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Secondary metabolites and related genes in *Vitis vinifera* L. cv. Tempranillo grapes as influenced by ultraviolet radiation and berry development

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Abstract

The effects of UV radiation on *Vitis vinifera* cv Tempranillo grapes were studied under field conditions as influenced by ultraviolet (UV) band (UV-A and UV-B), UV-B level (ambient vs enhanced), grape phenological stage (pea-size, veraison, and harvest), grape component (skin, flesh, and seeds), and fraction from which phenolic UV-absorbing compounds (UVACs) were extracted (soluble vs insoluble). Ambient UV-B levels caused stronger effects than ambient UV-A. These effects included increases in flavonol contents (particularly quercetins and kaempferols), the expression of flavonol synthase and chalcone synthase genes (*VvFLS4* and *VvCHS1*), and grape weight and size. In addition, the contents of flavanols and hydroxycinnamic acids increased under UV-B radiation at pea-size stage. All these compounds play physiological roles as antioxidants and UV screens. Synergic effects between UV-B and UV-A were observed. The responses of anthocyanins, stilbenes, and volatile compounds to UV were diffuse or nonexistent. Enhanced UV-B led to rather subtle changes in comparison with ambient UV-B, but differences between both treatments could be demonstrated by multivariate analysis. Pea-size and harvest were the phenological stages where the most significant responses to UV were found, while the skin was the most UV-responsive grape component. Soluble phenolic compounds were much more UV-responsive than insoluble compounds. In conclusion, UV radiation was essential for the induction of specific grape phenolic and volatile compounds. Given the physiological roles of these compounds, as well as their contribution to grape and wine quality, and their potential use as nutraceuticals, our results may have implications on the artificial manipulation of UV radiation.

1 | INTRODUCTION

Ultraviolet (UV) radiation is an important environmental factor influencing plant morphology and physiology (Robson et al., 2019; Verdaguer et al., 2017). Only UV-A (315–400 nm) and UV-B (280–315 nm) bands reach the Biosphere, always at wavelengths greater than 290 nm. The UV fraction represents around 5% of the solar radiation and is mostly composed (95%) by UV-A, whereas UV-B constitutes the remaining 5%. Although excess UV can cause diverse physiological damage to plants, the current prevailing opinion is that UV radiation represents, rather than a generic stressor, a specific regulator inducing a number of adaptive responses (Hideg et al., 2013).

Many studies have been carried out on the effects of UV radiation on grapevine, due to the importance of this crop all over the world (Anderson & Nelgen, 2020; Jackson, 2020). These studies have considered different experimental systems (cut-off filters, UV lamps, natural UV gradients, UV-A, and/or UV-B application, and so on), plant organs (mainly leaves and berries, but also flowers and stems), response variables (from gene expression to morphology, physiology, and secondary metabolism), cultivars, developmental stages, seasons, and even interactions of UV with other environmental factors and organisms (Carvalho & Amancio, 2019; Jordan, 2017; Jug & Rusjan, 2012). Thus, important background knowledge is already available for the scientific community, growers, and technicians.

In particular, studies on grapes have been more frequently carried out than those on leaves, because of their higher applicability. In this line, grapes' secondary metabolites have been the most used variable to evaluate the grapevine responses to UV radiation, due not only to their diverse physiological roles but also to their direct relationship with the quality of both grapes and wines. The most studied metabolites in this respect have been phenolic compounds and, to a lesser extent, volatile organic compounds (VOCs). Phenolic compounds have a great diversity in grapes and range from simple to highly polymerized molecules (Keller, 2020; Teixeira et al., 2013). Phenolic acids (hydroxycinnamic and hydroxybenzoic acids, and their derivatives) are simple phenols, some of which act as lignin precursors, determining the astringent and bitter mouthfeel of grapes and wines. Stilbenes, the most famous of which is resveratrol, are physiologically phytoalexins typically responding to abiotic and biotic stress, such as wounding or pathogens. The remaining phenolic compounds are flavonoids, which are characterized by the presence of the flavan nucleus in their structure. Flavonoids consist of a complex mixture of different flavanols, flavonols, and anthocyanins. Flavanols can have a monomeric or polymeric structure and are responsible for bitterness and astringency. Flavonols differ in their hydroxylation level (from trihydroxylated myricetins to dehydroxylated quercetins and monohydroxylated kaempferols, isorhamnetins, and syringetins), and can be glycosylated in different ways. They contribute to wine copigmentation with anthocyanins, and their accumulation in a number of grapevine genotypes under a great diversity of experimental conditions represents the most reliable response of grape skins to UV-B radiation (Berli et al., 2011; Carbonell-Bejerano et al., 2014; Del-Castillo-Alonso, Diago, et al., 2016, Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Martínez-Abaigar, et al., 2020; Downey et al., 2004; Koyama et al., 2012; Liu et al., 2015; Martínez-Lüscher et al., 2014; Spayd et al., 2002). Finally, anthocyanins can also be diversely substituted and glycosylated, and they are responsible for red, purple, and blue pigmentation of grapes and red wines. Many phenolic compounds can play a physiological role as UV screens and/or antioxidants against the UV excess, including in physiological disorders, such as grape sunburn (Gambetta et al., 2021). In addition, resveratrol or anthocyanins, among other metabolites, are considered healthy nutraceuticals because of their antioxidant properties, and their enhancement in grapes and wines would be relevant and desirable (Teixeira et al., 2013).

Regarding VOCs, they include a great diversity of compounds, such as alcohols, ketones, hydrocarbons, aldehydes, furans, terpenes, norisoprenoids, and fatty acids. In the plant, these compounds are involved in primary and secondary metabolic pathways, antioxidant functions, resistance to abiotic and biotic stressors, and structural roles (Gil et al., 2013; Keller, 2020). In grapes and (especially) wines, VOCs decisively contribute to the different aromas, flavors, and textures. Nevertheless, the excess of certain compounds (such as specific fatty acids or aldehydes), or their metabolic transformation during winemaking, leads to undesirable effects in wines, such as the appearance of rancid taste and smell (Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Núñez-Olivera, et al., 2020).

Most studies on the effects of UV radiation on grape metabolites have focused on individual factors due to the higher complexity of simultaneously dealing with several different factors. In addition, the results obtained have been diverse, probably due to the differences in the cultivar used and the experimental conditions applied, together with the specific responses of the different metabolites. This makes it difficult to generalize the physiological responses of grapes to UV radiation, and thus to apply the generated knowledge to improve the quality of grapes and wine. In particular, doubts still persist on key questions, such as the relative influence of UV-A and UV-B wavelengths, the effect of the developmental stage of the grape, the interannual changes, or the ecological relevance of the results obtained. Other aspects that remain underexplored are, for example, the link between metabolites and genes (Jordan, 2017; Liu et al., 2015), the effects of UV radiation on grape morphology, the relationship between grape and wine compounds (Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Martínez-Abaigar, et al., 2020; Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Núñez-Olivera, et al., 2020; Van Leeuwen et al., 2020), the influence of the cell compartmentalization of phenolic compounds (Del-Castillo-Alonso, Diago, et al., 2016), or the effects of climate change on grapes and wines (Fraga et al., 2016; Carvalho & Amancio, 2019).

In the context described, our aim was to somewhat synthesize several experimental scales on the effects of UV radiation on grapes. We used different combinations of lamps and filters to differentiate the effects of UV-A and UV-B wavelengths, and to study the effects of the enhanced UV-B levels which will probably reach the Biosphere as a consequence of ozone degradation and climate change (Bais et al., 2019). The application of enhanced UV-B may also be useful to

evaluate the options of manipulating UV in viticulture and enology. In addition, we measured variables directly related to the quality of grapes and wines (such as the contents of phenolic and volatile compounds), as well as the expression of associated genes, in three phenological stages (pea-size, veraison, and harvest) and three grape components (skin, flesh, and seeds). Finally, we considered the cell locations of the UV-absorbing compounds because this location may determine different photoprotection modalities (Agati et al., 2012) and different extractabilities in the enological process. The study was carried out using a major grapevine cultivar, given that Tempranillo is the third most used cultivar worldwide and the first world's fastest-expanding winegrape in the period 2000–2016 (Anderson & Nelgen, 2020). It occupies almost 220,000 ha globally (5% of the total), mostly in Spain. Tempranillo is also the most important cultivar in La Rioja Qualified Denomination of Origin (Spain), where the study was performed.

2 | MATERIALS AND METHODS

2.1 | Plant material, culture conditions, and experimental design

The present field experiment was conducted in the 2017 season in Logroño (La Rioja, northern Spain, 42°27′N, 2°25′W, 373 m elevation). The experiment was performed on 4-year-old *Vitis vinifera* L. cv. Tempranillo (clone 43) plants grafted onto 110R rootstock and planted in 50 L pots. An automatic drip irrigation system maintained the soil at water field capacity, and soil water content was continuously measured with a tensiometer (Watermark, Irrometer Company). The soil in the pot was slightly alkaline (pH 8.0) with a medium texture (55% sand, 29% silt, 16% clay) and 1.4% content in organic matter. Plants were kept in a good phytosanitary status throughout the experiment.

