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Effects of UV-B radiation on grapevine (Vitis vinifera cv. Tempranillo) leaf physiology and berry composition, framed within the climate change scenario (water deficit, elevated CO₂ and elevated temperature)

Johann Martínez Lüscher

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Doctoral dissertation performed under joint supervision between the University of Navarra and the University of Bordeaux, submitted by Johann Martínez Lüscher for the degrees of *Doctor en Ciencias Biológicas por la Universidad de Navarra* and *Doctorat en Biologie Végétale de l'Université de Bordeaux*.

Signature,

Pamplona , 3rd of July 2014

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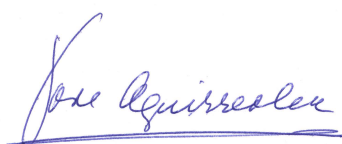
That the thesis entitled “Effects of UV-B radiation on grapevine (*Vitis vinifera* cv. Tempranillo) leaf physiology and berry composition, framed within the climate change scenario (water deficit, elevated CO₂ and elevated temperature)” was performed by Johann Martínez Lüscher under our supervision, for the degree of *Doctor en Ciencias Biológicas por la Universidad de Navarra* and *Doctorat en Biologie Végétale de l’Université de Bordeaux* and; considering it is finished, we authorize the submission for the evaluation by the corresponding jury.

And for the record, we issue this certificate in Pamplona, 3rd of July 2014.

Signatures,



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Jone Aguirreolea Morales



Eric Gomès

*"Life isn't about waiting for the storm to pass; it's about learning to dance in the rain."
Vivian Greene*

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Effects of UV-B radiation on grapevine (*Vitis vinifera* cv. Tempranillo) leaf physiology and berry composition, framed within the climate change scenario (water deficit, elevated CO₂ and elevated temperature)

SUMMARY

The aim of the thesis was to assess the effect of UV-B radiation on grapevine *Vitis vinifera* cv. Tempranillo leaf physiology and grape berry composition, framed within the climate change scenario. Experiments were conducted under glasshouse controlled conditions with fruit-bearing cuttings. Plants were exposed to three UV-B biologically effective doses (0, 5.98, 9.66 kJ m⁻² d⁻¹) either from fruit set or veraison to maturity. The combined effects of UV and water deficit, as well as, UV-B and elevated CO₂-temperature (700ppm, +4°C), applied from fruit set to maturity were also tested. Gas exchange, Chlorophyll *a* fluorescence, lipid peroxidation, antioxidant enzyme activity, UV-B absorbing compound levels and chlorophyll and carotenoid concentration were determined in leaves. Berry development was assessed quantitatively (e.g. elapsed time to reach phenological stages). Amino acid, anthocyanin and flavonol concentrations and profiles were analyzed in berries, as well as, transcript profiling of regulatory and structural genes involved in flavonoid biosynthesis.

The results show that initial down-regulation of photosynthesis was followed by an acclimation, mediated by the accumulation of UV-B absorbing compounds and antioxidant response elicitation (flavonoids and antioxidant enzymes). Water deficit and elevated CO₂-temperature did not alter UV-B acclimation process, however, UV-B did led to certain degree of cross-tolerance to elevated CO₂-temperature, avoiding the senescence-induced oxidative damage. Berry technological maturity (ca. 22°Brix) was delayed by UV-B exposure and water deficit, especially when combined, whereas it was hastened by elevated CO₂-temperature. In the last case, UV-B attenuated the effect of elevated CO₂ and temperature. Changes in berry ripening rates were associated with changes in photosynthetic performance.

UV-B radiation and water deficit induced lower grape must acidity, mediated by increases in relative skin mass or potassium levels rather than a decrease in organic acid concentration. In addition this increase in relative skin mass may have contributed to higher anthocyanin concentration in the must. Grape berry skin flavonol and anthocyanin concentration was increased by UV-B, mainly due to the up-regulation of the structural (*CHS*, *F3'H*, *FLS*, *UFGT* and *GST*) and regulatory genes (*MYBF1* and *MYBA1*) committed to their synthesis. Quantitative changes in flavonol concentration induced by UV-B were always associated with qualitative changes in flavonol profile (i.e. increased relative abundance of mono- and disubstituted flavonols), as a result of the competition of *FLS* with flavonoid hydroxylases (*F3'H* and *F3'5'H*) for the same substrates. The independent up-regulation of *FLS* and *F3'5'H* by UV-B radiation and water deficit, respectively, resulted in an interactive effect on the flavonol B ring hydroxylation pattern. Under elevated CO₂-temperature anthocyanin-sugar accumulation was decoupled. However, UV-B partially alleviated this uncoupling by up-regulating anthocyanin biosynthesis and modulating berry ripening rates.

UV-B radiation greatly influenced grapevine leaf physiology and berry composition. These responses to UV-B were modulated, to a greater or lesser extent, by other factors linked to climate change (water availability, atmospheric CO₂ levels and temperature).

Keywords: Grapevine, UV-B radiation, climate change, photosynthetic response, acclimation, flavonoid profile.

Efecto de la radiación UV-B sobre la fisiología de la hoja y la composición de la baya de vid (*Vitis vinifera* cv. Tempranillo), en un contexto de cambio climático (déficit hídrico, CO₂ elevado y alta temperatura)

RESUMEN

El objetivo de la tesis fue evaluar el efecto de la radiación UV-B en la fisiología de la hoja y la composición de la baya de vid (*Vitis vinifera* cv. Tempranillo), enmarcado dentro de un escenario de cambio climático. Los experimentos se llevaron a cabo con esquejes fructíferos bajo condiciones controladas en invernaderos. Las plantas se expusieron a tres dosis de radiación ultravioleta biológicamente efectiva (0, 5,98, 9,66 kJ m⁻² d⁻¹), bien desde el cuajado o desde el envero, hasta la madurez. Además, se estudiaron los efectos combinados de la radiación UV-B y el déficit hídrico, así como la combinación de UV-B y CO₂-temperatura elevados (700 ppm, +4°C), aplicados desde cuajado hasta madurez. En hoja se determinaron los siguientes parámetros: intercambio gaseoso, fluorescencia de la clorofila *a*, peroxidación lipídica, actividad de enzimas antioxidantes, nivel de compuestos absorbentes de UV-B y concentración de clorofilas y carotenoides. El desarrollo de las bayas fue evaluado cuantitativamente (e.g. tiempo transcurrido para alcanzar diferentes estados fenológicos). La concentración y perfil de aminoácidos, antocianinas y flavonoles fueron analizados en bayas, así como los niveles de expresión de los genes estructurales y reguladores involucrados en la síntesis de flavonoides.

Los resultados muestran un descenso inicial de la fotosíntesis, seguido de una aclimatación, mediada por la acumulación de compuestos absorbentes de UV-B y la exaltación de la respuesta antioxidante (flavonoides y enzimas antioxidantes). El déficit hídrico y los niveles elevados de CO₂-temperatura no alteraron el proceso de aclimatación a la radiación UV-B. No obstante, la radiación UV-B dio lugar a cierto grado de tolerancia cruzada a las condiciones de CO₂-temperatura elevados, evitando el daño oxidativo inducido por la senescencia. La madurez de las uvas se retrasó por la exposición a la radiación UV-B y el déficit hídrico, sobre todo cuando estos factores actuaron en combinación, mientras que la maduración se aceleró en condiciones de CO₂ y temperatura elevados. En este último caso, la radiación UV-B atenuó el efecto del CO₂-temperatura elevados, retrasando la maduración. Estos cambios fenológicos se asociaron a cambios en el rendimiento fotosintético.

La radiación UV-B y el déficit hídrico disminuyeron la acidez del mosto, debido a un aumento de la relación hollejo/pulpa o de los niveles de potasio, más que por un descenso en la concentración de ácidos orgánicos. Además, este aumento en la proporción de piel por peso de baya desencadenó un aumento en la concentración de antocianinas en el mosto. La concentración de flavonoles y antocianinas en el hollejo aumentó con la radiación UV-B, debido principalmente a la estimulación de genes estructurales (*CHS*, *F3'H*, *FLS*, *UFGT* y *GST*) y reguladores (*MYBF1* y *MYBA1*) de la ruta biosintética. Los cambios cuantitativos en estos metabolitos inducidos por la radiación UV-B estuvieron siempre relacionados con los cambios cualitativos en los perfiles de flavonoles (aumento de la abundancia relativa de flavonoles mono- y disustituídos), debido a la competencia de *FLS* con flavonoide hidroxilasas por los mismos sustratos. Se observó una interacción entre la radiación UV-B y el déficit hídrico sobre el perfil de hidroxilación de los flavonoles, debido a que cada uno de estos factores aumentó la expresión de diferentes enzimas de la vía de síntesis. En condiciones de CO₂ elevado - alta temperatura se produjo un desacoplamiento de la acumulación de antocianinas y azúcares.

Sin embargo, la radiación UV-B alivió parcialmente este desacoplamiento debido a la estimulación de la biosíntesis de antocianinas y a la modulación de las tasas de maduración de las bayas.

Los resultados muestran del trabajo un fuerte impacto de la radiación UV-B sobre la fisiología de la hoja y en la composición de la baya de vid. Estos efectos fueron modulados, en mayor o menor medida, por otros factores relacionados con el cambio climático, como son la disponibilidad de agua, los niveles de CO₂ atmosférico y la temperatura.

Palabras clave: Vid, radiación UV-B, cambio climático, respuesta fotosintética, aclimatación, perfil de flavonoides.

Etude des effets du rayonnement UV-B sur la physiologie foliaire et la maturation des baies chez la Vigne (*Vitis vinifera* L. cv Tempranillo), dans le contexte du changement climatique (déficit hydrique, température et taux de CO₂ élevés).

RÉSUMÉ

Ce travail de thèse porte sur l'effet du rayonnement UV-B sur la physiologie foliaire et la maturation des baies chez la Vigne (*Vitis vinifera* L. cv Tempranillo), dans le contexte du changement climatique en cours. Dans ce but, des expériences ont été menées en conditions contrôlées en serre, sur des boutures fructifères. Les plantes ont été exposées à trois doses de rayonnement UV-B biologiquement actifs (0, 5,98 et 9,66 kJ.m⁻².jour⁻¹), soit à partir de la nouaison, soit à partir de la véraison, et ce jusqu'à la pleine maturité. Le rayonnement UV-B a été appliqué seul ou en combinaison avec d'autres stress abiotiques (déficit hydrique, taux de CO₂ et température élevés). L'impact de ces stress sur l'activité photosynthétique, les contenus en pigments et en composés photoprotecteurs, ainsi que sur les activités des enzymes produisant des composés antioxydants, ont été étudiés. La phénologie, les profils d'accumulation des flavonols et des anthocyanes, ainsi que le contenu en acides aminés des baies ont également été analysés, de même que l'expression des principaux gènes régulateurs et structuraux de la voie de biosynthèse des polyphénols.

