, citrate, malate and maleate, and isoascorbate with its putative precursor gulonate, in addition to the phytohormone indol-3-acetate and its precursor tryptophan; and the amino acid alanine.

In a LC/MS-based PCA analysis (grape skin, Figure 3b), E and EW samples were separated on principle Component 1, explaining 26.8% of the overall variance. Metabolites contributing to this separation

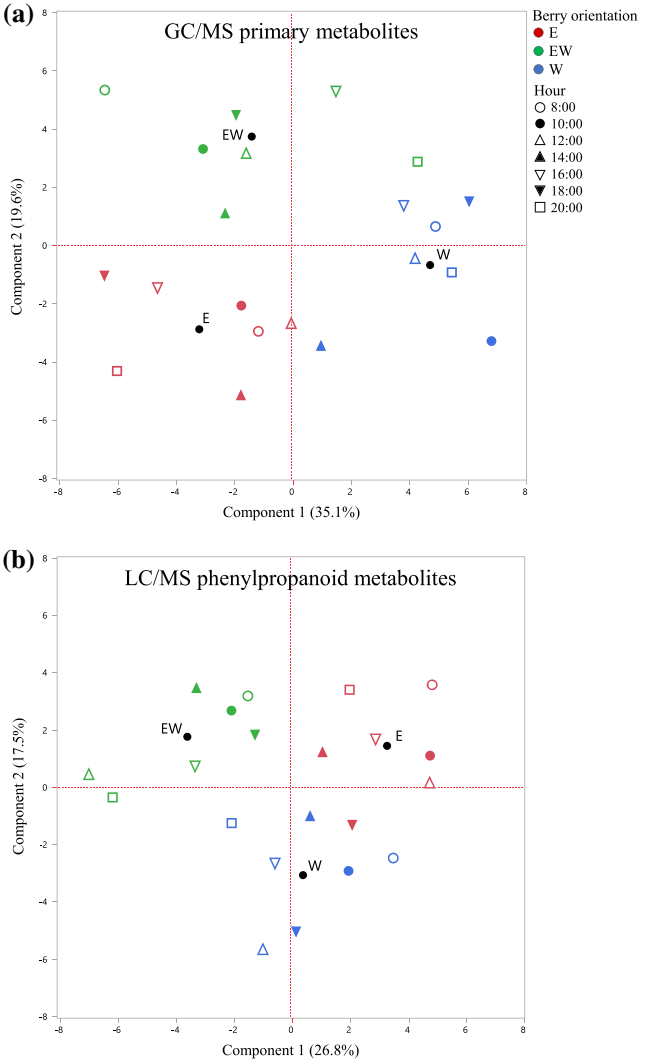

FIGURE 3 Principle component analysis (PCA) of primary metabolites detected in grape pulp using a GC/MS system (a) and phenylpropanoid metabolites detected in grape skin using an LC/MS system (b). Values for each berry orientation and time point are fold changes, relative to the values of the respective orientation at 6:00 a.m. [Colour figure can be viewed at wileyonlinelibrary.com]

(Table S2) include the glycosylated and coumaroylated anthocyanins (cyanidin, delphinidin, and petunidin), acetylated forms of peonidin and malvidin, and mal-3-glu, in addition to phenylalanine and p-coumarate, and the flavonols quer-3-glr and myricetin in its aglycon, galactose, and glucuronide conjugated forms. Principle Component 2 explained 17.5% of the total variance. It separated samples according to cluster location with berries from E and EW sampled from the same clusters having similar and positive values, and berries from W having negative values. These differed in the patterns of change of acetylated (cyanidin, delphinidin, petunidin, and peonidin) and glycosylated (malvidin, peonidin, and petunidin) anthocyanins and coumaroylated forms of peonidin and malvidin, in addition to the flavan-3-ols epicatechin, epigallocatechin, and procyanidin B2, and the stilbene piceid.

To delve further, we assessed the dissimilarities in the diurnal pattern of fruit metabolite profile between pairs of berry orientations, differing in the diurnal insolation pattern of the fruit (E and EW), and the diurnal CO_2 assimilation pattern of their adjacent leaves (EW and W). This was achieved by performing two-way repeated measures ANOVA, considering time and berry orientation. Only four metabolites differed in their diurnal pattern between EW and W, differing in the leaf CO_2 assimilation pattern, namely, glucose-6-phosphate, putrescine, serotonin, and the anthocyanin peo-3-coum. Conversely, 18 metabolites differed in their diurnal pattern between E and EW, experiencing different radiation regimes, including sugars, organic acids, flavonoids, and a set of nine amino acids and related compounds. Of these, glucose-6-phosphate, putrescine, and serotonin exhibited a significant interaction factor in both pair-wise comparisons.

On the basis of the number of metabolites showing a significant interaction factor, the diurnal CO_2 assimilation pattern of the adjacent leaves had a marginal effect on diurnal metabolic processes in the grape. Instead, this analysis highlighted the major impact of intercepted solar irradiance and fruit temperature.