TABLE 1 Total doses (in MJ m^{-2}) of photosynthetically active radiation (PAR), UV-A, UV-B, biologically effective ultraviolet radiation (UV_{BE}) and biologically effective UV-B radiation ($UV\text{-}B_{BE}$) received by plants of *Vitis vinifera* cv. Tempranillo during the period of study (6 April–5 September 2017) under the five radiation regimes imposed in the experiment: P (photosynthetically active radiation, PAR, alone), PA (PAR + UV-A), PB (PAR + UV-B), PAB (PAR + UV-A + UV-B), and PAB↑ (PAR + UV-A + enhanced UV-B). UV_{BE} and $UV\text{-}B_{BE}$ were calculated based on the action spectra of Flint and Caldwell (2003) and Caldwell (1971), respectively.

Radiation regime	PAR	UV-A	UV-B	UV_{BE}	$UV\text{-}B_{BE}$
P	840	6.2	0.04	0.03	0.01
PA	802	73.1	0.10	0.10	0.00
PB	832	7.0	1.91	0.76	0.76
PAB	834	77.3	1.90	1.40	0.37
PAB↑	833	78.2	2.12	1.50	0.44

A completely randomized block design was set up (Figure S1). Each block consisted of a frame built with metal profiles that allowed the positioning of different radiation filters. The dimensions of each block were: 1.7 m high, 1.5 m wide, and 1 m in depth. Block orientation was N–S, and the N side was covered with a shading mesh (PE/RF 70, Rombull Ronets), preventing sunlight from affecting the plants but allowing aeration. The S, E, and W sides of the blocks, together with the top part, were covered with specific cut-off filters. Lateral filters (1.5 m wide and 1.1 m high) were placed at 45° from the vertical axis of the blocks, while top filters were placed at 15° inclination to facilitate rainwater evacuation. Each block was separated by 1.5 m to the next block and a shading mesh was placed covering the top and S side of the gaps between the blocks. A total of 18 blocks were set up and divided into five radiation regimes (Table 1, Figure 1):

- P (only photosynthetically active radiation, PAR), using XT Vitroflex 395 Solarium Incoloro (Polimertecnic), which cut off UV radiation.
- PA (PAR + UV-A), using acetate Folex 320 (Folex GmbH), which cut off UV-B and UV-C radiation. As this filter was flexible, it was complemented with an additional polymetacrylate rigid filter

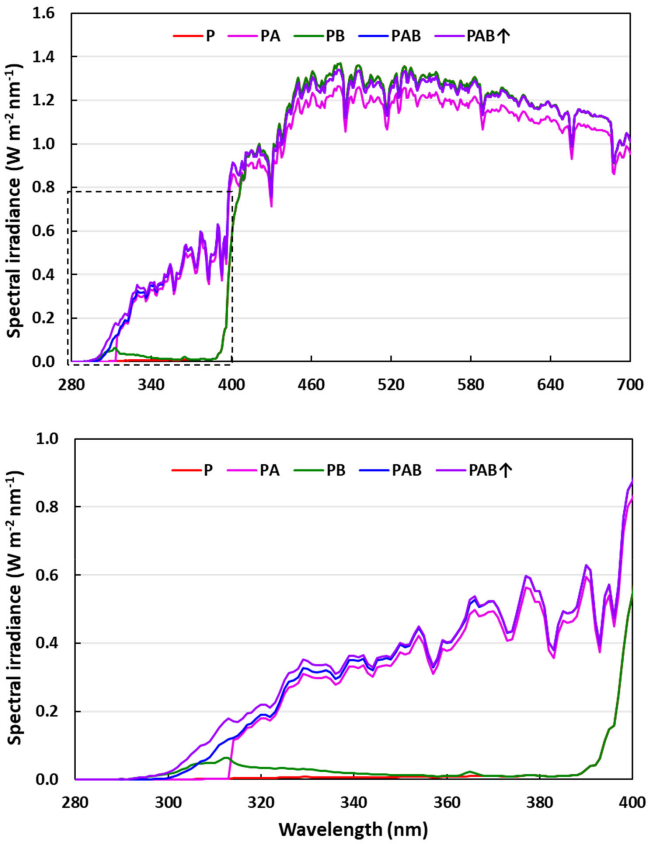

FIGURE 1 Spectral irradiances in the wavelength intervals 280–700 nm (top) and 280–400 nm (bottom), as measured in the different radiation regimes (P, PA, PB, PAB, and PAB↑) imposed in the experiment (see Table 1 and Section 2 for a full description of each regime). For wavelengths higher than 400 nm, spectral irradiances in the regimes P, PB, PAB, and PAB↑ were remarkably coincident; thus, their respective curves overlap

(PMMA XT Vitroflex 295, Polimertecnic), preventing the tear of the acetate film by adverse meteorological conditions.

- PB (PAR + UV-B), using a Vitroflex 395 filter and UV-B lamps (TL 40 W/12 UVB, Philips Lighting). Lamps were switched on during 10-min periods in the central hours of the day to provide the plants with the same UV-B that they would receive if exposed to ambient sunlight.
- PAB (PAR + UV-A + UV-B), using PMMA XT Vitroflex 295 (Polimertecnic), which cut off UV-C radiation. Among the regimes used, this was the most similar to the natural solar radiation.
- PAB↑ (PAR + UV-A + enhanced UV-B), using the same filter as in PAB and the same lamps as in PB, but providing 10% higher UV-B than that received in the PAB treatment by adjusting the time of functioning of the lamps.

Two plants were placed in each block and 3–4 replicates were established for each regime. UV-B lamps were placed in all the blocks to prevent differences in shading between the treatments. Filters were placed from 6 April (before bud break) to 5 September (harvest). Spectral irradiances under the filters were measured regularly (Macam SR9910 spectroradiometer, Macam Photometrics Ltd.) to confirm the stability of the filters. The biologically effective UV-B irradiance ($UV\text{-}B_{BE}$) and the biologically effective UV irradiance (UV_{BE}) were calculated following Caldwell (1971) and Flint and Caldwell (2003), respectively. In addition, ambient PAR, UV-A, and UV-B irradiances were continuously recorded close to the experimental plot by broad-band sensors (Skye Quantum SKP 215, SKU 420, and SKU 430, respectively, Skye Instruments Ltd.). Total doses of the different radiations received by the plants during the period of study are shown in Table 1. The air temperature outside and inside the blocks was measured using a digital thermometer.

2.2 | Grape sampling and analysis

A schematic representation of the experiment performed, including the phenological stages when grapes were collected, the grape components and fractions analyzed, and the variables measured, is shown in Figure S2. For each radiation regime and replicate, grapes were collected from the two plants around noon on sunny days in three different phenological stages: pea-size (16 June), veraison (14 July), and harvest (5 September). Grapes were immediately frozen in liquid nitrogen, transported to the laboratory, and kept at −80°C until further procedure. At harvest, grape diameter, and the fresh weight (FW) of the grape and its three components (skin, flesh, and seeds), were measured using 10 grapes per replicate. For the remaining analyses, we used 15 grapes per replicate.

Phenolic compounds were analyzed in the methanol-soluble fraction of grapes (skin and flesh together, discarding seeds) in three phenological stages (pea-size, veraison, and harvest). In addition, at harvest, phenolic compounds were separately analyzed in each of the three grape components (skin, flesh, and seeds), differentiating the methanol-soluble and methanol-insoluble fractions, which are presumably mainly located in the vacuoles and bound to the cell walls, respectively (Schnitzler et al., 1996). Methodological details of extraction and analysis can be found in Del-Castillo-Alonso et al. (2015). In brief, for extraction of phenolic compounds from the ensemble of skin and flesh, seeds were retired from the frozen grapes, and the remaining material was ground together in liquid nitrogen in a mortar to obtain a homogeneous powder, which was stored at −80°C. For each subsequent extraction, 200 mg FW and 2 ml of extractant were used. For separate extraction from each of the three grape components, frozen berries were allowed to partially thaw, skin, flesh, and seeds were separated and immediately submerged in liquid nitrogen, lyophilized, and ground (UltraTurrax T25 Basic homogenizer, IKA Labortechnik). For subsequent extraction, 50 mg dry weight (DW) of skins (and 4 ml of extractant), 200 of flesh, and 80 of seeds (and 2 ml of extractant in these two last cases) were used. Methanol:water:7 M HCl (70:29:1 v:v:v) was used for extraction (24 h at 4°C in the dark). To differentially extract the methanol-soluble and methanol-insoluble phenolic compounds, the extract was centrifuged at 6000*g* for 15 min and the supernatant and pellet were considered the source of soluble and insoluble compounds, respectively. The pellet was then hydrolyzed with 1 ml of 1 M NaOH for 3 h in a water bath at 80°C. Afterwards, 1 ml of HCl (5.6 N) was added and the sample was rinsed three times with ethyl acetate. The supernatant obtained from the rinsing process was then allowed to evaporate (Büchi R-200, Büchi Labortechnik) at 40°C and the remaining material was resuspended in absolute methanol up to a final volume of 1 ml (for flesh samples) and 2 ml (for the remaining samples).