Les résultats obtenus montrent que les effets des UV-B sur la physiologie foliaire peuvent être découpés en deux phases : une première phase transitoire de diminution de l'activité photosynthétique, suivie d'une phase d'acclimatation due à la production de composés photoprotecteurs (flavonoïdes essentiellement) et à l'activité d'enzyme de détoxification des formes actives de l'oxygène (superoxyde dimutase, catalase, ascorbate peroxidase). Le déficit hydrique, le stress thermique et un taux de CO₂ élevé (700ppm, +4°C) ne modifient pas le processus d'acclimatation aux UV-B ; en revanche on note une interaction positive entre les UV-B d'une part et la tolérance aux températures et au taux de CO₂ élevé d'autre part, atténuant les dommages oxydatifs dus à l'induction de sénescence par ces deux derniers facteurs. La maturité des baies est retardée par les UV-B et le déficit hydrique, et ce phénomène est amplifié lorsque ces deux stress sont appliqués en combinaison ; alors que les hautes températures et un taux de CO₂ élevé ont l'effet inverse. Dans ce dernier cas, le rayonnement UV-B atténue les effets du couple température/CO₂ élevés. Ces effets sur la phénologie de la Vigne ont pu être reliés à des modifications de l'activité photosynthétique des feuilles.

En ce qui concerne la composition des baies, l'augmentation du rayonnement UV-B et le déficit hydrique ont augmenté les concentrations en anthocyanes et en potassium des moûts et diminué leur acidité, ce qui peut s'expliquer en partie par une augmentation du ratio de masse pellicule/pulpe. L'augmentation des teneurs en anthocyanes et flavonols des pellicules observée en réponse aux UV-B a pu être liée à l'induction de gènes structuraux (*CHS*, *F3'H*, *FLS*, *UFGT* and *GST*) et régulateurs (*MYBF1* et *MYBA1*) de la voie de biosynthèse des flavonoïdes. Ces changements quantitatifs étaient toujours associés à des changements qualitatifs, avec une augmentation de la part relative des flavonols mono- et di-substitués, en raison d'une compétition de la flavonol synthase avec les F3' et F3'5' hydroxylases pour les mêmes substrats. Une interaction notable a été observée entre le rayonnement UV-B et le déficit hydrique, sur les profils d'hydroxylation des flavonols, ce qui s'explique par le fait que ces deux facteurs agissent sur deux étapes distinctes de la voie de biosynthèse. Enfin, lorsque les boutures fructifères ont été soumises à un stress combiné par une élévation de température (+4°C) et un taux de CO₂ élevé (700 ppm), cohérents avec les conditions climatiques futures, l'accumulation des anthocyanes et des sucres a été partiellement découplée ; cependant une dose élevée de rayonnement UV-B (9,66 kJ.m⁻².jour⁻¹) annule en partie cet effet en stimulant la biosynthèse des flavonoïdes et en retardant significativement la maturité technologique.

Les résultats de ce travail démontrent un fort impact du rayonnement UV-B sur la physiologie foliaire et la maturation des baies chez la Vigne. Ces effets sont modulés dans une plus ou moins grande mesure par des interactions notables entre le rayonnement UV-B et d'autres facteurs abiotiques liés au changement climatique en cours (disponibilité en eau, niveau de CO₂ atmosphérique, température ambiante).

Mots-clés: Acclimatation, changement climatique, rayonnement UV-B, réponse photosynthétique, Vigne.

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GENERAL INTRODUCTION

1. GRAPEVINE

Grapevine refers to a species within the genus *Muscadinia* and *Vitis*, both members of the family *Vitaceae*. While genus *Muscadinia* contains only 3 species, genus *Vitis* comprises 60-70 species (Keller, 2010). Grapevine species are predominantly distributed in temperate and subtropical climate zones of the northern hemisphere (Keller, 2010). Among them, the *Vitis vinifera* L. gave rise to the majority of the varieties cultivated nowadays. Given the sensitivity of *V. vinifera* to the aphid phylloxera, other *Vitis* species or crosses between them, such as *V. rupestris*, *V. riparia* and *V. berlandieri*, are used as rootstocks (Galet and Smith, 1998). These species, which may have a lower fruitfulness, usually show higher tolerance to phylloxera and diseases (Mullins et al., 1992). In addition, the rootstock choice may contribute to changes in plant vigour and grape composition (Dry et al., 2004).

The cultivar Tempranillo is mainly cultivated in Spain, where is the variety with the widest distribution (20% of the total vineyard surface). Recent studies locate its origin in the surroundings of river Ebro less than a thousand years ago, as a natural hybridization between the varieties Albillo Mayor and Benedicto (Ibáñez et al., 2012). It is regarded as a variety highly sensitive to pests and diseases and not very resistant to drought or high temperatures within the Spanish gradient. Its grapes are adequate to produce wines able to withstand long ageing periods, with a good balance of alcohol content, colour and acidity, and an honest, smooth, fruity mouth feel that turns velvety as it ages. However its quality may drop as yield increases, leading to a poor anthocyanin production and green, drying tannins (Appellation Rioja, 2014).

1.1. GRAPEVINE PHENOLOGY

In order to unify growth stage schemes, Coombe (1995) proposed a combination of both, the major stages proposed by Baillod and Baggioini (1993) and more detailed schemes, such as the Eichhorn and Lorenz (1977) and Hack et al. (1992).

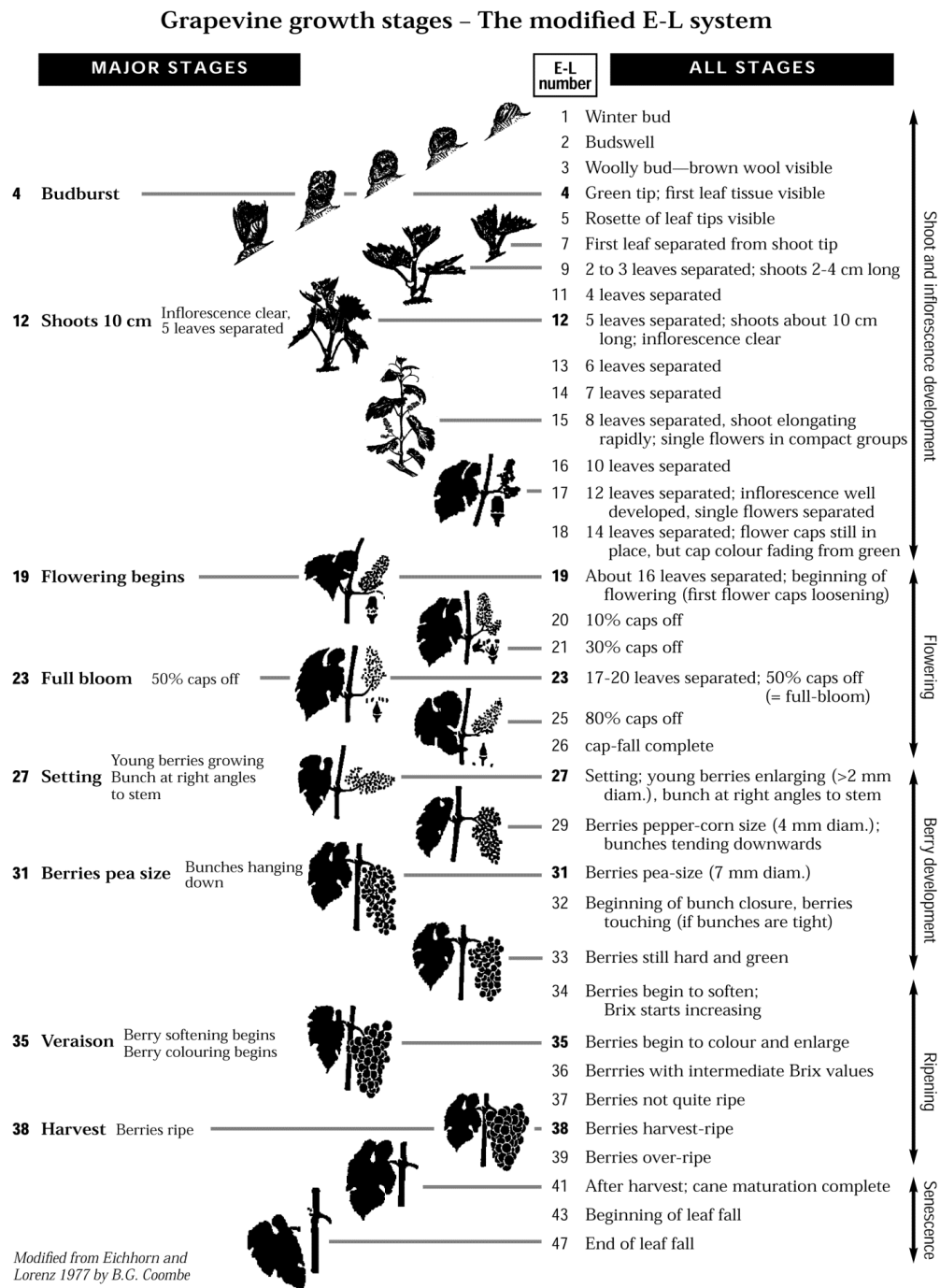


Figure 1. Grapevine growth stages (Coombe, 1995)

1.2. VEGETATIVE CYCLE

Grapevines coming from seeds spend several years before they reach the reproductive phase (Champagnol, 1984). However, vineyard plants are propagated asexually, as cuttings

grafted into rootstocks. As the year starts, dormant grapevines are pruned. In late winter or early spring pruning wounds start to exude sap (bleeding), which may last from several days to few weeks (Martínez de Toda, 1991). Dormant plants respond to spring rising temperatures which results in budburst (Reynier, 2012). The temperature threshold for this phenomenon is highly cultivar dependent. For instance, while Chardonnay is regarded to have an early budburst, Cabernet Sauvignon has generally a later budburst (Keller, 2010).

When buds break, these are highly pubescent and leaves may be red coloured while they are still expanding. This may respond to a photoprotective mechanism for the young tissues (Liakopoulos et al., 2006). At this stage, growth depends entirely on nutrient reserves stores in roots and stems formed in previous seasons (Keller, 2010). These reserves are used both to build structures and to be respired, until the new leaves become photosynthetically active. This period of reserve consumption coincides with bloom, which makes vines extremely sensitive to stress (Lebon et al., 2005; Lebon et al., 2008; Pallas et al., 2008). The initial growth stage is characterized by a strong apical dominance. However, this may be compromised in highly vigorous plants (Keller, 2010). Growth usually stops at midsummer, around the onset of grape ripening. Shoots then begin to form a periderm, then turning from green to yellowish-brown. Storage of nutrients for the next season may then commence (Reynier, 2012).

Even before grapes are harvested, day length trigger leaf senescence to reabsorb part of the nutrients invested in the leaves. Following the re-absorption, phloem is sealed and leaves fall from the base of the petioles, at the abscission zone. As conditions become more adverse for plant growth, shoots dehydrate as part of the acclimation to cold. Bud winter dormancy (endodormancy) is associated with a strong increase in abscisic acid levels in the buds and nodes (Rohde and Bhalerao, 2007). Internal signals will keep buds from bud breaking in response to autumn warm temperatures. A period of relatively low temperatures in

combination with short days induces a decrease in bud abscisic acid levels, thus allowing growth resume (Galet and Towey, 2000).