3.5 | Contrasting berry insolation patterns effected common carbon influx but modified metabolism

The total carbon content in the fruit revealed a common diurnal increase during early morning (6:00 a.m. to 10:00 a.m.) followed by a decrease towards sunset (4:00 p.m. to 8:00 p.m.; Figure 4b). The early morning increase in carbon in E berries was coupled with a significant decrease in sucrose, whereas sucrose levels were stable at that time of day in both west-facing (EW and W) berries. Throughout the day, sucrose and glucose-6-phosphate decreased dramatically in all berry orientations (Figure 4a). Although the diurnal decrease in sucrose was proportionally similar in all orientations, reaching in the afternoon between 20% to 25% (2.3–3.5 mg g^{-1} fresh weight) of its maximum daily levels in fresh tissues, the levels of glucose-6-phosphate decreased more dramatically in EW and W (70% and 76% decrease, respectively) compared with E berries (43% decrease). In contrast, the levels of the sugar alcohol erythritol decreased diurnally in E but increased in EW and W (Figure 4a).

Similar to that found for erythritol, the organic acids galactarate and gulonate, previously shown to be involved in the response to salt stress in leaves (Sanchez, Siahpoosh, Roessner, Udvardi, & Kopka, 2008), decreased in E berries, but did not change and even slightly increased in EW and W berries, respectively (Figure 5). Comparable patterns were found for the majority of amino acids and amines (Figure 6). Altogether, berries which experienced extended periods of direct insolation during the morning (i.e., E) had higher levels of several of those abiotic-stress related primary metabolites compared with berries insolated during the afternoon (i.e., EW and W). However, the levels of these metabolites decreased during the day in E, whereas stagnating or increasing in EW and W. For instance, alanine decreased by 42% (19 $\mu g\ g^{-1}$ FW) in E and increased by 91% (18 $\mu g\ g^{-1}$ FW) in EW. Leucine, serine, threonine, and valine decreased in E by 41%, 40%, 30%, and 32% during the course of the day, whereas the corresponding changes in EW and W were substantially lower.