In both soluble and insoluble fractions of the respective grape components, the bulk levels of UV-absorbing compounds (UVAC) were measured as the area under the absorbance curve (AUC) in the wavelength intervals 280–315 nm ($AUC_{280-315}$) and 280–400 nm ($AUC_{280-400}$), respectively, covering the UV-B and UV ranges (Perkin-Elmer λ 35 spectrophotometer). Soluble and insoluble individual phenolic compounds were analyzed in the respective grape components by UPLC/LC–MS (Waters Acquity UPLC system, Waters Corp.). The chromatographic conditions were those previously reported and specifically developed for grape flavonoids analysis (Del-Castillo-Alonso, Diago, et al., 2016; González-Hernández et al., 2014). Solvents were: A, water/formic acid (0.1%), and B, acetonitrile with 0.1% formic acid. The gradient program employed was: 0–7 min, 99.5–80% A; 7–9 min, 80–50% A; 9–11.7 min, 50–0% A; 11.7–15 min, 0–99.5% A. The UPLC system was coupled to a micrOTOF-QII-ESI-MS/MS high-resolution mass spectrometer (Bruker Daltonics) controlled by the Bruker Daltonics Data Analysis software. The electrospray (ESI) source was operated in positive or negative mode, in the range of *m/z* 120 and 1505. The optimized conditions of the ESI source were as follows: capillary potential 4 kV, ESI source temperature 180°C, desolvation temperature 200°C, gas flow 9 L min^{-1}; nebulizer gas 3.5 bar and 25°C. LC–MS and MS/MS were performed operating in continuum mode. The spectra were acquired at two scans per second. The fragmentor voltage for MS/MS acquisition mode was 35 eV. The identity assignation of compounds was carried out by combining different information: retention time, UV–vis data, MS spectra, and MS/MS fragmentation patterns of peaks of available pure compounds and/or published in previous studies (González-Hernández et al., 2014). For

quantification, DAD chromatograms were extracted at 520 nm for anthocyanins and 324 nm for the other compounds; the calibration curves of the respective standards were used. In absence of commercial standards, compounds with the same chromophore were used: stilbenes using *t*-resveratrol (Sigma-Aldrich); flavanols using catechin, epigallocatechin (Sigma-Aldrich), and procyanidin B1 (Fluka); flavonols using kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucuronide (Fluka), myricetin, quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucopyranoside, quercetin-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, and syringetin-3-*O*-glucoside (Sigma-Aldrich); hydroxycinnamic acids and its derivatives using caffeic, *p*-coumaric, and ferulic acids (Sigma-Aldrich); hydroxybenzoic acids using gallic acid, syringic acid (Sigma-Aldrich), and protocatechuic acid (Fluka); and anthocyanins using malvidin-3-*O*-glucoside (Extrasynthese).

Total phenols (Folin–Ciocalteu reagent) and total flavonoids were determined as in Farhadi et al. (2016). The antioxidant capacity of the different grape components was measured by generating the radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) and was expressed in terms of Trolox equivalent (TE) antioxidant capacity (Del-Castillo-Alonso, Castagna, et al., 2016).

VOCs were analyzed only at harvest in skin and flesh together, following Arroyo et al. (2018). For extraction, 0.5 g samples of frozen grape powder were used. Samples were heated at 30°C for 10 min, and 1.5 ml of a saturated $CaCl_2$ solution and 300 μl of EDTA 500 mM (pH 7.5) were added. After gentle mixing, 1.5 ml of the resulting mixture were transferred to a 10 ml headspace screw cap vial and subjected to headspace solid-phase microextraction (HS-SPME). A 65 μM PDMS/DVB fiber (Supelco) was used for the analysis. Pre-incubation and extraction were performed at 50°C for 10 and 20 min, respectively. Desorption was performed for 1 min at 250°C in splitless mode. VOCs trapped on the fiber were analyzed by GC–MS using an autosampler COMBI PAL CTC Analytics, a 6890 N GC Agilent Technologies and a 5975B Inert XL MSD Agilent, equipped with an Agilent J&W Scientific DB-5 fused silica capillary column (5%-phenyl-95%-dimethylpolysiloxane as stationary phase, 60 m length, 0.25 mm i.d., and 1 μm thickness film). Oven temperature conditions were 40°C for 2 min, 5°C min^{-1} ramp-up to 250°C, and then held isothermally at 250°C for 5 min. Helium was used as the carrier gas at 1.4 ml min^{-1} constant flow. Mass/z detection was obtained by an Agilent mass spectrometer operating in the EI mode (ionization energy of 70 eV; source temperature 230°C). Data acquisition was performed in scanning mode (mass range *m/z* 35–220). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC–MS (Agilent). Compound identification was based on the comparison between the MS for each putative compound with those of the NIST 2005 Mass Spectral library, as well as the match to a GC retention time and Mass Spectra custom library generated using commercially available compounds.

The analysis of gene expression was carried out in grapes of the three phenological stages: pea-size, veraison, and harvest. Gene selection (Table S1) was made on the basis of UV sensitivity and physiological importance in the metabolic pathways of phenolic compounds and terpenoids, as well as in UV signaling processes (Carbonell-Bejerano et al., 2014; Liu et al., 2015, 2018; Loyola et al., 2016). In particular, *VvCHS1* and *VvFLS4* were chosen because of their solid response to UV radiation and expression in every berry developmental stage, *VvHY5* due to its participation in the UV-B signaling pathway and significant induction by UV in grapes, *VvELIP1* because of induction by UV-B, and *VvbHLH* due to upregulation in grapes under UV radiation. Frozen grapes without seeds were crushed in a mortar with liquid nitrogen. The resulting powder was stored at −80°C until use. RNA was extracted according to Zeng and Yang (2002), and RNA from each sample was treated with DNase (RNasefree) according to the manufacturer's instructions (TURBO ADN-free Kit, Invitrogen) to eliminate contamination with genomic DNA. The concentration of RNA was quantified by a Nanodrop 2000c spectrophotometer (ThermoScientific). Final RNA purification was carried out using the Spektrum Plant Total RNA kit (Sigma-Aldrich) according to standard protocols. cDNA was synthesized from 2 to 4 μg of the treated RNA samples using the NZY First-Strand cDNA Synthesis kit (NZYTech). Oligonucleotide primers used in real-time (RT) qPCR analysis were taken from previous studies or designed by the Primer3 program (https://primer3.ut.ee/), and synthesized from Invitrogen (Table S1). Transcript levels were measured by quantitative RT-PCR using an ICycler Bio-Rad instrument and iTaq Universal SYBR Green Supermix (Bio-Rad). Triplicates of PCR reactions of each sample were performed, and relative gene expression levels were calculated according to the $2-\Delta\Delta CT$ methods using *ACTIN* as a control gene to normalize individual gene expression.

2.3 | Statistical analysis

The global effects of the radiation regime and the grape phenological stage on phenolic composition, antioxidant activity, and gene expression were tested using a two-way analysis of variance (ANOVA), once proved that the data met the assumptions of normality (Shapiro–

TABLE 2 Grape morphometric variables in the five radiation regimes (P, PA, PAB, PB, and PAB↑) imposed in the experiment (see Table 1).

Variable	P	PA	PB	PAB	PAB↑	Statistical significance
Berry fresh weight (FW) (mg)	1404 ± 52^{a}	1465 ± 44^{a}	1759 ± 32^{b}	1578 ± 81^{ab}	1668 ± 69^{ab}	**
Diameter (cm)	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.4 ± 0.1^{b}	1.2 ± 0.1^{ab}	1.4 ± 0.1^{b}	**
Skin FW (mg)	218 ± 9	233 ± 10	273 ± 21	241 ± 16	267 ± 9	ns
Flesh FW (mg)	1085 ± 41^{a}	1129 ± 38^{a}	1377 ± 16^{b}	1237 ± 60^{ab}	1294 ± 57^{ab}	**
Seed FW (mg)	101 ± 64	103 ± 1	109 ± 2	100 ± 4	106 ± 2	ns

Note: For each variable, the statistical significance of a one-way ANOVA test is shown, and different letters mean significant differences between radiation regimes (Tukey's test). Means ± SE are shown ($n = 10$). **$P<0.01$; ns, not significant.

Wilks's test) and homoscedasticity (Levene's test). In addition, one-way ANOVA was applied (1) to test the global effect of the radiation regime for each phenological stage; and (2) to test the global effect of radiation regime on the variables measured only at harvest (grape morphology, phenolic compounds, and VOCs), testing separately the three grape components (skin, flesh, and seeds). In the case of significant differences, means were then compared by Tukey's test. Pearson correlation coefficient (r) was used to examine the relationships between selected variables. The grape samples were ordinated through two different principal components analysis (PCA), using both the individual phenolic compounds contents (as measured in the five radiation regimes and the three grape phenological stages: Table S2), and the contents of phenolic and volatile compounds grouped per families (as measured in the five radiation regimes at harvest: Tables S2 and S4). In both cases, the biological replicates of the respective radiation regimes were used for ordination. All the statistical procedures were performed with SPSS 24.0 for Windows (SPSS Inc.).