1.3. REPRODUCTIVE CYCLE

After budburst, as the apex expands, leaves are deployed and the inflorescence becomes visible. When caps open, pollen is released into the air and both self- and cross-pollination takes place. The speed at which the pollen tubes grow toward the ovules is critical for fruit set, and this is greatly influenced by temperature (Callis, 1995). Fertilization is followed by a period of cell division, which is most likely stimulated by cytokinins and auxins (Chacko et al., 1976). The rate and duration of these cell divisions is determined by the seeds (unfertilized ovules in the case of seedless varieties), so the final size of the berries is determined, to a great extent, by the number of seeds (Gillaspy et al., 1993). While mesocarp cells stop dividing after 2 to 4 weeks after anthesis, exocarp cells may continue to divide longer (Pratt, 1971). These processes are comprehended in *Stage I* (early fruit development) (Fig. 2). At this stage, berry texture is firm, while its colour is green due to chlorophyll. The sugar content of the berry remains low while organic acids start to accumulate (Fig. 2) (Dokoozlian, 2000). By the end of this phase berries may reach half of their final volume. Even though the main process leading to fruit enlargement at this stage is regarded to be cell division, the vast majority of berry growth is due to cell expansion at any stage (Keller, 2010). During *Stage II* (lag phase) (Fig. 2), embryos grow and by the end of this phase seeds reach their maximum size (Pratt, 1971). The influx of abscisic acid suppresses embryos growth by blocking gibberellins production. The content of auxins, a ripening inhibitor, peaks at this phase, followed by a sharp decline. Ethylene and brassinosteroids are regarded as the key hormones leading to changes in cell wall composition, which result in berry softening and expansion. Organic acids reach their maximum at this point.

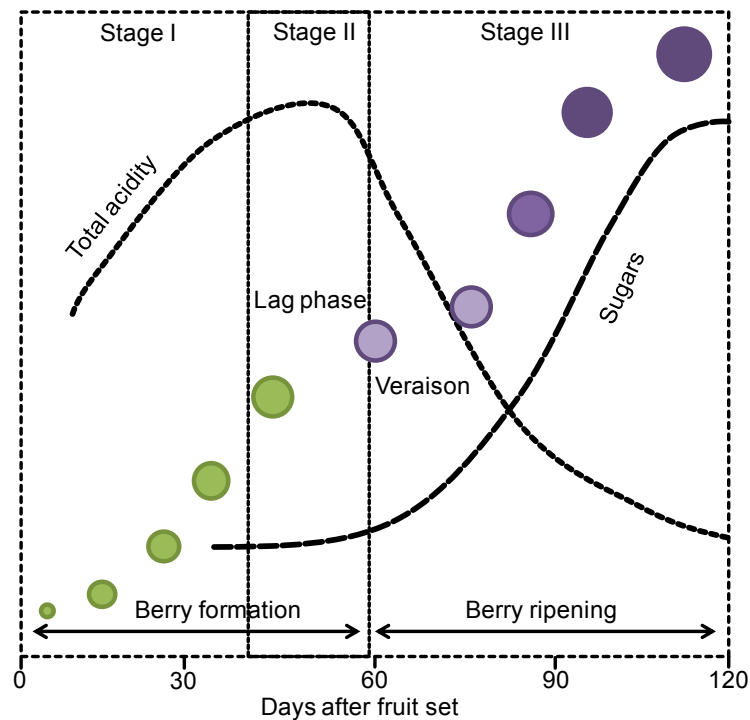


Figure 2. Diagram showing the evolution of relative berry size, colour and main metabolites (sugars and organic acids) throughout development. Adapted from Coombe (2001) and Dokoozlian (2005)

Veraison marks the beginning of the second phase of berry growth, *Stage III* (ripening). This phase is characterized by a berry softening, increase in sugar content and a change in colour (Fig. 2). In addition, aroma and flavour compounds accumulate during this phase (Dokoozlian, 2000). During *Stage III* berries stop being photosynthetic organs to be entirely heterotrophic. Water inflow through the xylem declines progressively and the import of water (as solvent for sugars) is carried out through the phloem. Contrarily to other fruits, grape berries may continue growing in size during this phase (Gillaspy et al., 1993), due to cell expansion. As the other non-climacteric fruits, ethylene does not play a key role in grape ripening, and, in fact, the hormonal regulation of this process remains unclear. Three hormones, abscisic acid, ethylene and brassinosteroids, appear to play a major role in grape ripening (Conde et al., 2007). Grape maturity at harvest is a concept subject to market-based criteria. Basically, a proper skin coloration and a certain level of sugars are essential for

wine-making, but also seed mature coloration due to tannin oxidation is a well-valued trait for wine production (Keller, 2010).

1.4. GRAPE MORPHOLOGY

Grape berries are fleshy fruit constituted from a single ovary and carpel. Seeded cultivars grapes contain usually one to four seeds, which contain embryos. As observed in Figure 3, the commonly called “pulp” or “flesh” is constituted by the endocarp and mesocarp layer. The innermost part of the pericarp, the endocarp, surrounds the seed however this is rather hard to differentiate from the mesocarp (Mullins et al., 1992). The cells of mesocarp layer are responsible for most of the berry enlargement and their vacuoles may represent 99% of the total cell volume (Diakou and Carde, 2001).

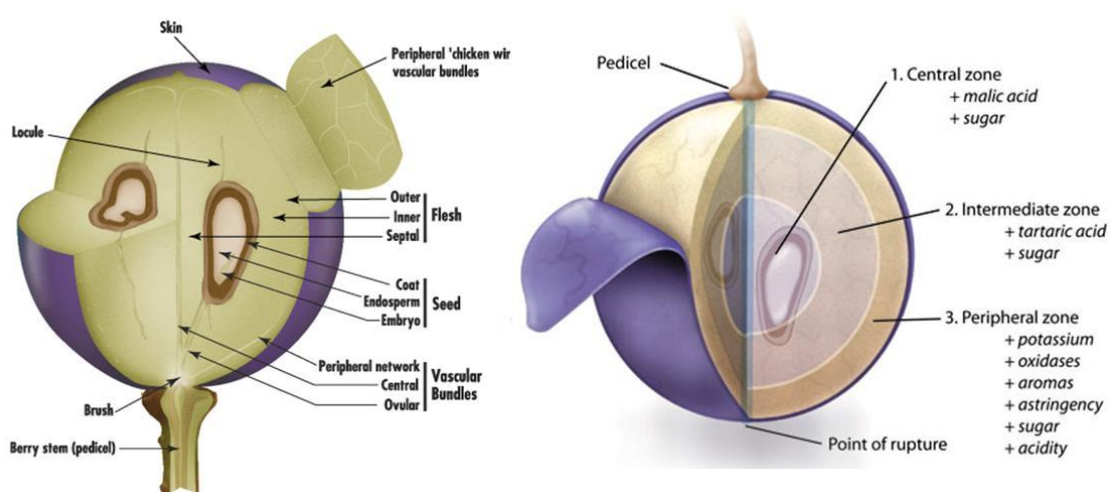


Figure 3. Structure of a ripe grape berry partially sectioned on the long and the central axis to show internal parts (Illustration by Winetitles)

The exocarp constitutes the outermost layer, also referred as “skin”. Depending on the variety, developmental stage and growing conditions, it may represent up to 24% of the total fresh weight. The exocarp develops from a single cell layer of the mesocarp that divides.

Although cells of this layer are several times smaller than pulp cells, their cell walls are 20 times thicker, which contributes to reduce water loss, but it is also a limitation to berry enlargement. The outermost layer of cells of the exocarp (epidermis) is covered by wax (cuticle) that may protect berries from dissection and pathogen invasion (Rosenquist and Morrison, 1988).

1.5. GRAPE COMPOSITION

1.5.1. Water

Water is the most abundant constituent of grape berries. Volumetric growth of the berries is mainly driven by water accumulation. Thereby, the water availability in the soil is one of the factors determining final berry size, but also the dilution of the rest of the constituents of the berry (Conde et al., 2007). Water inflow into the berry takes place predominantly through the xylem up to veraison. However, during ripening, water inflow takes place through the phloem (Dokoozlian, 2000).

1.5.2. Sugars

Sugars present in grape berries are mainly glucose and fructose. Whereas, during grape growth, glucose predominates, at maturity, the proportion of glucose and fructose is similar (Conde et al., 2007). Sugars are imported into the berry from the leaves through the phloem. They are predominantly accumulated in the vacuoles of the “flesh” cells and provide carbon skeletons for other compounds such as organic acids and amino acids (Dokoozlian, 2000).

1.5.3. Organic acids

Tartaric and malic acids usually constitute approximately 90% of the organic acids present in the berry. Others, such as citric, ascorbic or succinic, may be found in grapes. Berry tartaric acid content is synthesized and accumulated during the early stages of fruit development, and its concentration declines during ripening due to dilution, as no enzyme found in the

berry is able to degrade it. Contrarily, malic acid is readily respired. Malate content is a result of the balance among its synthesis before veraison and breakdown and dilution, which mostly takes place during ripening (Dokoozlian, 2000). The concentration of the organic acids, as well as the balance between the two major organic acids, tartaric and malic acid, have major implications for the wine-making process and the ulterior wine quality (Conde et al., 2007).

1.5.4. Phenolic compounds

Phenolic compounds encompass a wide spectrum of components. The accumulation of most of them takes place in seeds and skins. They can be divided into: non-flavonoids (with a simple C6 backbone), such as hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols and stilbenes, and flavonoid compounds, such as flavones, flavanones, flavonols, flavan-3-ols, proanthocyanidins and anthocyanins. Non-flavonoid phenolics, except hydroxycinnamic acids, are found in grapes and wine in low concentrations (Kennedy et al., 2006; Conde et al., 2007). Flavonoids make up a significant portion of the phenolic material in grapes and are found primarily in the skins and seeds (Conde et al., 2007). They are C6–C3–C6 polyphenolic compounds, in which two hydroxylated benzene rings, A and B, are joined by a three-carbon chain that is part of a heterocyclic C ring (Fig. 4). According to the oxidation state of the C ring, these compounds are divided into structural classes: flavonols, flavan-3-ols monomers (catechins), proanthocyanidins (condensed tannins) and anthocyanins (Teixeira et al., 2013).

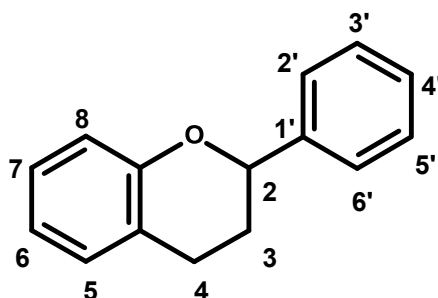


Figure 4. Flavonoid ring structure and numbering

Flavan-3-ols monomers (catechins) are found in the seeds and are responsible for bitterness in wine, and may also have some associated astringency (Conde et al., 2007). Flavonols are found in the epidermis of the grape. Although they are not associated with a sensory component in wine yet, they are known as co-factors for the colour-enhancing phenomenon known as co-pigmentation. Anthocyanins are largely responsible for the red colour of grapes and young wines (He et al., 2010). They are found only in the skin, except “teinturier” varieties (with skin and pulp coloured). Proanthocyanidins (condensed tannins) are polymers of flavan-3-ol subunits responsible for bitterness and astringency. They appear in white and red varieties and are located both in the skin and seeds. Besides their key role in determining the flavour and organoleptic properties of wine (Dokoozlian, 2000), flavonoids exhibit a great nutraceutical potential due to their antioxidant, antitumoral, cardioprotective and anti-inflammatory activities (Nassiri-Asl and Hosseinzadeh, 2009).

1.5.5. Nitrogen compounds

Grape berries contain inorganic nitrogen mostly as free ammonia (NH_4^+) and organic nitrogen compounds such as amino acids, proteins and amines (Conde et al., 2007). Aside from proteins, amino acid metabolism constitutes the basis of other biosynthetic pathways, such as phenolic compounds (via phenylalanine) and aromas (via valine and leucine). Arginine and proline are the most abundant amino acids in grape berries. However, their abundance may vary depending on the variety and water availability (Deluc et al., 2009).