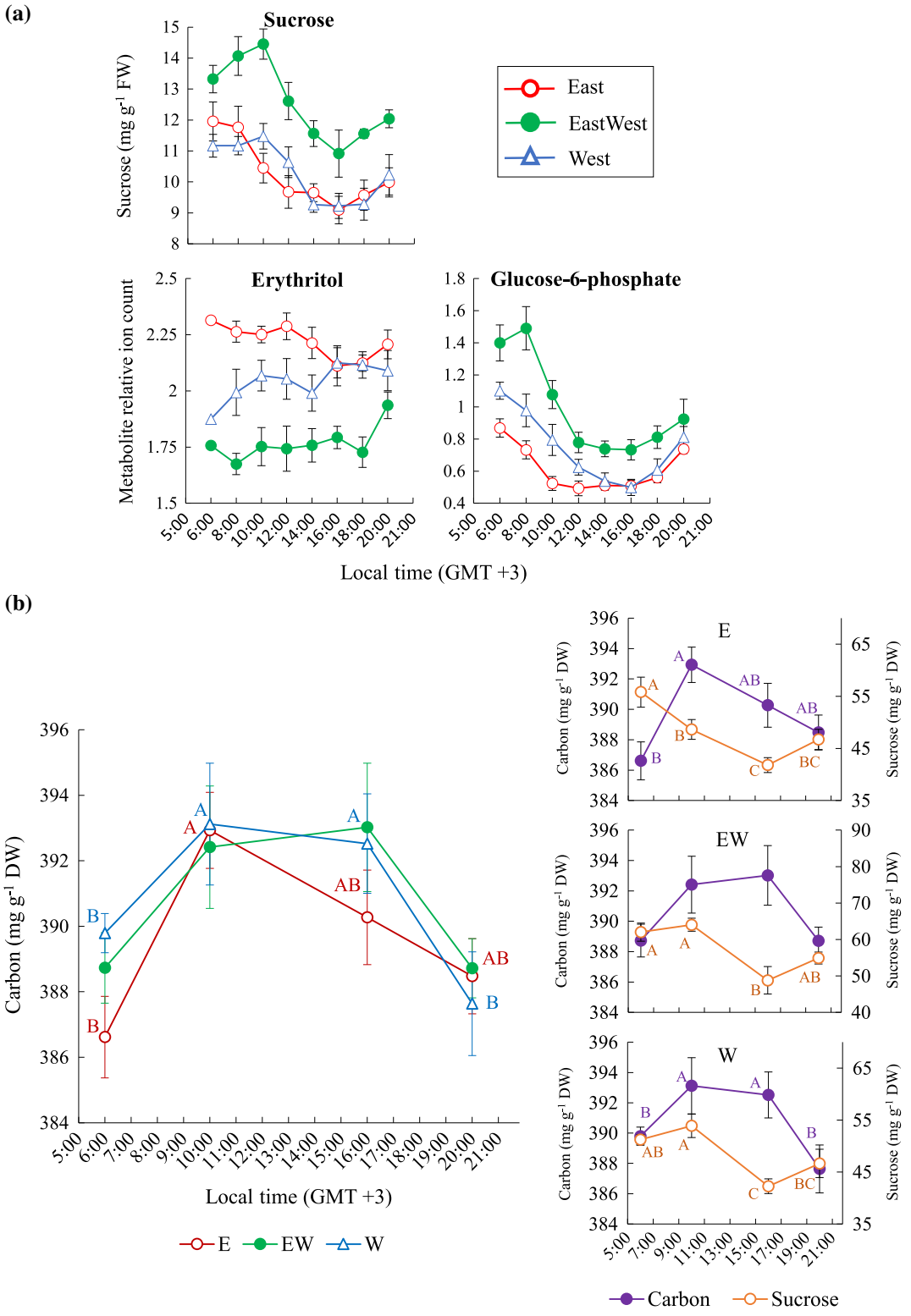

FIGURE 4 (a) Sugars detected in grape pulp tissues showing a significant interaction factor in their diurnal pattern in a two-way repeated measures ANOVA between berry orientations. Error bars represent standard error (n = 6). (b) Total carbon levels during the diurnal campaign in grape pulp tissues, and berry carbon levels superimposed with sucrose levels. Data points marked by different letters represent significant differences (p < 0.05, n = 6) [Colour figure can be viewed at wileyonlinelibrary.com]

Among the flavonoids showing a significant interaction factor between time and berry orientation (Figure 7), the levels of the upstream glycosylated anthocyanins, cyan-3-glu and delph-3-glu, were found to quickly respond to solar irradiance. Both increased in E berries by 33% and 31%, respectively, corresponding with the insolation period (8:00 a.m. to 10:00 a.m.), and decreased thereafter. In

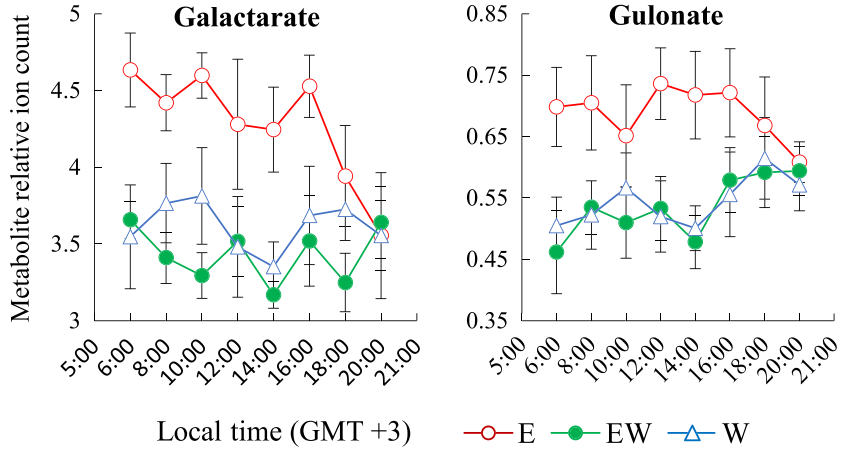

FIGURE 5 Organic acids detected in grape pulp tissues showing a significant interaction factor in their diurnal pattern in a two-way repeated measures ANOVA between orientations of sampled berries. Error bars represent standard error (n = 6) [Colour figure can be viewed at wileyonlinelibrary.com]