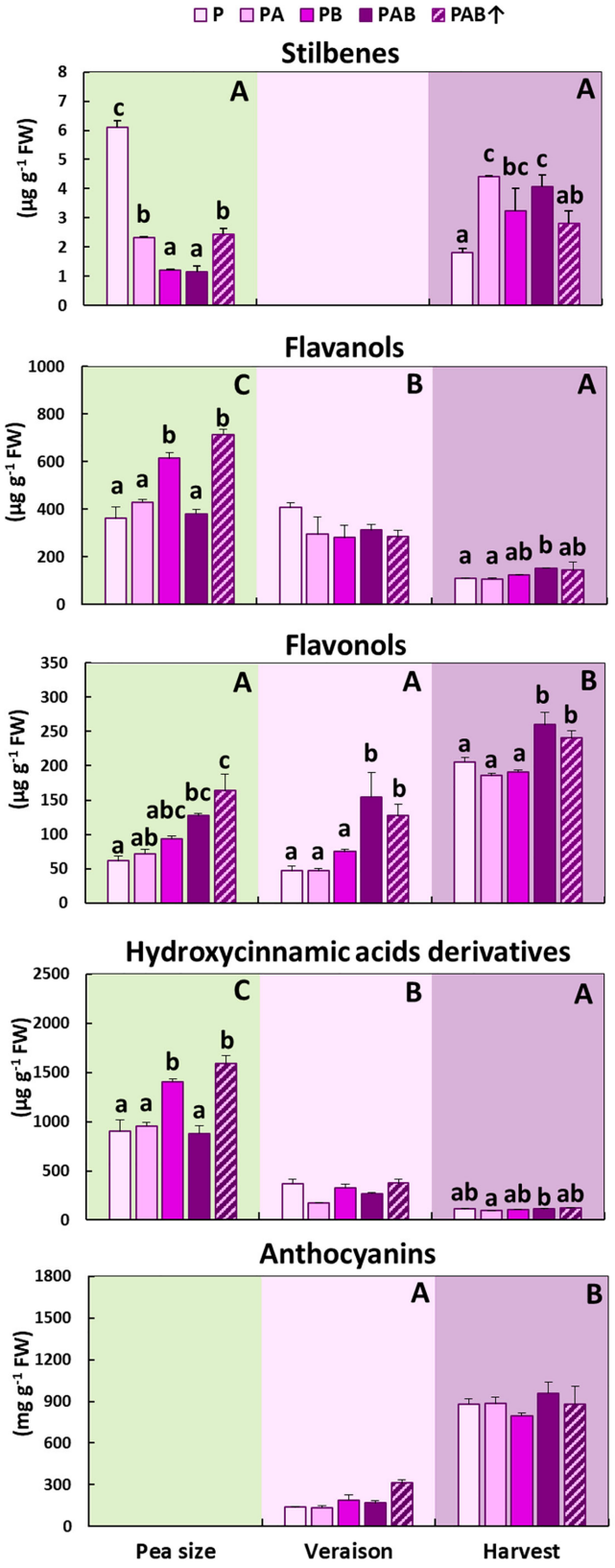

FIGURE 2 Contents of phenolic compounds in three grape phenological stages (pea-size, veraison, and harvest) and five radiation regimes (P, PA, PB, PAB, and PAB↑: see Table 1), measured in the methanol-soluble fraction of the ensemble of skin and flesh. Different capital letters mean significant differences between phenological stages, and different lower-case letters between radiation regimes for each phenological stage (results of post-hoc Tukey's tests after a two-way analysis of variance (ANOVA) test using phenological stage and radiation regime as main factors). Means ± SE are shown ($n = 3$ replicates, using 15 berries from two plants for each replicate). FW, fresh weight

3 | RESULTS

3.1 | Radiation and temperature conditions

The PAR doses received by the plants during the experiment were similar in all the regimes considered (Table 1, Figure 1). UV-A dose in P and PB regimes was around 9% of that in PA, PAB, and PAB↑ regimes. UV-B dose was relatively high and similar in PB and PAB regimes, and around 10% higher in PAB↑, whereas plants in P and PA only received around 2% of that in PB and PAB. Thus, the P regime was not totally deprived of UV-A and UV-B radiation, and PA and PB regimes were not totally deprived of UV-B and UV-A, respectively. In addition, UV_{BE} doses (and also UV-B_{BE} doses) were different between PB and PAB regimes, due to the facts that (1) the UV-B source was different in PB (lamps) and PAB (sunlight), and different sources imply different spectral irradiances (in this sense, the PAB regime was the most similar to natural solar radiation among the regimes used); and (2) the action spectra applied to calculate UV_{BE} and UV-B_{BE} doses were different (Flint & Caldwell, 2003 and Caldwell, 1971, respectively), and only the first action spectrum takes into account the UV-A wavelengths. Consequently, although UV-B dose was similar in PB and PAB regimes, UV_{BE} dose was around 50% lower in PB than in PAB, whereas UV-B_{BE} dose was around 2-fold higher in PB than in PAB.

Air temperatures recorded outside the blocks during the period of study varied between −0.3°C and 36.5°C. The means of the

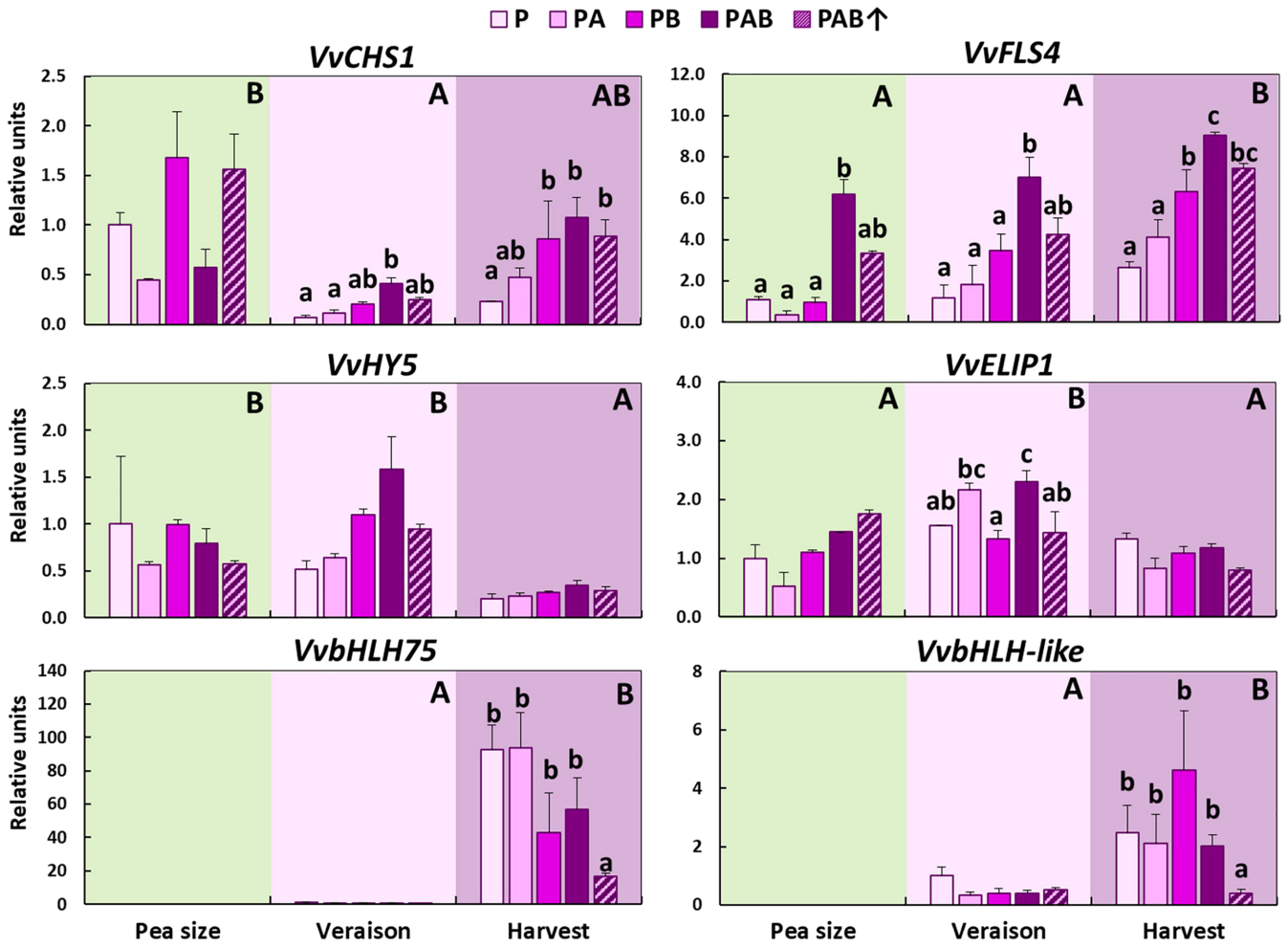

FIGURE 3 Relative expression of the specified genes (chalcone synthase, flavonol synthase, early light-induced protein, and HY5, bHLH75, and bHLH-like transcription factors: see Table S1) in three grape phenological stages (pea-size, veraison, and harvest) and five radiation regimes (P, PA, PB, PAB, and PAB↑: see Table 1), measured in the ensemble of skin and flesh. Gene expression is shown in relative units, normalized using the expression of P samples in the pea-size stage (or in absence of it, in veraison). Different capital letters mean significant differences between phenological stages, and different lower-case letters between radiation regimes for each phenological stage (results of post-hoc Tukey's tests after a two-way analysis of variance (ANOVA) test using phenological stage and radiation regime as main factors). Means ± SE are shown ($n = 3$ replicates, using 15 berries from two plants for each replicate)

TABLE 3 Relative abundance (percentage) of the main groups of methanol-soluble and methanol-insoluble phenolic compounds in the three grape components (skin, flesh, and seeds) at harvest.

	Soluble compounds			Insoluble compounds		
Compounds	**Skin**	**Flesh**	**Seed**	**Skin**	**Flesh**	**Seed**
Resveratrols	100	–	-	–	–	–
Catechins	20.7	1.1	76.7	–	–	1.5
Procyanidins	35.9	1.7	62.4	–	–	–
Myricetins	100	–	–	–	–	–
Quercetins	96.8	0.4	2.8	–	–	–
Kaempferols	100	–	–	–	–	–
Isorhamnetins	100	–	–	–	–	–
Syringetins	100	–	–	–	–	–
Hydroxybenzoic acids	–	–	–	47.5	4.8	47.7
Hydroxycinnamic acids	–	–	–	95.4	0.8	3.8
Hydroxycinnamic acid derivatives	63.5	20.0	16.5	–	–	–
Anthocyanins	100	–	–	–	–	–

minimum, mean, and maximum temperatures were, respectively, 13.3°C, 19.1°C, and 26.2°C. Air temperature inside the blocks was 4.0 ± 0.6°C higher than outside. In addition, in the blocks of the treatments using lamps (PB and PAB↑), air temperature was 1.0 ± 0.1°C higher than that found in the remaining blocks.