1.5.6. Inorganic minerals

Minerals taken through the roots from the soil can be directly accumulated in the berries. Principal mineral ions in grapes are potassium, calcium, sodium, phosphate and chloride. Cations, mainly potassium, accumulate during ripening. Potassium plays a key role in enzyme activation, transmembrane potential control, and osmotic potential regulation (Conde et al., 2007).

1.5.7. Aroma compounds

Several hundreds of aroma compounds can be found in grape berries. These volatile metabolites are classified into groups depending on the biosynthetic pathways they arise. Their concentrations may vary according to the variety and grape development. For instance, methoxypyrazines arise from the metabolism of free amino acids and accumulate in Cabernet Sauvignon before veraison, conferring grapes and wine an herbaceous aroma (Bayonove et al., 1975). Closely related to carotenoid metabolism, monoterpenes accumulate at the end of ripening and give the floral aromas to varieties such as Muscat (Oliveira et al., 2004).

1.6. FLAVONOID BIOSYNTHESIS REGULATION

Flavonoids arise from the general phenolic compound biosynthetic pathway, which, in turn, arises from the shikimate pathway (Fig. 5). Inside the flavonoid biosynthetic pathway, three different kind of enzymes are described: i) general flavonoid enzymes that are committed to synthesize undetermined precursors of flavonoids, such as chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3 hydroxylase (F3H), dihydroflavonol reductase (DFR) and leucoanthocyanidin dioxidase (LDOX); ii) specific enzymes of each flavonoid group such as UDP: glucose flavonoid glucosyl transferase (UFGT), flavonol synthase (FLS), anthocyanin reductase (ANR) and leucoanthocyanin reductase (LAR); iii) and diversifying enzymes, that are in charge of adding substituents in the B ring of the general flavonoid backbone, such as flavonoid 3' hydroxylase (F3'H), flavonoid 3'5' hydroxylase (F3'5'H) and flavonoid O-methyl transferase (OMTs).

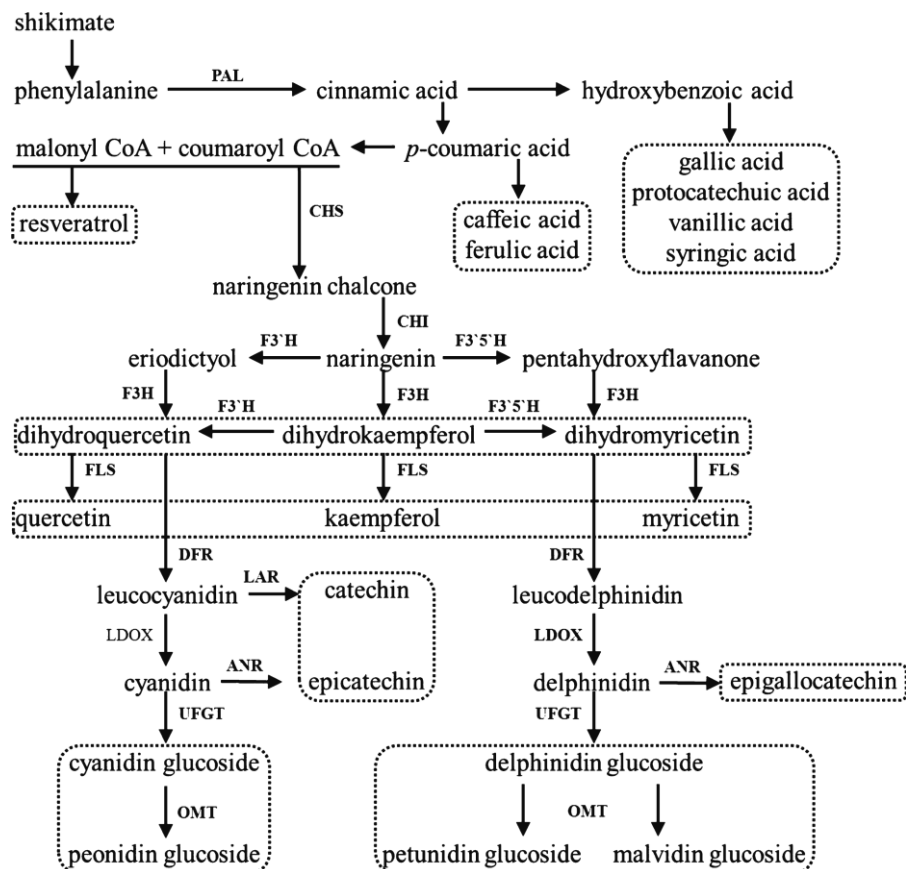


Figure 5. Biosynthetic pathways of the major phenolic compounds of grape (modified from Berli et al., 2011)

The expression of the genes encoding for enzymes involved in the biosynthesis of flavonoids is regulated at the transcriptional level. Transcription factors are proteins able to find and bind to the promoter region of specific genes, to promote the recruitment of RNA polymerase to these genes. Specific signaling pathways result in the up-regulation of each transcription factor to promote the synthesis of each flavonoid group. In the last decade, several transcription factors within the R2R3-MYB family, responsible for flavonoid biosynthesis, have been isolated and characterized (Czemmel et al., 2012).

The vast majority of proanthocyanidins biosynthesis takes place between fruit set and the onset of veraison (Downey et al., 2003) (Fig. 6). The transcription factors MYBPA1, MYBPA2 (and probably MYB5a) promote the expression of genes encoding the flavonoid pathway

enzymes (CHS, CHI, DFR and LDOX) and proanthocyanidin specific enzymes (LAR and ANR).

At the onset of veraison, the transcription factors MYBA1 and MYBA2 initiate specifically the expression of *UFGT* gene (Ageorges et al., 2006; Kobayashi et al., 2002; Walker et al., 2007). The enzyme UFGT mediates in the glucosylation of anthocyanidins, coloured but unstable compounds, to anthocyanins, resulting in grape coloration. The so called white-skinned cultivars, which do not accumulate anthocyanins, have the anthocyanin specific transcription factors as well as *UFGT* gene. However, due to mutations in genes encoding MYBA1 and MYBA2, *UFGT* gene does not express and therefore, these varieties do not synthesize anthocyanins either in their fruits or in any other organ (Walker et al., 2007). During grape ripening, the transcript induction of general flavonoid biosynthesis pathway genes is necessary for anthocyanin biosynthesis. Given the developmental expression pattern of MYBPA1 and MYB5b, these are likely promoting the biosynthesis of anthocyanin precursors during the ripening phase (Bogs et al., 2007; Deluc et al., 2008).

Flavonols are the smaller fraction of flavonoids; however, they play a key role protecting pollen and young berries from ultraviolet (UV) radiation damage (Keller, 2010). In addition, flavonols also may bind covalently to anthocyanins enhancing colour intensity and modulating their colour hue *in planta* (Bloor and Falshaw, 2000; Saito et al., 2011; Zhu et al., 2012). Their developmental regulation is regarded to have two peaks of biosynthesis, around fruit set and close to maturity (Fig. 6) (Downey et al., 2003); although, the great majority of grape flavonols are accumulated during ripening (Fujita et al., 2006). Recently isolated and characterized in grapevine, MYBF1 is the transcription factor promoting gene expression of the flavonol specific enzyme FLS1 (and to a lesser extent FLS2) and the general flavonoid enzymes CHS and CHI (Czemmel et al., 2009). FLS enzyme mediates in the conversion of dihydroflavonols into flavonols. Contrarily to anthocyanins, flavonols are colourless but stable without the need of glucosylation. Flavonols are glycosylated by several glycosyl

transferases, leading to a higher diversity of compounds according to their hexose substituent in 3 position of the C-ring (Ono et al., 2010).

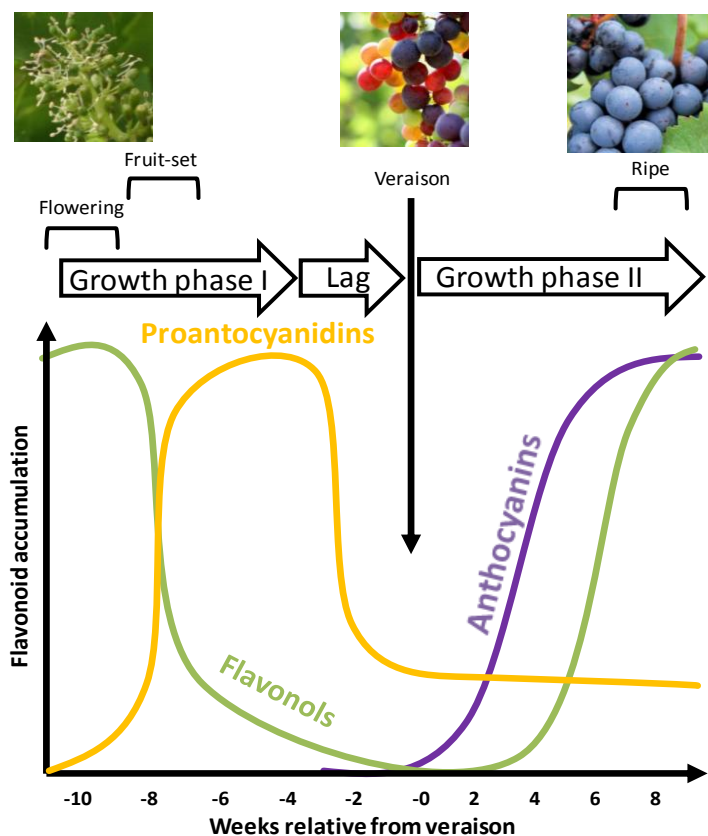


Figure 6. Developmental berry flavonoids (anthocyanins, flavonols and proanthocyanidins) accumulation (adapted from Czemplak et al., 2012)

According to their substituent in 3' and 5' position of the B ring, flavonoids may diversify (Fig. 7), and in the case of anthocyanins this leads to major changes in colour (Tanaka and Ohmiya, 2008). Diversification of flavonoids takes place through the transcriptional regulation of F3'H, F3'5'H and OMTs enzymes (Bogs et al., 2006; Fournier-Level et al., 2011). Their expression levels are, to a large extent, pre-defined constitutively (Castellarin and Di Gasparo, 2007). In addition, *F3'5'H* and *OMTs* genes are also regulated by water availability, leading to changes in flavonoid profile (Castellarin et al., 2007a; Castellarin et al., 2007b). However, most of the work related to flavonoid diversification has been performed with

anthocyanins, which may not be always representative of what happens to flavonol profile (Mattivi et al., 2006).