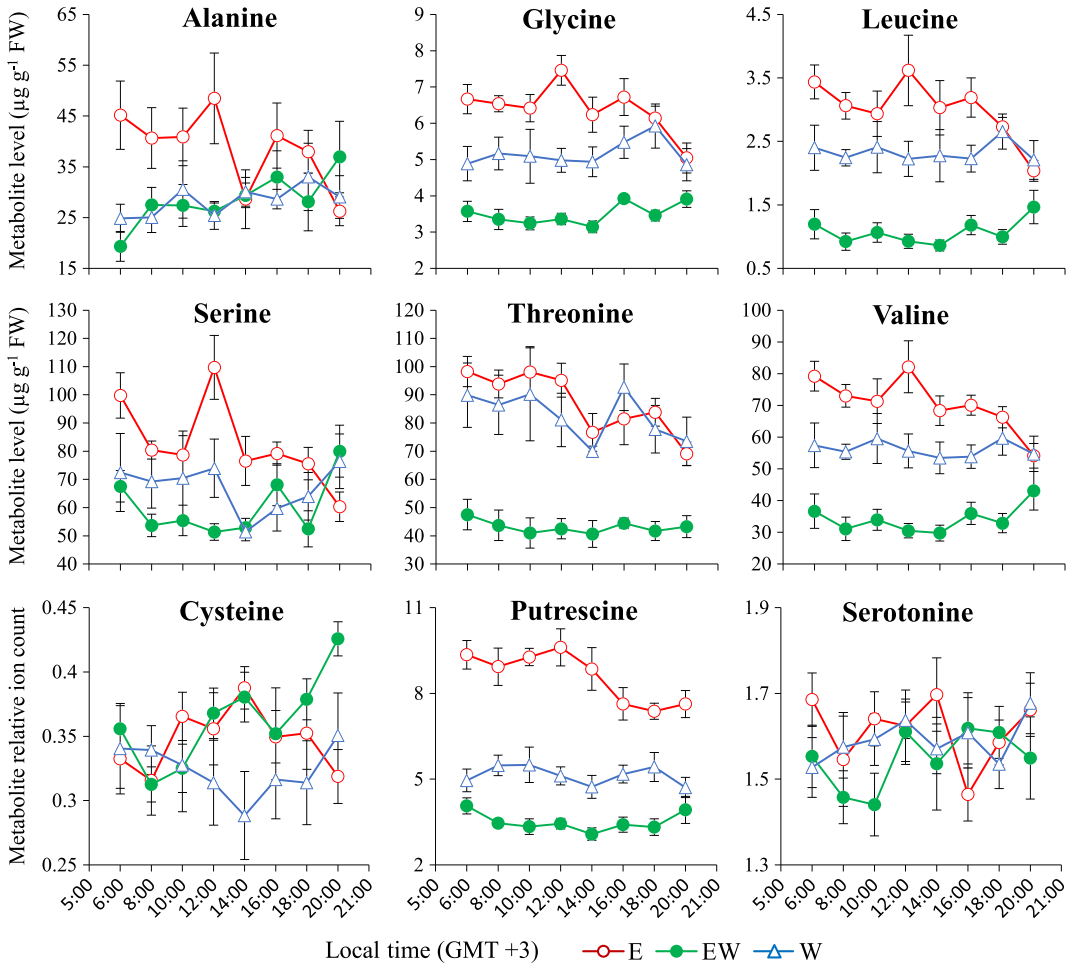

FIGURE 6 Amino acids and polyamines detected in grape pulp tissues showing a significant interaction factor in their diurnal pattern in a two-way repeated measures ANOVA between orientations of sampled berries. Error bars represent standard error (n = 6) [Colour figure can be viewed at wileyonlinelibrary.com]

EW and W berries, the levels of delph-3-glu increased by up to 60% during direct insolation (6:00 p.m.) and dramatically decreased thereafter, whereas changes in the levels of cyan-3-glu were more minute. Quercetin aglycon showed a similar diurnal pattern, indicating a positive effect of insolation on its levels in the fruit.

3.6 | Diurnal changes in fruit metabolic profiles highlighted correlated metabolite groups

The diurnal changes in metabolite levels from all orientations were used to construct a metabolite–metabolite correlation matrix