3.2 | Grape morphology

The radiation regime significantly influenced grape and flesh FW, and grape diameter, but not skin and seed FW (Table 2). Grape morphology was similar in P and PA samples, but grape and flesh FW, together

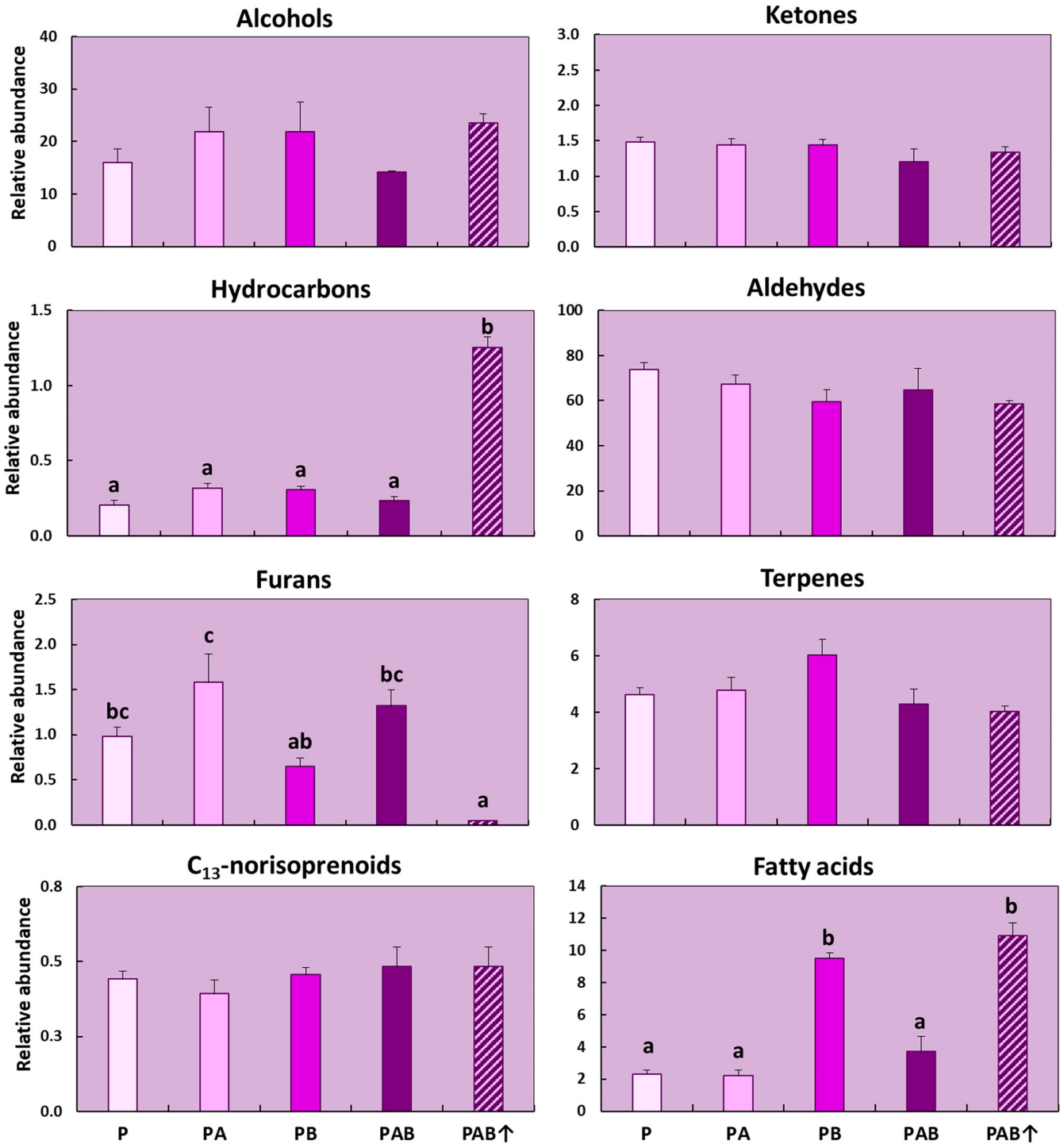

FIGURE 4 Relative abundance (percentages) of families of volatile organic compounds (VOCs) in the ensemble of skin and flesh in the five radiation regimes used (P, PA, PB, PAB, and PAB↑: see Table 1) at harvest. For each variable, different letters mean significant differences between radiation regimes (post-hoc Tukey's test after a one-way analysis of variance (ANOVA) test using radiation regime as main factor). Means ± SE are shown ($n = 3$ replicates, using 15 berries from two plants for each replicate)

with grape diameter, were significantly higher in PB than in P and PA samples. There was a similar trend, although mostly nonsignificant, in PAB and PAB↑ samples.

3.3 | Phenolic composition and associated genes

Total phenols and total flavonoids responded to the radiation regime, but only at the pea-size stage (Table S2). P samples showed lower values than the remaining samples, but differences were significant only in PAB↑ samples (and also in PB samples, but only for total phenols). The bulk levels of UVAC, and antioxidant capacity did not respond to the radiation regime, whereas the phenolic families and individual compounds showed diverse responses (Figure 2, Table S2). The most consistent responses were those of flavonols, which increased in PAB and PAB↑ samples at veraison and harvest. UV-A alone did not cause any effect on flavonols, but contributed to a higher increase in PAB and PAB↑ samples in comparison to PB samples in both developmental stages. Among flavonols, quercetins showed the strongest response to UV, either at pea-size stage or at harvest. Flavanols and hydroxycinnamic acid derivatives increased in PB and PAB↑ samples, but only at pea-size stage. Stilbenes showed opposite responses in pea-size and harvest stages, with P samples showing the highest and the lowest contents, respectively. Anthocyanins showed no significant response to radiation regimes.

VvFLS4 was the gene most consistently responding to UV radiation. It was upregulated by UV-B alone (PB samples) at harvest and by the combination of UV-B and UV-A (PAB and/or PAB↑ samples) at every phenological stage (Figure 3). *VvCHS1* also showed a higher expression in PAB samples in comparison with P samples at veraison, and in PB, PAB, and PAB↑ samples at harvest. There was a significant linear regression between the content of flavonols and the expression