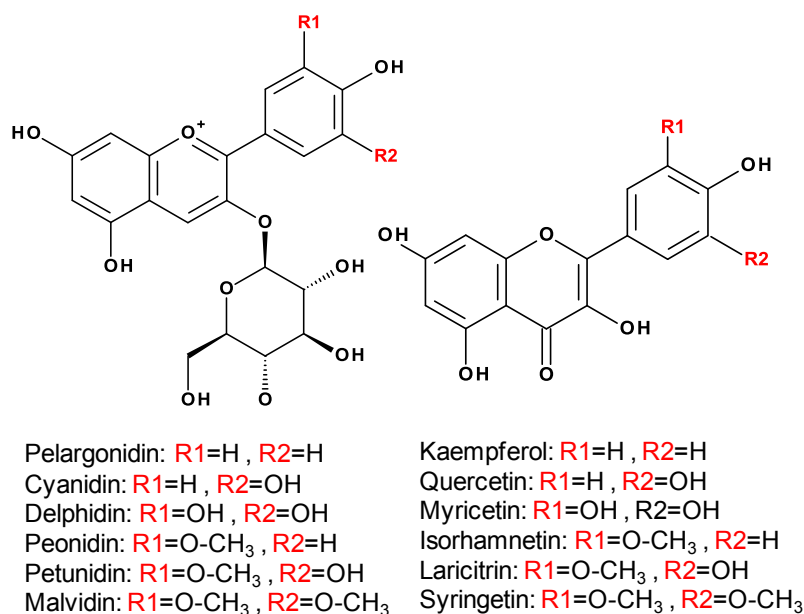


Figure 7. Flavonol and anthocyanin glucoside general structure with the subsequent name according to their substituents at 3' and 5' position

Even though the synthesis of flavonoid is tightly induced by developmental events, the expression of flavonoid transcription factors may be affected by environmental conditions (Koyama et al., 2012), cultural practices (Matus et al., 2009) and pathogens (Gutha et al., 2010). Given the contribution of flavonoids to the wine organoleptic properties and their health-promoting effects, the regulation of their synthesis has called the attention of the research community. But also the wine industry, which may benefit from the knowledge gained in the last years.

2. ULTRAVIOLET-B (UV-B) RADIATION

2.1. UV-B RADIATION REACHING THE EARTH

Accounting for less than 0.5%, UV-B radiation (280-315 nm) is a minor fraction of the solar radiation reaching the earth surface. This is due to the action of the stratospheric ozone layer. The extinction coefficient of ozone increases several orders of magnitude as wavelength decreases. This property confers to the stratospheric ozone layer the role of protecting earth's life form solar most-harming UV radiation wavelengths. Stratospheric ozone sustains a cycle in which three forms of oxygen are involved. Molecular oxygen (O_2) photodissociates into two oxygen radicals (O) in response to solar radiation around 240 nm. Oxygen radicals react spontaneously with oxygen molecules to produce ozone (O_3), which absorbs UV radiation between 200 and 315 nm. Such reaction leads to the splitting of ozone into a molecule of oxygen and an atom of oxygen. This atom of oxygen may form ozone newly, or react with another oxygen radical to produce molecular oxygen and close the cycle (Harrison and Hester, 2000).

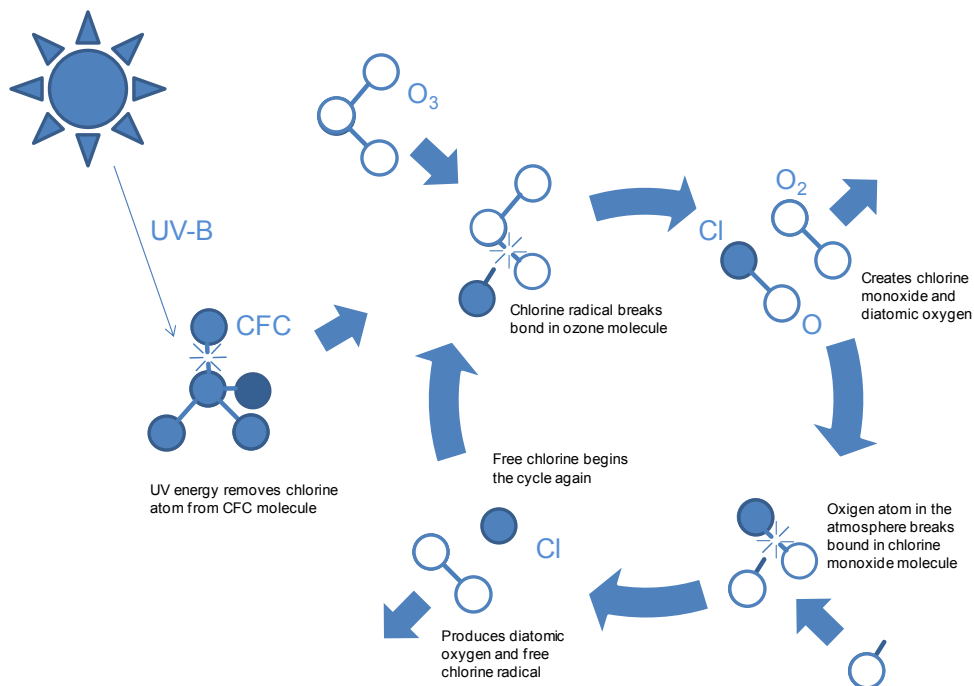


Figure 8. Ozone depletion process

Substances, such as chlorofluorocarbon compounds (CFCs), are dissociated by UV radiation, releasing chlorine atoms (Fig. 8). These act as catalyst, consuming several ozone molecules before they exit the stratosphere. Due to the anthropic release of ozone-depleting substances, stratospheric ozone layer has been thinned at a rate of 4% per decade up to the late 1980s (McKenzie et al., 2007). The successful implementation of the Montreal Protocol (1987) contributed to reduce to a great extent the emission of ozone depleting substances. However, due to the resilience of ozone depleting substances, ozone levels may not recover until 2050 (UNEP, 2012). Ozone depletion simulations reveal the relevance of the success of Montreal Protocol (Fig. 9). Under an scenario of constant CFCs emissions, UV levels would have increased several times before the end of the present century, which would have been devastating for plant life (Newman et al., 2009).

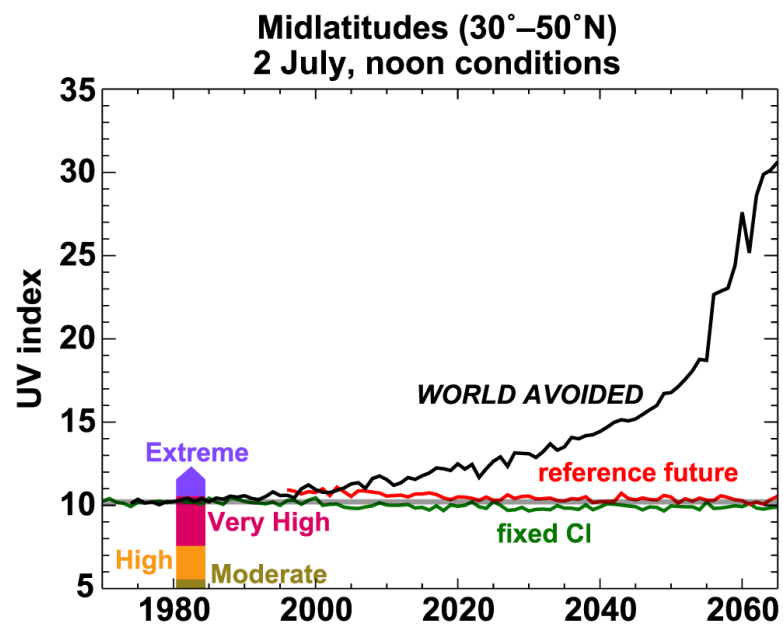


Figure 9. UV index versus year for simulated scenarios: scenario where CFCs never released (fixed CI); current scenario where CFCs were phased out after Montreal Protocol (reference future) and scenario in which ozone depleting substances were never regulated (*WORLD AVOIDED*) (Newman et al., 2009)

2.2. PLANT RESPONSES TO UV-B RADIATION

Plant responses to UV-B can be classified into stress responses and photomorphogenic responses, usually coinciding with expositions to high and low fluent rates of UV-B radiation, respectively (Fig. 10). The plants genotypic characteristics (e.g. constitutive levels of UV shielding compounds) and their previous acclimation are important factors determining whether a plant is UV-B stressed or not. Usually plants growing within their natural distribution are acclimated to UV-B (Jansen et al., 2012).

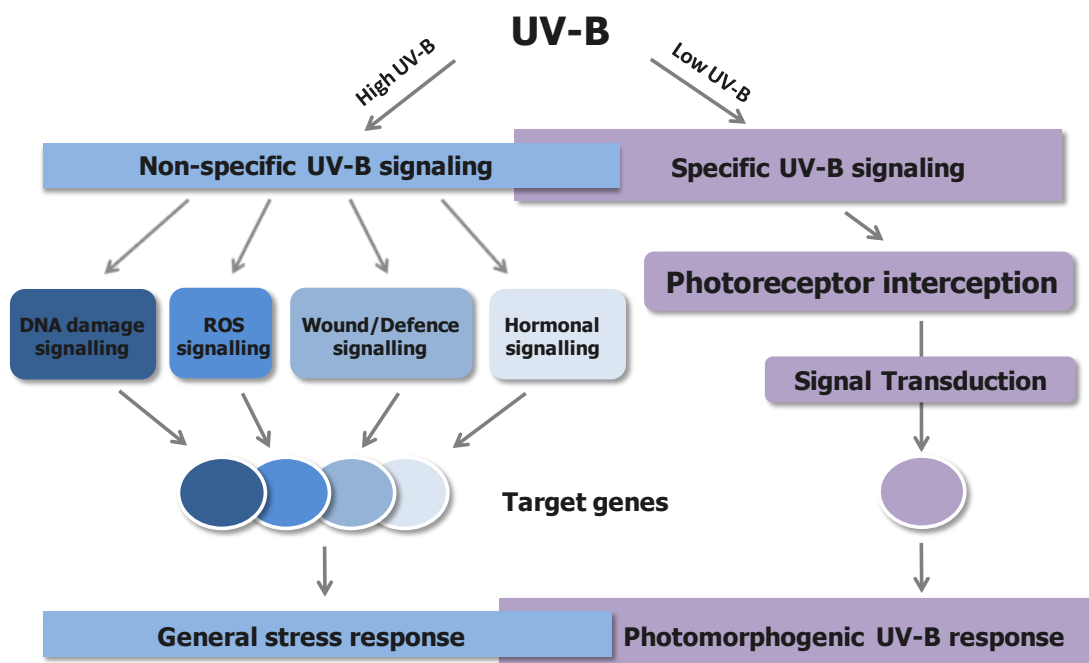


Figure 10. UV-B signal transduction pathways (Adapted from Jenkins et al., 2009 and Bandurska et al., 2013)

UV-B radiation has been traditionally considered as an environmental stress factor, able to induce damaging effects on plants. This “narrow perspective” resulted from the use of high UV-B fluent rates in experiments associated to ozone depletion (reviewed by Kakani et al., 2003); and it dominated the field of UV-B research for decades (Jansen and Bornman, 2012). Plant UV-B stress response is characterised by the activation of non-specific

signalling pathways also triggered by other stresses such as DNA damage, pathogen defence or wounding (Jenkins 2009) (Fig. 9). Although UV-B radiation may target mainly nucleic acids and proteins, the oxidizing side of photosystem II (i.e. degradation of D1 and D2 proteins) and reaction centres may get significantly damaged (Bornman, 1989; Jansen et al., 1998). Non-specific pathways may be mediated by cellular damage sub-products such as ROS, leading antioxidant enzyme up-regulation (Jansen et al., 2012), but also, hydrogen peroxide, nitric oxide, abscisic acid, jasmonic acid, ethylene and salicylic acid participate in the activation of defence mechanisms (reviewed by Bandurska et al., 2013). In addition, UV-B radiation may also trigger antioxidant enzyme genes expression, through signal cascades downwards of UV-B photoreception without the stimulus of oxidative stress (Hideg et al., 2013). Therefore, plant responses to UV-B are often the result of a combination of specific and non-specific pathways (Fig. 10).