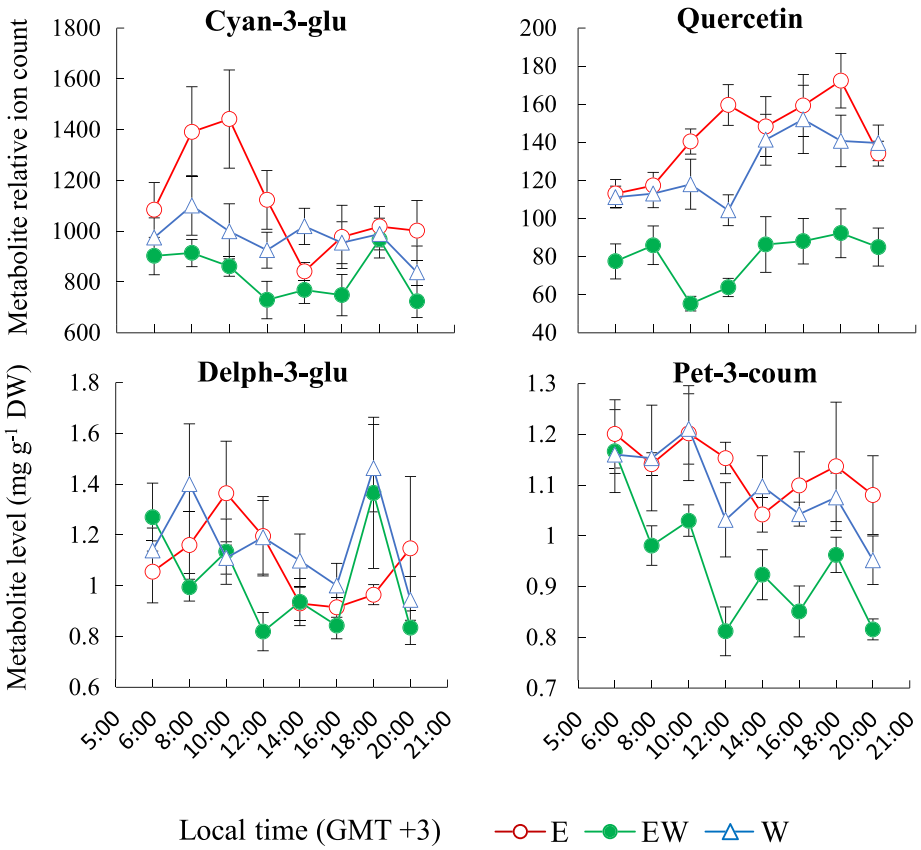

FIGURE 7 Flavonoids detected in grape skin tissues showing a significant interaction factor in their diurnal pattern in a two-way repeated measures ANOVA between orientations of sampled berries. Error bars represent standard error (n = 6) [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 8). An important group of primary metabolites showing strong positive correlation included the amino acids beta-alanine, glycine, leucine, proline, serine, threonine and valine, and the polyamine putrescine; the sugar alcohols erythritol, galactitol, and threitol; and the sugars galactinol, galactose, melibiose, turanose, and trehalose. This group consists, to a considerable extent, of metabolites associated with plant response to solar irradiance, heat, and other abiotic stresses (Alcazar et al., 2006; Conde et al., 2015; Garg et al., 2010; Obata & Fernie, 2012; Pillet et al., 2012; Reshef et al., 2017). Another group showing strong positive correlations included ribulose, glycolate, glycerate, and pyruvate, associated with photorespiration; isoascorbate and its metabolite threonate; and indole-3-acetate and its precursor tryptophan. In addition, pyroglutamate, sucrose, and glucose-6-phosphate were strongly correlated. This may be associated with the translocation of assimilates from source tissues through the phloem sap, in which sucrose and glutamate predominate (Gholami, Coombe, & Robinson, 2004). In the secondary metabolites, the glycosylated and coumaroylated anthocyanin forms of malvidin and peonidin were tightly correlated yet showed minor coordination with the corresponding correlated group of cyanidin, delphinidin, and petunidin. The latter group was tightly correlated with the large majority of detected flavonols. Conversely, among the acetylated forms, all anthocyanins grouped together and showed positive correlation with the flavan-3-ols metabolites.

3.7 | R_c and T_{air} were strongly correlated with the diurnal pattern of fruit metabolite levels

Correlation analysis between diurnal metabolite profiling and micrometeorological measurements highlighted T_{air} and R_c as the two leading factors associated with changes in metabolite levels (Figure 9). This was counter to our expectations that BST would outperform T_{air} as BST is a more direct indicator of the thermal state of the fruit. With the threshold level set at p value < 0.05 and r > 0.3 or r < −0.3, 30 metabolites correlated with T_{air} and 21 with R_c, with six common to both. Of the metabolites showing correlation with T_{air}, 24 were negatively correlated, of which the majority were flavonoids, several amino acids and related metabolites (putrescine, pyroglutamate), and the sugars sucrose and glucose-6-phosphate, that showed exceptionally strong negative correlation. Conversely, six metabolites were positively correlated with T_{air}, including the amino acids alanine and GABA and quercetin aglycon.

Of the metabolites showing correlation with R_c, delph-3-glu and cyan-3-glu that represent the first step of the two main branches of the anthocyanin biosynthesis, were positively correlated. Similarly, the sugars trehalose and melibiose and the amino acids glycine, alanine, and GABA positively correlated with R_c. As found for T_{air},