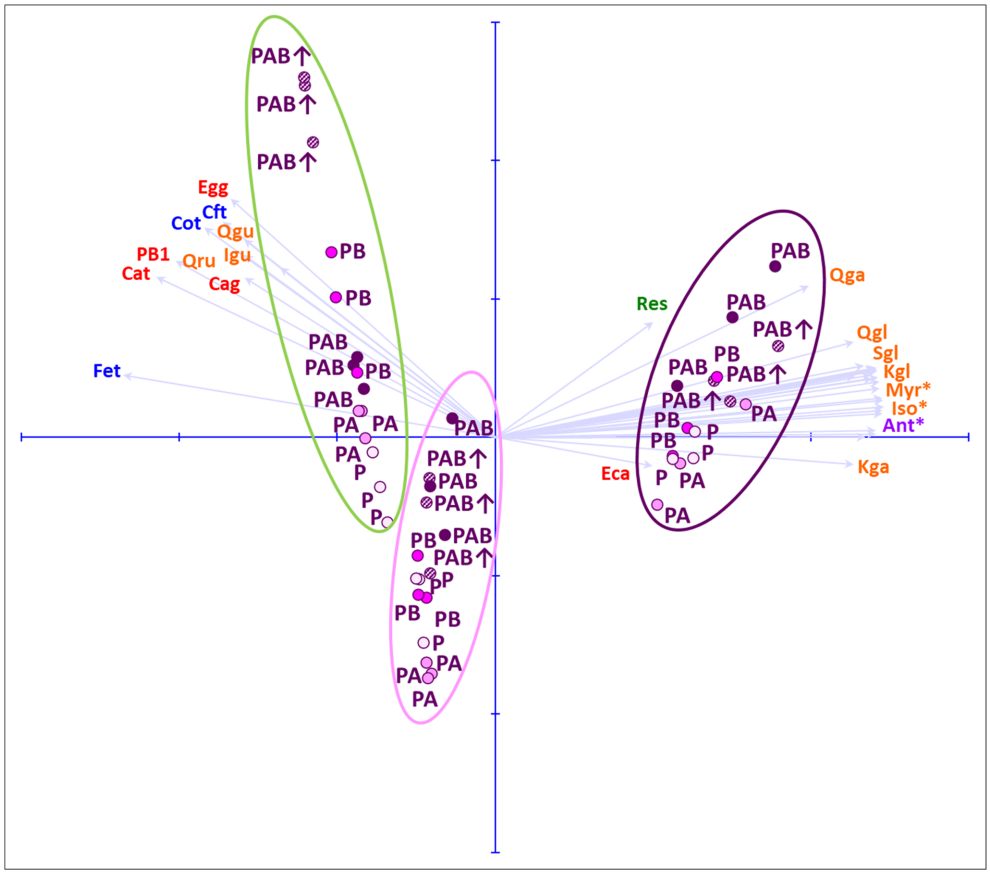

FIGURE 5 Ordination, through principal components analysis (PCA), of grape samples exposed to the five different radiation regimes applied in the experiment (P, PA, PB, PAB, and PAB↑: see Table 1), based on the content of individual phenolic compounds as measured in each regime in three different phenological stages (pea-size, green oval; veraison, pink oval; harvest, purple oval). Biological replicates of treatments and phenological stages were used for ordination. Significant loading factors are shown as arrows. Ant*, all the anthocyanins; Cag, catechin gallate; Cat, catechin; Cft, caffeoyl tartaric acid; Cot, coumaroyl tartaric acid; Eca, epicatechin; Egg, epigallocatechin gallate; Fet, feruloyl tartaric acid; Igu, isorhamnetin-3-*O*-glucuronide; Iso*, isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-galactoside; Kga, kaempferol-3-*O*-galactoside; Kgl, kaempferol-3-*O*-glucoside; Myr*, all the myricetins; PB1, procyanidin B1; Qga, quercetin-3-*O*-galactoside; Qgl, quercetin-3-*O*-glucoside; Qgu, quercetin-3-*O*-glucuronide; Qru, quercetin-3-*O*-rutinoside; res, resveratrol; Sgl, syringetin-3-*O*-glucoside. The different phenolic families are shown in different colors: Stilbenes in green, flavanols in red, flavonols in orange, hydroxycinnamic acid derivatives in blue, and anthocyanins in purple. Axis I is the horizontal one, and axis II is the vertical one

of *VvFLS4* and *VvCHS1* genes, as well as between the content of anthocyanins and *VvCHS1* (Figure S3). The remaining genes did not respond consistently to UV radiation at any phenological stage.

3.4 | Phenological stage

Most phenolic variables, together with antioxidant capacity, showed significant differences between phenological stages (Figure 2, Table S2). The highest diversity and content of phenolic compounds were found at harvest, with 37 compounds (15 anthocyanins, 13 flavonols, five flavanols, three hydroxycinnamic acids, and one stilbene). The only stilbene was found at pea-size and harvest, but not at veraison. Flavanols and hydroxycinnamic acids decreased along the season. Total flavonols showed similar contents at pea-size and veraison stages and significantly increased at harvest. Among flavonols, quercetins, and one isorhamnetin appeared at pea-size stage and then followed different patterns (two quercetins and the isorhamnetin decreased along the season, whereas the other two quercetins increased). Kaempferols and one myricetin appeared in veraison and significantly increased at harvest, whereas other myricetins, isorhamnetins, and syringetin appeared only at harvest. Anthocyanins were not detected at pea-size stage and then increased from veraison to harvest. *VvFLS4* and the bHLH transcription factors showed the highest levels of expression at harvest, whereas HY5 showed rather the contrary (Figure 3). At veraison, *VvCHS1* showed the lowest expression and *VvELIP1* the highest.

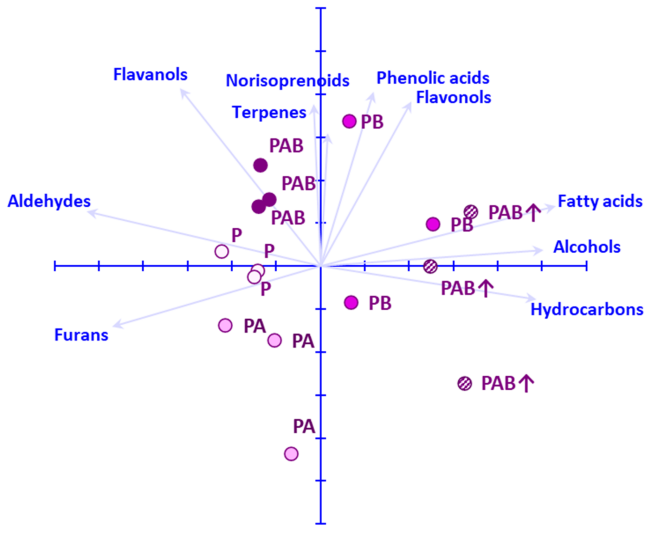

FIGURE 6 Ordination, through PCA, of the grape samples exposed to the five different radiation regimes applied in the experiment (P, PA, PB, PAB, and PAB↑: see Table 1), based on their contents of phenolic and volatile compounds (grouped per families) as measured in each regime at harvest. Biological replicates ($n = 3$) of treatments were used for ordination. Significant loading factors are shown as arrows. Axis I is the horizontal one, and axis II is the vertical one

3.5 | Grape components and cell fractions

A total of 44 phenolic compounds were identified in skins, 16 in flesh, and 15 in seeds (Table S3). The highest levels of total phenols, total flavonoids, and UV-absorbing compounds, together with the highest antioxidant capacities, were found in skins, followed by seeds (-Figure S4). This occurred in both the methanol-soluble and—insoluble fractions. Flesh showed the lowest values of all the variables.

Most compounds were mainly (or only) located in the skins, except catechins and procyanidins, which were predominantly found in the seeds (Table 3, Table S3). Only minor amounts of specific compounds were found in the flesh, with the exception of hydroxycinnamic acid derivatives (20%). The insoluble fraction contained the totality of hydroxybenzoic and hydroxycinnamic acids. Hydroxycinnamic acids were mainly located in the skins, while hydroxybenzoic acids were equally distributed between skins and seeds. The remaining compounds were only found in the soluble fraction, except a small proportion of catechins.

The skin was the grape component showing the strongest response to the radiation regime, given that skin flavanols, and flavonols from the methanol-soluble fraction, together with total phenols and the bulk level of UVACs, were significantly affected by radiation (-Table S3, Figure S4). Flavanols did not show a consistent response to UV radiation, whereas some kaempferols and quercetins, as well as total phenols and UVACs, increased under UV-B and/or UV-A radiation. Anthocyanins and hydroxycinnamic acid derivatives from skins, and most compounds from the soluble fraction of flesh and seeds, did not respond to the radiation regime. The compounds found in the methanol-insoluble fraction were almost completely insensitive to UV radiation.

3.6 | VOCs

A total of 57 VOCs were identified: 14 aldehydes, 13 alcohols, 10 terpenes, six fatty acids, five hydrocarbons, four ketones, three C_{13}-norisoprenoids, and two furans (Table S4). VOCs response to UV radiation was slight (Figure 4, Table S4). Only one hydrocarbon (heptane,2,2,4,6,6-pentamethyl) increased in PAB↑ samples, while four fatty acids (2-ethylhexanoic, heptanoic, octanoic, and nonanoic acids) increased in both PB and PAB↑ samples. On the other hand, furans strongly decreased in PAB↑ samples.

3.7 | Synthesizing the effects of UV radiation on grapes

In the PCA performed using the individual phenolic compounds of the grapes in the three phenological stages (Figure 5), the accumulated variance by the first two axes was 87% (77% for axis I, and 10% for axis II). Samples were clearly ordinated by their phenological stage. The ordination of the different radiation regimes within each stage was more or less clear, depending on the stage. Pea-size samples were

placed towards the negative part of axis I, mainly due to their higher contents of flavanols and hydroxycinnamic acids in comparison with the other stages, together with the lack of anthocyanins and the presence of three flavonols synthesized at pea-size stage and then decreasing (isorhamnetin-3-*O*-glucuronide, quercetin-3-*O*-glucuronide, and quercetin-3-*O*-rutinoside). Veraison and, especially, harvest samples were displaced to the less negative or positive parts of axis I, because of their higher contents of anthocyanins and most flavonols (those synthesized at veraison or harvest, or at pea-size stage and then increasing). In addition, veraison and harvest samples showed lower contents of flavanols and hydroxycinnamic acids. Within the pea-size stage, there was a clear distinction between the radiation regimes, particularly between PAB↑ and the remaining samples. PB samples were the closest to PAB↑ samples, and then PAB, PA, and P samples, showing a progressively lower influence of UV radiation. Again, flavanols, hydroxycinnamic acids, and some flavonols, were responsible for this ordination because they increased in PAB↑ and, to a lesser extent, PB samples. At veraison, there were less differences between the samples than at pea-size stage. In addition, P, PA, and PB samples were somewhat intermixed, whereas PAB and PAB↑ samples formed a more or less differentiated second group. This relatively ambiguous ordination probably reflects the different increasing (flavonols, anthocyanins) or decreasing (flavanols, hydroxycinnamic acids) trends shown by phenolic compounds in this intermediate developmental stage. Finally, at harvest, the radiation regime distribution was as compact as at veraison, and some mixing persisted. Nevertheless, PAB and PAB↑ samples were separated from the remaining samples, probably due to their higher flavonol contents. In conclusion, the combination of UV-B and UV-A caused stronger effects than UV-B alone on grape phenolic composition, while UV-A alone showed similar responses to PAR. These effects were modulated by the grape developmental stage.

For the second PCA (Figure 6), we used the groups of phenolic and volatile compounds measured in the five radiation regimes at harvest. The accumulated variance by the first two axes was 54% (34% for axis I, and 20% for axis II). Despite the radiation regimes were mostly clearly separated, the relatively low variances accumulated indicated that the factors responsible for the ordination were relatively weak, in line with the slight influence of UV radiation on VOCs.