UV-B is now regarded as a specific modulator, not so much as a generic stress factor, and current research is mainly driven to investigate the regulatory effects of UV-B radiation within the natural light environment (Jansen and Bornman, 2012). The recent elucidation of the role of ULTRAVIOLET RESISTANCE LOCUS 8 (UVR8; Rizzini et al., 2011) in UV-B signalling is one of the major contributions to consider UV-B as a specific modulator. UVR8 protein forms homodimers maintained by salt-bridges interactions between charged amino acids at the dimeric interface. Interception of UVR8 dimers by UV-B radiation causes the neutralization of salt bridges leading to monomerization (Christie et al., 2012). UVR8 monomers interact directly with CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1, Fig. 11) for the transcriptional activation of the ELONGATED HYPOCOTYL 5 (HY5). This transcription factor controls the regulation of around 20% of the light-regulated genes and triggers photomorphogenic responses under a wide range of wavelengths (i.e. far red, red and blue light) (Koornneef et al., 1980; Oyama, et al., 1997; Osterlund et al., 2000), including those required for UV-B acclimation (Heijde and Ulm, 2012). REPRESSORS OF UV-B

PHOTOMORPHOGENESIS proteins (RUP1 and RUP2) mediate in the re-dimerization of UVR8. This terminates COP1-UVR8 interaction (inactivates the signalling pathway) and regenerates UVR8 dimers pool for a UV-B perception (Tilbrook et al., 2013).

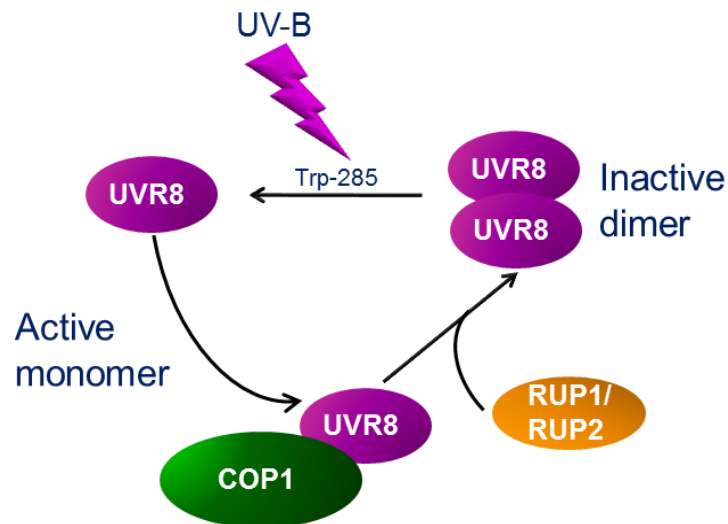


Figure 11. The UVR8 photocycle (Heijde and Ulm, 2013)

Among the photomorphogenic effects caused by UV-B are reduced leaf expansion, increased leaf thickness, and finally, accumulation of leaf phenolic compounds and cuticle waxes, which in turn may result in altered morphology and metabolic cost, which may lead to biomass reduction (reviewed by Wargen and Jordan, 2013). One of the most relevant photomorphogenic responses to UV-B radiation is the up-regulation of flavonoid biosynthesis. UVR8-COP1 conjugate is able to transactivate the transcription factor VvMYBF1 (AtMYB12; Mehrtens et al., 2005), which activates the promoter of *CRUY* (encoding for a putative lyase), *CHS* and *FLS*. CHS and FLS enzymes are directly involved in flavonoid synthesis. While CHS enzyme synthesizes precursors for the general flavonoid biosynthesis, FLS is exclusively committed to the synthesis of flavonols, which is the group of flavonoids that more efficiently absorbs UV-B radiation (Cerovic et al., 2002). In grape berries, UV-B may up-regulate the biosynthesis of several flavonoids (Berli et al., 2011). As

described in section 1.5.4., these compounds are well-valued components of wine grapes, but in turn, the phenolic compounds may reduce the plant susceptibility to pathogens (Keller et al., 2003).

3. CLIMATE CHANGE RELATED FACTORS

3.1. ATMOSPHERIC CO₂ CONCENTRATION, AIR TEMPERATURE AND WATER AVAILABILITY

Since the beginning of industrial development, gases such as water vapour, carbon dioxide (CO₂), methane (CH₄), mono-nitrogen oxides (NO_x) and CFC compounds have accumulated in the atmosphere. These gases are the so-called heat-trapping gases, due to their absorption of infrared radiation emitted by the sun. As a consequence, present average air temperature is 0.6°C higher (compared to pre-industrialization record reference) due to the anthropic release of these gases (IPCC, 2013). CO₂ is regarded to cause most of this warming, mainly due to its larger concentration, as other compounds may have a higher heat-trapping ability. Even though plants are able to fixate atmospheric CO₂ and oceans act as gigantic CO₂ traps, the half-life of a CO₂ molecule in the atmosphere is higher than any other green-house gas (IPCC, 2007b). Since the preindustrial period the concentration of CO₂ has rose from 280 ppm to the present 400 ppm.

Under a “business as usual” modelling of CO₂ emissions, atmospheric CO₂ levels may eventually rise up to 700 ppm by the end of the 21st century (IPCC, 2007a). Such level of atmospheric CO₂ may result in an increase of up to 4.8°C in average air temperature, depending on future CO₂ emissions (Fig. 12) (IPCC, 2013).

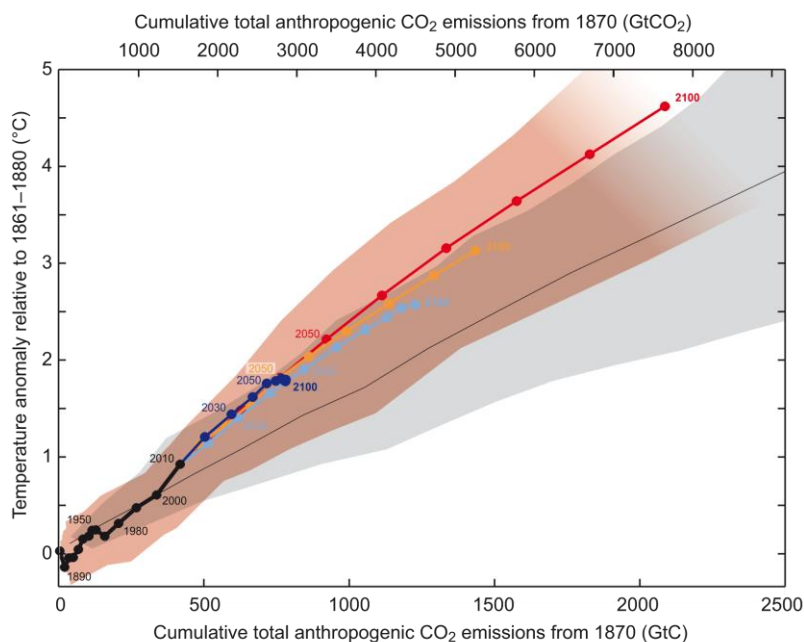


Figure 12. Cumulative CO₂ emissions versus the predicted temperature increase. Different simulations (colour lines) respond to emission policies. For more details see IPCC (2013)

Due to the increase in temperature, global evaporative demand is expected to change, which in turn may alter cloud patterns. Higher evaporation may induce more precipitations, but not necessarily uniformly distributed. For the Mediterranean region, long-term simulations (up to 2100 A.C.) show a decrease in precipitations of up to 30%, in part due to a reduction of cloud coverage, thus leading to an increase in solar radiation reaching the earth surface (IPCC, 2013; Trenberth and Fasullo, 2009). In addition to changes in global CO₂, temperature and precipitation trends, extreme weather and climate events will be more likely in the future.

3.2. EFFECTS OF WATER DEFICIT, ELEVATED TEMPERATURE AND ELEVATED CO₂ ON GRAPEVINE

The effect of water deficit on grapevine has been well documented due to the seasonal drought experienced in the main viticultural regions. Nevertheless, contradictory effects may be found in the literature due to the wide range of situations encompassed by water deficit studies (Chaves et al., 2010). A mild or moderated water deficit may promote abscisic acid

biosynthesis, a phytohormone involved in ripening control (Coombe and Hale, 1973). Water deficit also promotes the accumulation of flavonoids, and other wine quality-related traits (Castellarin et al., 2007a; Deluc et al., 2009; Olle et al., 2011; van Leeuwen et al., 2009). The effect of water deficit on berry acidity is not clear. However, some hints suggest that it may promote organic acid (mostly malate) breakdown (Conde et al., 2007 and references therein). As water deficit becomes more severe, grapevine may suffer a dramatic reduction in carbon assimilation and canopy leaf area, which may compromise grape quality and yield; and, in extreme cases, plant survival (Chaves et al. 2010 and references therein).

Modelling of future conditions has also been focused in assessing the impact of global temperature rise on the distribution of wine grape producing areas (Hannah et al., 2013; Webb et al., 2007; White et al., 2006). These models predict major reductions in the extension of the current premium wine regions and major changes in grapevine phenology due to temperature rise. Changes in phenology may not constitute a problem itself. However, they are tightly associated to the decoupling of sugar accumulation from the synthesis / breakdown of important quality-related metabolites, leading to changes in the sensory traits such as, colour, acidity and bitterness (Bonada et al., 2013; Sadras and Moran, 2012; Sadras et al., 2013).

For C_3 plants such as grapevine, carbon fixation is limited by CO_2 levels at least up to 800 ppm (Long et al., 2006). Thus, the increased carbon assimilation under elevated CO_2 often leads to an over-accumulation of carbohydrates in the leaves, which results in photosynthesis inhibition (Paul and Pellny, 2003). Additional carbon sinks, such as leguminous nodules may prevent photosynthesis down-regulation under elevated CO_2 (Bertrand et al., 2007; Erice et al., 2006). In addition, it remains unclear to what extent the higher carbohydrate levels in the leaves may be translocated to berries altering the ripening process (Mira de Orduña, 2010).

Whereas grapevine responses to elevated CO₂, temperature or water deficit as single environmental stress factors have been documented, little is known about grapevine responses to their combined effects. In this respect, Salazar-Parra (2011) performed several experiments aiming to unravel the interactive effects of the most characteristic factors of climate change, elevated CO₂ (700 ppm), elevated temperature (+4°C) and water deficit. Results show slightly higher preponderance of CO₂ above temperature inducing phenological changes, having the combination of the two factors the strongest effect. It is generally conceived that the shorter grapevine life cycle observed in the last 30 years due to climate change is correlated with changes in wine composition (Mira de Orduña, 2010). Adaptive strategies have been successfully implemented to counteract or mitigate these effects (Martínez de Toda and Balda Manzanos, 2011, 2014; van Leeuwen et al., 2013). Therefore, understanding the underlying mechanisms affecting grape ripening in response to environmental conditions may provide the hints to design the best adaptive strategies.

4. RESEARCH UNDER CONTROLLED CONDITIONS

4.1. USING GREENHOUSES TO SIMULATE CLIMATE CHANGE CONDITIONS

Assessing the impact of climate change factors, temperature, CO₂, water availability and UV-B radiation on plants, has been one of the main goals in plant biology research for the last two decades (Long et al., 2004). With the aim of studying in depth the mechanisms involved in the responses of plants to these environmental factors, both individually or in combination, it is necessary to control carefully the different variables. This is the main limitation of field studies. In this regard, the growth chamber-greenhouses have been proposed as suitable facilities for such studies (Morales et al., 2014) (Fig. 14).