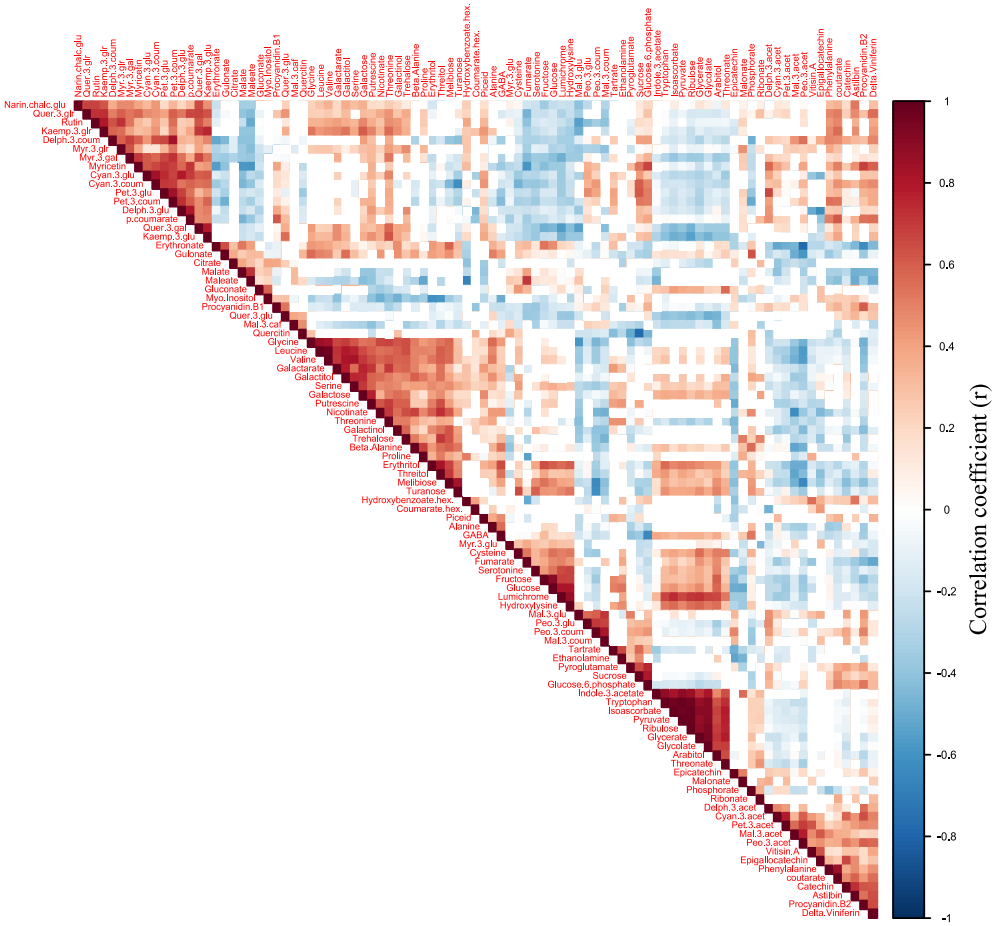

FIGURE 8 Correlation matrix based on diurnal changes in grape metabolic profile, using Pearson's method. Data was scaled to daily mean of each metabolite, log transformed, and the mean values of biological replicates (n = 6) were used [Colour figure can be viewed at wileyonlinelibrary.com]

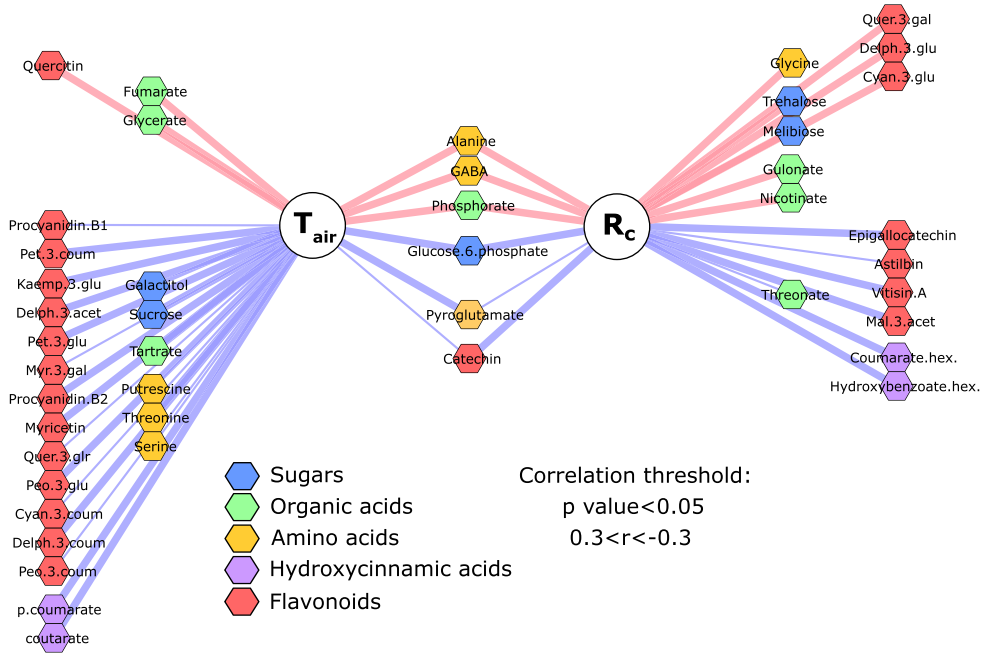

FIGURE 9 Network visualization of correlations between solar irradiance intercepted at the sampled cluster sections (R_C) and measrued air temperature, and both primary and secondary grape metabolites of the entire dataset, that is, all berry orientations and time points obtained during a diurnal sampling campaign. Correlations were based on Pearson's method [Colour figure can be viewed at wileyonlinelibrary.com]

negatively correlated metabolites mainly comprised the major flavonoids, as well as hydroxycinnamic acids and glucose-6-phosphate.

A model using these two factors, air temperature and solar irradiance, in a multilinear regression analysis, significantly explained the diurnal trends of 19 metabolites: 13 phenylpropanoids, three sugars, trehalose, sucrose, and glucose-6-phosphate, and the amino acids GABA, threonine, and pyroglutamate (Table S3). T_{air} accounted for 87%, and 93% of the total variance in the levels of glucose-6-phosphate and myricetin, explained by the model (86% and 50%, respectively). R_c accounted for 74% and 93% of the total variance in the levels of delph-3-glu and epigallocatechin, explained by the model (57% and 54%, respectively).