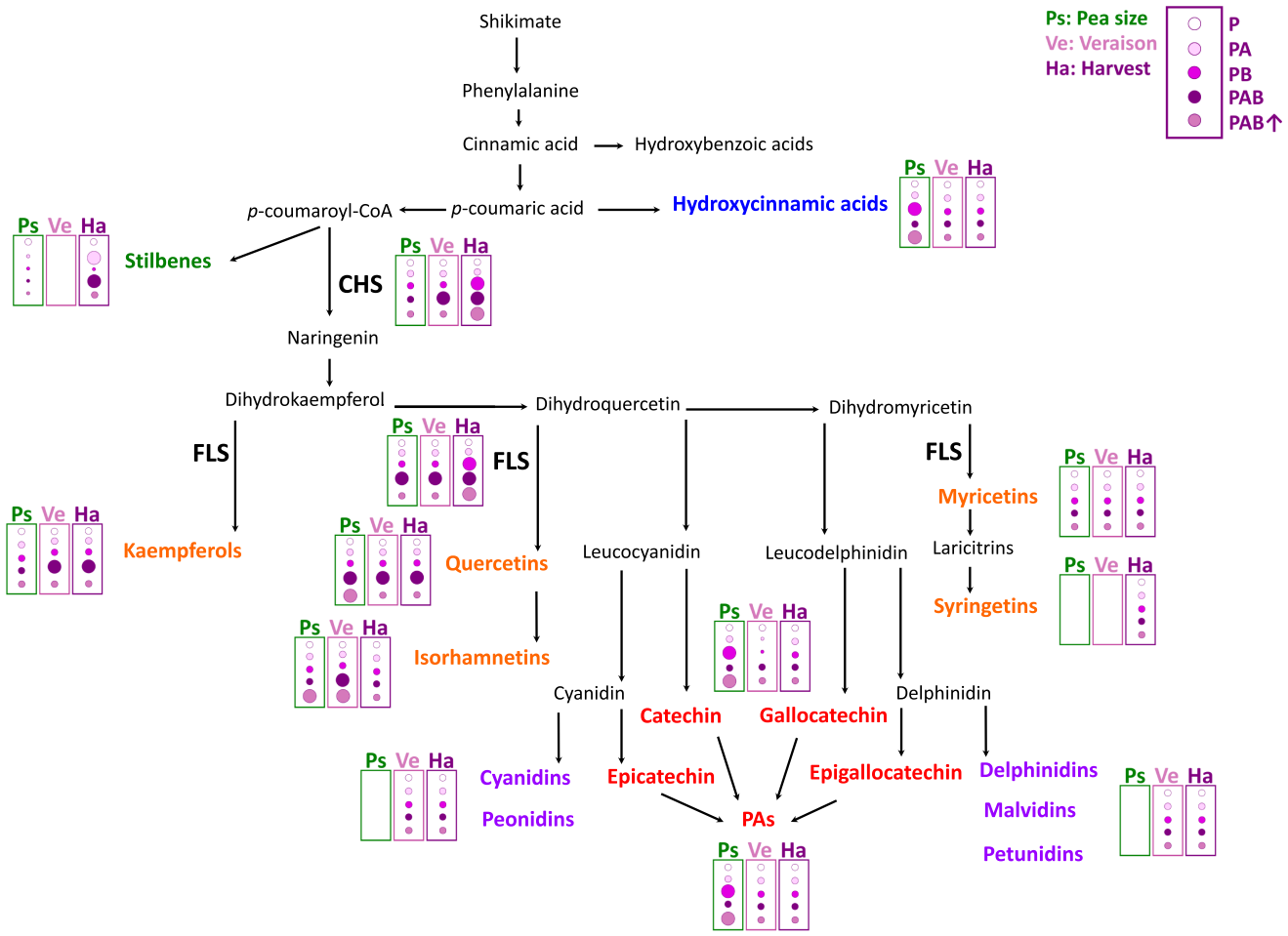

FIGURE 7 Simplified pathway of phenolic compounds synthesis showing the influence of the grape phenological stage (pea-size, veraison, and harvest) and the radiation regime applied (P, PA, PB, PAB, and PAB↑: see Table 1) on compounds (grouped by families) and genes. For each phenological stage, compound type, and gene, the influence of the radiation regime is indicated by circles of different sizes, using values in P regime as the unit. The different phenolic families are shown in different colors: Stilbenes in green, flavanols in red, flavonols in orange, hydroxycinnamic acid derivatives in blue, and anthocyanins in purple. CHS, chalcone synthase. FLS, flavonol synthase

PAB↑ samples were placed to the most positive part of axis I, based on their higher contents of fatty acids and hydrocarbons and their lower contents of furans. PB samples were also placed to the positive part of axis I, mainly due to their high contents of fatty acids. P, PA, and PAB samples were located to the slightly negative part of axis I, because of their higher contents of furans and lower contents of fatty acids. These samples were distributed along axis II, with PAB samples towards the positive part, due to their higher contents of flavanols, flavonols, and hydroxycinnamic acids, in comparison with P and PA samples.

Figure 7 shows a synthetic picture of the effects of the different treatments applied on the grape phenolic composition and associated genes, as influenced by the phenological stage.

4 | DISCUSSION

In the present study, we have evaluated the effects of UV radiation on Tempranillo grapes considering different response variables (morphology, phenolic composition and associated genes, antioxidant capacity, and VOCs) as influenced by diverse factors: UV band and level, grape phenological stage (pea-size, veraison, and harvest), grape components (skin, flesh, and seeds), and cell location of UVACs based on their methanol solubility.

4.1 | Grape morphology

Grape diameter, together with grape and flesh FW, increased in PB samples and showed similar (although mostly nonsignificant) trends in PAB and PAB↑ samples. In this regard, previous comparative results are diverse. Contrary to our results, Berli et al. (2011, 2015) found larger and heavier grapes when ambient UV-B was excluded, using Malbec cultivar grown at high elevations. However, also using Malbec, Alonso et al. (2016) found no effect of ambient UV-B on berry FW in well-watered plants. Using Tempranillo grapes under close-to-ambient UV-B provided by lamps, Martínez-Lüscher et al. (2014) did not find significant changes in diameter or weight, but Martínez-Lüscher et al. (2016) found a weight loss in UV-B-treated plants under similar experimental conditions. Clearly, further research is needed to explain these different results, which could initially be attributed to differences in the genotype used and/or the experimental conditions applied. Nevertheless, our results could be partially explained by (1) the higher (although non significantly) compactness of the skin in PB and PAB↑ samples, which could have reduced berry water loss, and/or (2) the slightly higher temperatures recorded inside PB and PAB↑ blocks (see Section 3.1), which could have promoted a greater growth (Keller, 2020).

4.2 | Phenolic composition and associated genes

The increase in flavonol content and the upregulation of *VvFLS4* and *VvCHS1* genes under the combination of UV-B and UV-A radiation (PAB and PAB↑ samples) (Figure 7) was expected. Similar effects were previously found in several grapevine cultivars, including Tempranillo, under diverse experimental designs (Berli et al., 2011; Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Martínez-Abaigar, et al., 2020; Jordan, 2017; Kolb et al., 2003; Liu et al., 2018; Martínez-Lüscher et al., 2016). Quercetins were the flavonols showing the strongest response to UV, and this response depended on the specific quercetin and the developmental stage (pea-size or harvest). Flavonols, as other phenolic compounds, play a role as antioxidants and UV-screens in plants (Agati et al., 2020); in addition, they contribute to grape and wine quality through color stabilization (Blancquaert et al., 2019) and are potentially healthy nutraceuticals (Pezzuto, 2016). Thus, the confirmation of the concomitant increase of both genes and metabolites in grapes under UV radiation may open new options to manage grapevine for a better quality of grapes and the resulting wines.

Anthocyanins did not show significant differences between radiation regimes, probably because they are more reactive to the interaction of PAR and temperature than to UV (Cortell & Kennedy, 2006; Del-Castillo-Alonso, Diago, et al., 2016; Mori et al., 2007). Regarding stilbenes, these phytoalexins respond to biotic and abiotic stress in general, including air pollution, wounding, and pathogens (Keller, 2020), whereas the effect of UV radiation may be more diffuse (Del-Castillo-Alonso et al., 2015; Del-Castillo-Alonso, Diago, et al., 2016; Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Núñez-Olivera, et al., 2020). Thus, in our study, differences in stilbene responses between pea-size stage and harvest would rather reflect the influence of uncontrolled factors than the effect of UV radiation, or a combination of both factors. In addition, the absence of stilbenes in veraison could be due to the accumulation of anthocyanins at this stage (Figures 2 and 7), given that stilbene synthase and chalcone synthase compete for the same substrates (Keller, 2020).

Most genes and transcription factors considered in the present study did not seem to be clearly regulated by UV radiation. This was strange for HY5 and bHLH transcription factors, which are involved in many abiotic stress responses, as well as in flavonol and anthocyanin biosynthesis (Jordan, 2017; Loyola et al., 2016). The lack of results was also surprising regarding *EARLY LIGHT INDUCED PROTEINS* (*ELIP*), which are induced by low UV in a UVR8- and HY5-dependent manner in Arabidopsis (Brown & Jenkins, 2008). Probably, the fact that we evaluated gene expression under field conditions, where variability is much higher than under controlled conditions, obscured the differences between treatments.