The use of artificial UV-B sources is required to study the effect of UV-B on plants under controlled conditions. UV-B lamps spectrum may differ significantly from one model to another, and from solar UV-B reaching the earth surface. In order to make UV-B doses applied with different lamps quantitatively comparable to solar radiation doses, several Biological Spectral Weighing Functions (BSWF) have been proposed, being Caldwell (1971), as formulated by Green et al. (1974), the most widely reported. This is a way of weighing the irradiance emitted by the source according to the effectiveness of each wavelength in eliciting a photobiological response (Fig. 13). In the case of Caldwell (1971) this model is based on the growth inhibition of the first leaf in cucumber seedlings (*Cucumis sativus*).

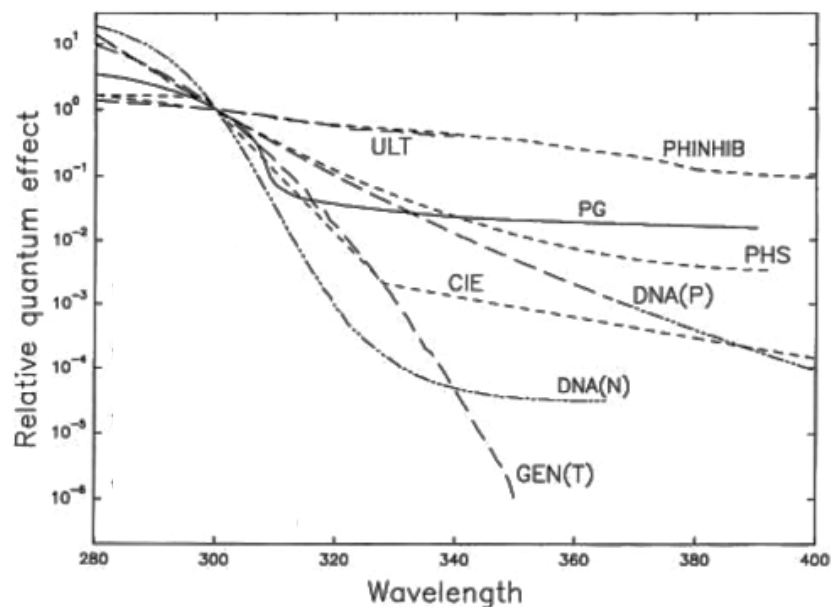


Fig. 13. Different equations to weigh UV wavelengths according to their effect on biological processes. E.g. GEN(T) stands for general plant action spectrum based on growth inhibition of the first leaf in cucumber seedlings (Flint and Caldwell, 2003 and references therein)

4.2. FRUIT-BEARING CUTTINGS MODEL

Grapevines are woody species and it takes several years before they become reproductive. In the vineyards, their roots tend to penetrate several meters into the soil and they need a great volume of substrate when potted. Therefore the maintenance of a full grown vine within

an experimental facility may be inconvenient when space and time are limited. The fruit-bearing cuttings model for grapevine (fruiting plants developed from rooted cuttings; Mullins, 1966, adapted to Tempranillo by Santa María, 2004) provides a feasible system that allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines, under fully controlled environmental conditions (Fig. 14). The plants obtained in this way respond like vineyard plants to different cultural and environmental conditions (Lebon et al., 2005; Ollat et al., 1998). It constitutes a reliable model for studies regarding grape metabolomic and transcriptomic (Carbonell-Bejerano et al., 2013; Dai et al., 2013). In addition, this technique has been successfully used to test the effect of different environmental factors on grapevine physiology (Antolin et al., 2010; Niculcea et al., 2014; Niculcea et al., 2013; Salazar-Parra et al., 2012a, b; Salazar Parra et al., 2010).



Figure 14. Fruit-bearing cutting and experimental set up inside the glasshouse with UV-B lamp array placed above the plants.

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OBJECTIVES

The main objective of the thesis was to assess the effect of UV-B radiation on grapevine leaf physiology and berry composition, framed within the climate change scenario (water deficit, elevated CO₂ and elevated temperature).

The general objective was divided into the following partial objectives and their respective tasks:

Objective 1. Characterize the adaptive response of grapevine to UV-B radiation, as a single factor or combined with other climate change-related factors (water deficit, elevated CO₂ and elevated temperature)

1.1. Characterize leaf photosynthetic performance (gas exchange and chlorophyll a fluorescence) and leaf photosynthetic pigments profile

1.2. Study the activity of key enzymes involved in the antioxidant protective system and the accumulation of UV-B absorbing compounds in leaves

Objective 2. Evaluate the changes in grape berry composition induced by the exposure to UV-B radiation, as a single factor or combined with water deficit, elevated CO₂ and elevated temperature

2.1. Determine the main general quality traits of the must

2.2. Characterize anthocyanin, flavonol and amino acid profiles of grape berries

Objective 3. Study the mechanisms underlying the changes induced by UV-B, as a single factor or in interaction with climate change-related factors, on the concentration and profile of anthocyanins and flavonols in grape berries

3.1. Determine gene expression of key enzymes involved in the anthocyanin and flavonol biosynthetic pathways

Objective 4. Assess whether changes induced by UV-B, as a single factor or in interaction with CO₂ and temperature, on grapevine leaf physiological status have significant effects on grape ripening rates

4.1. Study leaf photosynthetic performance

4.2. Determine yield and yield components

4.3. Assess the ripening rates of grapes

The above exposed objectives have been developed in the following chapters:

Chapter 1. Short- and long-term physiological responses of grapevine leaves to UV-B radiation (Objective 1)

Chapter 2. Ultraviolet-B radiation modifies the quantitative and qualitative profile of flavonoids and amino acids in grape berries (Objective 2)

Chapter 3. Characterization of the adaptive response of grapevine (cv. Tempranillo) to UV-B radiation under water deficit conditions (Objectives 1 and 4)

Chapter 4. Ultraviolet-B radiation and water deficit interact to alter flavonol and anthocyanin profile in grapevine berries through transcriptomic regulation (Objectives 2 and 3)

Chapter 5. Climate change conditions (elevated CO₂ and temperature) and UV-B radiation affect grapevine (*Vitis vinifera* cv. Tempranillo) leaf carbon metabolism, altering fruit ripening rates (Objectives 1 and 4)

Chapter 6. UV-B alleviates the uncoupling effect of climate change conditions (elevated CO₂-temperature) on grape berry (*Vitis vinifera* cv. Tempranillo) anthocyanin-sugar accumulation (Objectives 2 and 3)

CHAPTER 1

Short- and long-term physiological responses of grapevine leaves to UV-B radiation

Short- and long-term physiological responses of grapevine leaves to UV-B radiation

ABSTRACT

The present study aimed at evaluating the short- and long-term effects of UV-B radiation on leaves of grapevine *Vitis vinifera* (cv. Tempranillo). Grapevine fruit-bearing cuttings were exposed to two doses of supplemental biologically effective UV-B radiation (UV-B_{BE}) under glasshouse-controlled conditions: 5.98 and 9.66 kJ m⁻² day⁻¹. A 0 kJ m⁻² d⁻¹ UV-B treatment was included as control. The treatments were applied either for 20 days (from mid-veraison to ripeness) or 75 days (from fruit set to ripeness). The main effects of UV-B were observed after the short-term exposure (20 d) to 9.66 kJ m⁻² d⁻¹. Significant decreases in net photosynthesis, stomatal conductance, sub-stomatal CO₂ concentration, the actual photosystem II (PSII) efficiency, total soluble proteins and de-epoxidation state of the VAZ cycle were observed, whereas the activities of several antioxidant enzymes increased significantly. UV-B did not markedly affect dark respiration, photorespiration, the maximum potential PSII efficiency (F_v/F_m), non-photochemical quenching (NPQ), as well as the intrinsic PSII efficiency. However, after 75 d of exposure to 5.98 and 9.66 kJ m⁻² d⁻¹ UV-B most photosynthetic and biochemical variables were unaffected and there were no sign of oxidative damage in leaves. The results suggest a high long-term acclimation capacity of grapevine to high UV-B levels, associated to a high accumulation of UV-B absorbing compounds in leaves, whereas plants seemed to be tolerant to moderate doses of UV-B.

Keywords: *Vitis vinifera* L., photosynthesis, UV-B radiation, UV-B absorbing compounds, antioxidant enzymes, water deficit

1. INTRODUCTION

Over the last 50 years, stratospheric ozone has decreased 3-6% (UNEP, 2012), primarily due to the chlorine- and bromine-containing compounds released into the atmosphere by anthropogenic activities (Kakani et al., 2003, and references therein). Reductions in the ozone column since 1980 led to an increase of 6-14% in UV-B radiation at the surface of the Earth, which is expected to revert to the pre-1980 level by 2040-2070 if all member countries implement the Montreal Protocol (UNEP, 2012). Nevertheless, predicted scenarios of climate change over the next decade include enhanced levels of incident UV-B radiation because of fluctuations in cloud patterns (IPCC, 2007).

UV-B radiation has been considered historically as an environmental stress factor, and the specific effects on plants include inhibition of photosynthesis, biomass reduction, alterations in pigment and protein synthesis, damage to DNA or alteration of reproductive biology (Jordan, 1996; Kakani et al., 2003). Nevertheless, UV-B is not necessarily a damage-inducing source of stress. Moderate non-damaging levels of UV-B act as an important environmental signal in higher plants, regulating several key developmental plant responses (Pontín et al., 2010) that can be termed as an induced acclimation (Jenkins, 2009). Many recent experimental approaches reflect a renewed focus on the regulatory effects of low fluence rates of UV-B. Low UV-B rates promote the expression of an array of genes involved in UV-B protection or repair of UV-B damage, including the accumulation of UV-B absorbing phenolic compounds in epidermal tissues and the enhancement of the cellular antioxidant system (Brosché and Strid, 2003; Jordan, 2002; Pontín et al., 2010; Rozema et al., 1997).

Grapevines are usually grown in the Mediterranean area, thus being often exposed to high UV-B intensities because of a cloudless summer sky (Doupis et al., 2011). UV-B doses can rise up to $6.94 \text{ kJ m}^{-2} \text{ d}^{-1}$ under clear sky conditions at the experimental location (northern of Spain), estimated according to the Tropospheric Ultraviolet and Visible Radiation Model (TUV Model, University Corporation for Atmospheric Research). However, other grapevine

suitable locations in the southern hemisphere (i.e. Cape point in South Africa and Mendoza, Argentina) can reach up to 10.24 and 10.30 kJ m⁻² d⁻¹ respectively under the same conditions. Besides, given the progressive global warming and its potential deleterious effect on the grape berry composition (Buttrose et al., 1971), many growers consider the possibility of moving their vineyards to higher and thus cooler areas. Such variation in altitude would result in higher fluence rates and dosages of UV-B reaching vineyard in the coming years.