Albeit daily changes in the levels of phenylpropanoid metabolites were largely found to be statistically insignificant, the tight correlation between their patterns of change and that of T_{air} and R_c emphasized their responsiveness to the diurnal fluctuations in these key environmental signals.

4 | DISCUSSION

Grape berries exhibit a clear diurnal pattern of assimilate translocation and metabolism in the fruit, partly in response to micrometeorological conditions. Total carbon levels in the fruit exhibited significant fluctuations during the day, common to all berry orientations. Sucrose was outstanding in showing a dramatic diurnal decrease in content, irrespective of fruit orientation, whereas most compounds that were found to change diurnally showed a clear orientation-dependent pattern, highlighting the significant role of solar irradiance and fruit temperature in triggering fruit diurnal metabolism.

The diurnal decrease in grape sucrose levels measured here is, to the best of our knowledge, unprecedented. Our findings are in contrast with previous studies that found no significant diurnal changes in sucrose levels in glasshouse-grown fruiting cuttings of grapevines (Hawker, Ruffner, & Walker, 1976) nor in other fruits such as apples and tomatoes (Benard et al., 2015; Klages, Donnison, Wunsche, & Boldingh, 2001; Mills, Behboudian, & Clothier, 1997). In the case of grape berries, this discrepancy may have resulted either from the use of small rooted cuttings or from differences in temperature and light intensity levels in the glasshouse that have not been characterized in depth. In apples and tomatoes, the role of sucrose in sink-strength and source-sink assimilate translocation was suggested as secondary to that of sorbitol and starch, respectively (F. Wang, Sanz, Brenner, & Smith, 1993; Watari et al., 2004). Correspondingly, diurnal changes in the level of starch in tomatoes were found to be significant (Benard et al., 2015). Here, we found that berries lost between 2.3 and 3.5 mg g^{-1} fresh weight of sucrose during the day, equivalent to roughly 20%–25% of their maximum daily level of sucrose, present during early morning. Fruit sucrose levels showed no correlation with the diurnal pattern of leaf CO_2 assimilation, nor with fruit-incoming solar irradiance, but had a strong negative correlation with air temperature. These results suggest leaf-photosynthesis and source-sink translocation as processes occurring at separate timeframes. During the day, when photosynthesis is active, leaves accumulate a proportion of assimilated carbon as starch. This is a highly regulated process that serves as storage for carbohydrates to allow for growth, respiration, and the translocation of carbohydrates to sink tissues during the night (Geiger, Servaites, & Fuchs, 2000; Smith et al., 2004; Zeeman, Smith, & Smith, 2007). The accumulation of starch in leaves competes with that of sucrose, and thus may come at the expense of sucrose translocation towards sink tissues (Geigenberger, 2011; Sulpice et al., 2014). Indeed, sucrose levels in the phloem sap of grapevine were found to be lowest during the day and highest during the night (Gholami et al., 2004). The diurnal fall in the level of grape berry sucrose measured here, is in accordance with the findings of Gholami et al. (2004), regarding the circadian pattern of assimilate levels at the adjacent vascular tissues. The fact that ripening grape berries have limited starch storage (Hunter, Ruffner, & Volschenk, 1995; Zhu et al., 2017) strengthens their dependency on sucrose translocation.

What would be the metabolic fate of the degraded sucrose within the berry? It is accepted that sucrose is readily transported to the vacuole for storage, prior or following enzymatic hydrolysis to glucose and fructose (Davies & Robinson, 1996; Davies, Wolf, & Robinson, 1999). However, glucose and fructose levels did not significantly change diurnally, and their levels showed no correlation with sucrose, resulting in a major discrepancy. This discrepancy may have arisen from the fact that diurnal changes of several milligrams in high abundant compounds such as glucose and fructose were concealed by natural variability. In contrast, sucrose was found to be tightly correlated with glucose-6-phosphate, which suggests that the latter participated in a biochemical reaction involved, at least in part, in fruit sucrose depletion. Glucose-6-phosphate participates in the glycolysis and is the substrate of the main regulatory enzyme of the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH). G6PDH catalyzes the oxidation of glucose-6-phosphate and, consequently, reduces NADP to NADPH—a process that plays an important role in the response of plants to abiotic stress in general, and maintenance of cellular redox homeostasis in particular (Esposito, 2016; Kruger & von Schaewen, 2003; X. Wang et al., 2008). Additional routes of sucrose daily depletion are likely respiration and ethanolic fermentation, which remain active during grape ripening (Famiani et al., 2014). Together, these cellular processes may explain the decrease in grape total carbon levels (common pattern, significant only in W berries) measured between 4:00 p.m. and 8:00 p.m. (Figure 4b). This possibility is further strengthened by the fact that berry mass did not change significantly (data not shown), and therefore a backflow of solutes towards the plant vascular tissues may be considered negligible. Although our results cannot provide a quantitative estimate of sucrose depletion in the abovementioned processes, we can postulate their contribution to the overall diurnal sucrose depletion, maintaining the energy and redox balance in fully exposed grape berries.