A realistic 10% UV-B enhancement (as predicted from global change models: Bais et al., 2019) was applied in PAB↑ samples, and the results obtained were mostly similar to the ones obtained with PAB. Thus, this enhancement was well-tolerated by grapes, which can be related to the Mediterranean origin of the cultivar used (Ibáñez et al., 2012) and its inherent adaptation to the relatively high UV-B levels typical of Mediterranean climate. Despite the similar effects caused, PAB and PAB↑ samples were separated through PCA, although only at pea-size stage (Figure 5). This was due to the contents of flavanols and hydroxycinnamic acid derivatives increasing at pea-size stage in PB and PAB↑ samples, but not in PAB samples. This

could be explained because UV-B radiation was provided, in both PB and PAB↑ treatments, by UV-B lamps, whose UV spectrum and pattern of application were different from those applied in PAB treatment (natural solar radiation). The bulk levels of UVACs did not respond to enhanced UV-B, neither did the content of flavonols (which is the most reliable response to UV-B) nor the most UV-B-responsive gene analyzed (*VvFLS4*). In line with these results, Martínez-Lüscher et al. (2015) did not found any response of UVACs to enhanced UV-B in Tempranillo grapes, as occurred in the gene responses of Sauvignon Blanc (Liu et al., 2015). Probably, the subtle changes caused by enhanced UV-B could be due to the saturation of grape responses with ambient UV-B doses. Thus, increasing UV-B beyond this limit would be irrelevant for the plant. Probably, stronger responses of UV-B-responsive variables to enhanced UV-B would rather have required a higher short-term peak irradiance than just a slightly higher dose applied in the long term (Del-Castillo-Alonso, Monforte, Tomás-Las-Heras, Núñez-Olivera, et al., 2020).

The effects of UV-A alone were much more diffuse than those of UV-B alone and closer to those of PAR. Nevertheless, we found a synergic effect between UV-A and UV-B on the flavonol content and the expression of *VvFLS4* and *VvCHS1* genes, which were higher in PAB than in PB samples. In leaves, the effects of UV-A on phenolic compounds depend on the species, the specific compound, the experimental conditions applied, and synergy between UV-A and UV-B has been observed in a few cases (Verdaguer et al., 2017). In Bacchus grapes, Kolb et al. (2003) found that flavonol accumulation was stimulated by ambient UV-A, although the highest stimulation occurred under the combination of UV-A and UV-B. Conversely, Gregan et al. (2012) did not find any UV-A effect on flavonols in Sauvignon blanc, but again the combination of UV-A and UV-B increased their contents. Thus, the synergy between UV-A and UV-B on flavonols accumulation seems to be a solid effect in grapes.

4.3 | Phenological stage

Antioxidant capacity, total phenols, total flavonoids, and UVACs showed the highest values in pea-size stage, decreased at veraison and slightly increased at harvest. Flavanols and hydroxycinnamic acids decreased from pea-size stage to harvest, whereas flavonols and anthocyanins increased from veraison to harvest. Stilbenes showed irregular changes along with the berry development. These are common temporal patterns of phenolic compounds in grapevine (Keller, 2020). In addition, the decrease of hydroxycinnamic acids from pea-size onwards and the concomitant increase of flavonols and anthocyanins could be related to the competition of phenolic acids and flavonoids for the same precursors (Del-Castillo-Alonso, Diago, et al., 2016).

Responses of phenolic compounds and gene expression to UV radiation were strongly influenced by the phenological stage (Figure 7), which was more important than the radiation regime in the ordination of the samples by PCA (Figure 5). For example, flavonol content and *VvFLS4* responded at every stage, while *VvCHS1* only responded at veraison and harvest.

Overall, strong responses to UV took place at every phenological stage, depending on the compound considered. Nevertheless, in our study, UV did not modify the natural evolution of phenolic compounds in grapes from pea-size to harvest. Identifying the influence of phenology on the UV effects is important to design an adequate temporal pattern of artificial UV application to increase specific compounds at a determinate phenological stage.

4.4 | Grape components and cell fractions

Among the three berry components (skin, flesh, and seeds), skin was the most UV-responsive, especially by the increase in flavonols. This was probably due to the skin directly receiving UV radiation, but also to the higher diversity of compounds found in skins in comparison with flesh or seeds. In particular, the most UV-responsive compounds (flavonols) were mostly located in the skin.

Regarding the influence of cell location, the methanol-soluble fraction (presumably mainly located in the vacuoles: Schnitzler et al., 1996) was more UV-responsive than the cell wall-bound methanol–insoluble fraction. The higher responsiveness of the soluble fraction would be justified by its higher diversity and amount of phenolic compounds, and especially by the exclusive presence of the UV-responsive flavonols. The insoluble fraction was insensitive to UV because it consisted of non-UV-responsive compounds (mostly hydroxybenzoic and hydroxycinnamic acids). Insoluble compounds would be rather immobilized in the cell walls, limiting their reaction capacity to UV. Other studies, although based on solar UV exclusion experiments, have also shown that the skin soluble fraction of Graciano and Tempranillo grapes was more UV-responsive than the insoluble fraction (Carbonell-Bejerano et al., 2014; Del-Castillo-Alonso et al., 2015; Del-Castillo-Alonso, Diago, et al., 2016). These coincident results would suggest a different physiological role for each fraction. The insoluble cell wall-bound fraction would represent an efficient UV screen, rather constitutive than UV-inducible, whereas the soluble vacuolar fraction could be more related to antioxidant defense. In addition, phenolic compounds in the cell walls could protect cells against pathogens and constitute a physical barrier to fungal penetration (Agati et al., 2012). Nevertheless, the content of the insoluble *p*-coumaric acid was higher in the skins exposed to ambient solar UV than in non-exposed skins (Carbonell-Bejerano et al., 2014; Del-Castillo-Alonso et al., 2015). This increase would require new deposits on the cell wall. Thus, even in the usually non-UV-reactive insoluble fraction, some compounds could increase their contents in response to UV radiation. Much effort will be required to disentangle the relationships between cell location, function, and UV responsiveness of phenolic compounds in grapes.

On the other hand, from an enological perspective, the insoluble fraction is much less important than the soluble fraction because extraction of insoluble phenolic compounds cannot be achieved using usual enological methods as they are covalently linked to the cell wall polysaccharides.

4.5 | VOCs

Only one hydrocarbon and four fatty acids (2-ethylhexanoic, heptanoic, octanoic, and nonanoic acids) increased their contents in response to UV radiation. In addition, apocarotenoid showed a similar although nonsignificant trend. However, furans decreased and ketones, aldehydes, terpenes, and the remaining C_{13}-norisoprenoids showed no response.

Fatty acids are constituents of phospholipids (De Carvalho & Caramujo, 2018) but also play a role in plant defense and stress responses (Gil et al., 2013). In grapes, they can be transformed into alcohols, aldehydes, or ketones, affecting berry aromas (González-Barreiro et al., 2015; Reynolds, 2010). In wines, octanoic and decanoic acids are generally associated with cheesy and rancid notes, and they can also form esters affecting wine flavor (Jackson, 2020; Perestrelo et al., 2019; Song et al., 2015). Although the contents of the UV-responsive fatty acids were low in our study, they could influence grape and wine characteristics, and thus further research on their response to UV radiation would be needed to better understand this influence.

Apocarotenoid compounds participate in essential functions like photoprotection, photosynthesis, pigmentation, and signaling (Felemban et al., 2019), and they also contribute to the floral and fruity attributes of grapes and wine (Lashbrooke et al., 2013). Although we found low contents of this compound, its potential reactiveness to UV can be important for grape quality.

There is little comparative data in the literature on the effects of UV radiation on grape VOCs (but see Matus, 2016 and Jordan, 2017). Specifically, Gil et al. (2013) found opposite results to ours, since in their study, monoterpenes, aldehydes, alcohols, and ketones increased under UV-B radiation. In addition, Miao et al. (2020) demonstrated the influence of ambient UV-B on terpenes. Interestingly, UV-B-induced terpenoids could increase protection against oxidative damage due to their antioxidant capacity (Gil et al., 2013), but no effect of UV radiation on terpenoids was found in our study. In line with our results, Joubert et al. (2016) suggested that VOCs are more responsive to global radiation than to UV. Again, differences in cultivar and experimental conditions could justify the different results obtained. The responses of VOCs to UV radiation should be better studied because of their importance in grape and wine aroma.

In our study, VOCs showed much more modest responses to UV than phenolic compounds and related genes, and thus, their influence in the ordination of the radiation regimes through PCA was lower (Figures 5-6).

5 | CONCLUSIONS

Our integrative study confirms the complexity of the responses of grapes to UV radiation, although some responses were well characterized under the specific experimental conditions used. Differences between cultivars and experimental conditions have repeatedly been mentioned in the literature to justify the variability of the results obtained in the different studies. Thus, experiments using several cultivars under the same experimental conditions are badly needed. Overall, although considerable progress has been achieved in the study of the effects of UV radiation on grapes, further research is needed to understand better both the physiological effects per se and their consequent applications on the artificial management of UV radiation for a better quality of grapes and wines.

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AUTHOR CONTRIBUTIONS

Encarnación Núñez-Olivera and Javier Martínez-Abaigar conceived the research. María-Ángeles Del-Castillo-Alonso conducted the experiment. All authors (María-Ángeles Del-Castillo-Alonso, Laura Monforte, Rafael Tomás-Las-Heras, Annamaria Ranieri, Antonella Castagna, Javier Martínez-Abaigar and Encarnación Núñez-Olivera) participated in methodological tasks and data analysis. Encarnación Núñez-Olivera and Javier Martínez-Abaigar wrote the manuscript with suggestions of all authors (as previously mentioned). All authors (as previously mentioned) read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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

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