Apart from sucrose, discernable diurnal changes were observed for a large number of amino acids and polyamines in the grape. The dramatic decrease in amino acid levels in E berries, in contrast with the results of L. Wang et al. (2014), was likely due to the prolonged exposure and higher radiation intensities characterizing the

orientation and conditions in this desert site. Current knowledge suggests that amino acid carbon skeleton replenishes the TCA cycle to maintain respiration under limited carbohydrate supply (Hildebrandt, Nesi, Araujo, & Braun, 2015). Accordingly, the induction of amino acid catabolism-related gene expression was observed upon sugar depletion in *Arabidopsis* (Thimm et al., 2004). The onset of the decrease in amino acid levels in our study coincided with minimum daily levels of sucrose in the grape, and therefore supports the above evidence.

On the other hand, amino acids and polyamines are known to play an important role in plant resistance to abiotic stress (Alcazar et al., 2006; Hildebrandt et al., 2015; Obata & Fernie, 2012), and the pool size of free amino acids was found to increase in grapes as a response to strong solar irradiance and elevated temperatures (Reshef et al., 2017; Sweetman, Sadras, Hancock, Soole, & Ford, 2014). In line with the above, the levels of free amino acids at dawn were significantly higher in E compared with EW and W orientations. Nevertheless, the progressive diurnal decrease in E berries resulted in equal or inferior levels to that measured in EW and W at dusk. This implies that the pool of free amino acids and polyamines in E berries underwent nocturnal replenishment. Accumulation of free amino acids under abiotic stress was suggested to result either from the anaplerotic flux from malate towards intermediates of the TCA cycle or from the degradation of proteins (Araujo, Tohge, Ishizaki, Leaver, & Fernie, 2011; Obata & Fernie, 2012; Sweetman et al., 2014). The lower malate levels in E, compared with EW and W berries, supports the former. We thus hypothesize that an extensive oscillatory turnover occurs in carbon and nitrogen metabolism in the grape between day and night. Carbon skeleton as malate may be utilized during the night for the replenishment of amino acids, which are subsequently utilized (i.e., degraded) during the day to protect the berry from the consequences of prolonged exposure to solar irradiance.

The levels of phenylpropanoids have been found to fluctuate diurnally in leaves, in association with solar irradiance levels (Tattini et al., 2015; Veit, Bilger, Muhlbauer, Brummet, & Winter, 1996). However, to the best of our knowledge, this had not been studied in fruits. In this study, the number of phenylpropanoids found to exhibit statistically significant diurnal changes was small, yet their diurnal course significantly correlated with that of air temperature and solar irradiance. The observed patterns of correlation support current understanding that solar irradiance triggers the biosynthesis of the phenylpropanoid pathway, whereas elevated temperatures inhibit phenylpropanoid gene expression and even accelerate the degradation of metabolites (Azuma, Yakushiji, Koshita, & Kobayashi, 2012; Mori, Goto-Yamamoto, Kitayama, & Hashizume, 2007; Tarara, Lee, Spayd, & Scagel, 2008). Of all the phenylpropanoid metabolites, quercetin aglycon was the only one found to increase along the day, suggesting its potential role as a carbon pool for the biosynthesis of flavonoids, readily used upon the nocturnal replenishment of carbohydrates. This agrees with recent evidence showing that phenylpropanoid-related gene expression is largely regulated during the night in ripening grape berries (Rienth et al., 2014).

To conclude, diurnal changes in environmental conditions were found to have an indirect effect on fruit carbon levels. On the other hand, amino acid and phenylpropanoid metabolism was directly affected by the diurnal pattern and duration of fruit insolation, implying that fruits remain highly responsive to changes in their environment, regardless of their oscillatory nature. As a result, harvest time must be considered as an important factor in the chemical phenotype, and sensorial quality of the grape. Given the gap in knowledge, additional diurnal experiments are required to resolve the regulatory processes driving the metabolic changes reported here.

AUTHOR CONTRIBUTION

N.R., N.A., and A.F. conceived and planned the study. N.R. has led and conducted the research, N.A. has overseen the field experiment and the environmental conditions monitoring, and A.F. has supervised the metabolomics analyses. N.R. wrote the body of the paper with A.F. and N.A. All authors reviewed and approved the manuscript.

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CONFLICT OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Hierarchical clustering of grape pulp primary metabolites.

Figure S2. Hierarchical clustering of grape skin phenylpropanoid metabolites.

Table S1. Eigenvectors in PCA of grape pulp primary metabolites.

Table S2. Eigenvectors in PCA of grape skin phenylpropanoid metabolites.

Table S3. Metabolites which variance was significantly explained by multi-linear analysis combining solar irradiance and air temperature.

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