Many studies on the effect of UV-B on grapevine leaf physiology have been carried out after short-term UV-B exposures (Berli et al., 2010; Doupis et al., 2011; Majer and Hideg, 2012; Núñez-Olivera et al., 2006). Nevertheless, long-term UV-B effects may take longer to manifest and they are not well understood yet. Therefore, the objective of the present work was to characterize the response of grapevine (*Vitis vinifera* L.) cv. Tempranillo to short- and long-term supplemental UV-B exposure, focusing on the leaf photosynthetic performance, pigment composition and antioxidant defenses. The study tries to understand what are the mechanisms underlying the acclimation of grapevine to UV-B radiation. The fruit-bearing cuttings model system for grapevine (fruiting plants developed from rooted cuttings) has been used providing a much more feasible system to study environmental effects on grapevine physiology, independently from environmental fluctuations, than the use of vines in the field. This model allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines, under fully controlled environmental conditions. The plants obtained in this way respond like vineyard plants to different cultural factors (Dai et al., 2013; Lebon et al., 2005; Ollat et al., 1998). Indeed, the fruit-bearing cuttings model has been previously useful in the evaluation of the physiological response of grapevine as well as berry ripening to environmental or developmental factors (Dai et al., 2013; Lebon et al., 2005; Ollat et al., 1998; Salazar-Parra et al., 2012a, b; Salazar Parra et al., 2010).

2. MATERIAL AND METHODS

Plant material and growth conditions

Dormant cuttings of *Vitis vinifera* L. cv. Tempranillo were obtained from an experimental vineyard of the Station of Viticulture and Enology of Navarra (Olite, Spain). Cuttings were selected to get fruit-bearing cuttings according to Mullins (1966) and modified by Ollat et al. (1998) and Santa María (2004). Rooting was induced using indol butiric acid, in a heat-bed (27°C) kept in a cool room (5°C). One month later (December 2010), rooted cuttings were planted in 4 L plastic pots containing a mixture of peat and perlite (2:1, v/v) and transferred to a glasshouse. Only a single flowering stem was allowed to develop on each plant. Shoots were fixed to horizontal rods compelling leaves facing upwards. Vegetative growth was controlled by pruning, thus maintaining in all the treatments a leaf area to grape mass ratio optimal for berry ripening (around 10 leaves per plant) (Jackson and Lombard, 1993). Growth conditions in the glasshouse throughout the whole experiment were 25/15°C and 50/90% relative humidity (RH) (day/night) and natural daylight (photosynthetic photon flux density, PPF, was on average 850 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at mid-day) supplemented with high-pressure sodium lamps (SON-T Agro Phillips, Eindhoven, Netherlands) to extend the photoperiod up to 14 h and ensure a minimum PPF of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from 7 AM to 9 PM (minimum of 18 $\text{mol m}^{-2} \text{day}^{-1}$ PAR). Humidity and temperature were controlled using M22W2HT4X transmitters (Rotronic Instrument Corp., Hauppauge, USA). PPF was monitored with a LI-190SZ quantum sensor (LI-COR, Lincoln, USA). Plants were irrigated with the nutrient solution detailed by Ollat et al. (1998).

Experimental design

Fruit-bearing cuttings (4-6 plants per treatment) were exposed to two doses of biologically effective UV-B (UV-B_{BE}) irradiance, calculated using the generalized plant action spectrum (Caldwell, 1971): 5.98 and 9.66 $\text{kJ m}^{-2} \text{day}^{-1}$ (0.55 W m^{-2} irradiance for 3 h and 4 h 51 min, respectively). These treatments were applied either for 20 days, short-term exposure from

mid-veraison to ripeness (March 2010) or 75 days, long-term exposure from fruit set to ripeness (January-March 2010) (Figure 1). A treatment with $0 \text{ kJ m}^{-2} \text{ day}^{-1}$ UV-B was included as control and it was achieved by the screening effect of the glasshouse (>99% decrease of solar UV-B light). Supplemental UV-B was applied using Philips TL 100W/01 tubes (311 to 313 nm spectrum peaking, Philips, Eindhoven, The Netherlands) suspended 0.35 m above the canopy. The lamps in the $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ treatment were unlit. Vertical polyester film (SKC199, Nuenka, Spain) curtains were hung between treatments to prevent the UV-B radiation reaching the control plants. Spectral distribution of UV lamps as well as UV irradiance was measured using a double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd, Livingstone, Scotland). The applied biological effective dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ corresponded to an 86% of the maximum daily dose in the study site. The dose of $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ was chosen to test plant's response to elevated UV-B radiation and ensure contrasting effects on photosynthesis and accumulation of UV-B screening compounds, if any, rather than to attempt realistic simulation of field conditions.

Given the importance of comparing leaves of the same age in UV studies (Majer and Hideg, 2012), young fully expanded leaves of the same physiological age (around four week old) were chosen for gas exchange, chlorophyll fluorescence and biochemical determinations. In the treatment with UV-B for 20 d, the leaves chosen for measurements were fully developed before the beginning of the UV-B treatments, under the glasshouse conditions described. In the treatment of 75 d, the leaves measured fully developed during the treatments with UV-B.

Gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll (Chl) fluorescence measurements were conducted using a portable photosynthesis system (GFS-3000, Walz, Germany) with a 3 cm^2 cuvette. Dark respiration (R_D) measurements were performed 2 h before the beginning of the light period, with the CO_2 concentration set at 375 ppm, the temperature in the measurement chamber at 25°C , and 50% RH. Gas exchange characteristics in illuminated leaves were measured 3 h

after the beginning of the light period, under a photon flux density of $1200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 375 ppm CO_2 , 25°C , and $50\% \text{ RH}$. Dark respiration, net photosynthesis (A_{net}), stomatal conductance (g_s) and sub-stomatal CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). Chl fluorescence was measured immediately after gas exchange measurements (in the dark and in the light) with a fluorescence module (PAM-fluorometer 3055-FL, Walz, Germany) attached to the photosynthesis equipment. The minimal and maximal fluorescence (F_o and F_m , respectively) were measured in dark-adapted leaves, whereas F_o' and F_m' were measured in the same leaves, with a photon flux density of $1200 \mu\text{mol m}^{-2} \text{ s}^{-1}$, also measuring steady-state fluorescence signal (F_s). The maximum potential PSII efficiency was calculated as F_v/F_m , where F_v is $F_m - F_o$ (Morales et al., 1991). Actual (Φ_{PSII}) and intrinsic ($\Phi_{\text{exc.}}$) PSII efficiencies were calculated as $(F_m' - F_s)/F_m'$ and F_v'/F_m' (where F_v' is $F_m' - F_o'$), respectively. Photochemical quenching (qP) was calculated as $(F_m' - F_s)/F_v'$, and non-photochemical quenching (NPQ) as $(F_m/F_m') - 1$ (Larbi et al., 2004). Electron transport rate (ETR) was calculated according to Krall and Edwards (1992) as $\Phi_{\text{PSII}} \times \text{PPFD} \times 0.5 \times 0.84$, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII (Ogren and Evans, 1993) and 0.84 is the fractional leaf absorptance, considered the most common absorptance coefficient for *Vitis vinifera* leaves under a wide range of environmental conditions and ages (Schultz, 1996). Multiplying 0.84×0.5 gives a value of 0.42, a value very similar to the α term used by other researchers to calculate ETR, which includes the product of leaf absorptance and the partitioning of absorbed quanta between PSI and PSII, and determined as the slope of the relationship between Φ_{PSII} and Φ_{CO_2} (i.e. the quantum efficiency of gross CO_2 fixation), obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing $1\% \text{ O}_2$ (Valentini et al., 1995). For grapevine cv. Tempranillo, α was reported to be 0.425 (Pérez-Martín et al., 2009). Photorespiration (R_L) was estimated as $1/12(\text{ETR} - 4 \times (A_{\text{net}} + R_D))$, according to Valentini et al. (1995).

Sampling for biochemical assays

The same leaves used for photosynthesis and Chl fluorescence measurements were used for the biochemical analysis. Two leaf disks of 1 cm² were harvested immediately after gas exchange and Chl fluorescence measurements, cut with a calibrated cork borer, wrapped in aluminum foil, immediately plunged into liquid N₂ and stored at -80°C until photosynthetic pigments and methanol extractable UV absorbing compounds (MEUVAC) analyses. Afterwards, the leaf was detached and frozen in liquid N₂ for lipid peroxidation (thiobarbituric acid reacting substances, TBARS), antioxidant enzyme activities and protein determinations.

Photosynthetic pigments and MEUVAC

Leaf photosynthetic pigments were extracted with 100% acetone in the presence of Na ascorbate, filtered through a 0.45-µm filter and quantified by HPLC (Larbi et al., 2004). Two steps were used: mobile phase A (acetonitrile:methanol, 7:1, v/v) was pumped for 3.5 min, and then mobile phase B (acetonitrile:methanol:water:ethyl acetate, 7:0.96:0.04:8 by volume) was pumped for 4.5 min. To both solvents, 0.7% (volume) of the modified triethylamine (TEA) was added to improve pigment stability during separation (Hill and Kind, 1993). Depoxidation state of the xanthophyll cycle (DES) was calculated as the sum of antheraxanthin (A) and zeaxanthin (Z) concentrations divided by the sum of violaxanthin (V), A and Z concentrations. For extraction of UV-absorbing compounds, frozen leaf disks were extracted with 10 mL of methanol:water: 7M HCl, equivalent to 70:29:1 (v/v/v). After 15 min at 85°C in the dark, the extract was centrifuged for 15 min at 2,000 g. The absorbance spectrum was measured in a spectrophotometer UV/Vis (UVMMini 1240, Shimadzu, Kyoto, Japan). The amount of methanol-extractable UV-B absorbing compounds (MEUVAC) was expressed in arbitrary units as the area under the absorbance curve in the UV-B interval (AUC₂₈₀₋₃₁₅) calculated per unit of leaf area (Dai et al., 2004).

Determination of total soluble protein and antioxidant enzymes

Leaf tissue (1 g) was homogenized with 10 mL of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA- Na_2 , 0.5 mM ascorbate and 1% PVPP (polyvinyl polypyrrolidone) in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min. The supernatant was used for determinations of protein content and antioxidant enzyme activity. Total soluble protein concentration was determined as described by Bradford (1976) using bovine serum albumin as standard. Superoxide dismutase (SOD, EC 1.15.1.1) was determined by the nitroblue tetrazolium (NBT) method (Dhindsa et al., 1981) with some modifications. The reaction mixture contained 4 mL of 2.2 μM riboflavin, 14 mM methionine, 80 μM NBT diluted in 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA- Na_2 and 0.1 mL of extract. The mixtures were illuminated with fluorescent lamps in a chamber at 25°C. The reduction of NBT was determined at 560 nm. Blanks and controls were performed without illumination and extract, respectively. Results were expressed in units of activity. One unit of SOD activity was defined as the amount of enzyme necessary to inhibit by 50% the NBT photoreduction under the assay conditions. Guaiacol peroxidase (GPX, EC 1.11.1.7) assay was performed using the method described by Pütter (1974). The mixture assay contained 0.1 mL of extracts, 50 μL of 12 mM H_2O_2 and 50 μL of 20 mM guaiacol, diluted in 2.4 mL of 0.1 M potassium phosphate buffer (pH 7). GPX activity was calculated following the oxidation of guaiacol at 436 nm using an extinction coefficient of 26.6 $\text{mM}^{-1} \text{cm}^{-1}$. Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of H_2O_2 disappearance at 260 nm as described by Aebi (1984) with some modifications. The incubation mixture contained 50 μL of extracts and 50 μL of 300 mM H_2O_2 , diluted in 1 mL of 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA- Na_2 . A calibration curve was set with H_2O_2 . Ascorbate peroxidase (APX, CE 1.11.1.11) activity was determined as described by Nakano and Asada (1981) with some modifications. The reaction mixture contained 50 μL of extracts, 0.1 mL of 5 mM H_2O_2 and 1 mL of 1 mM ascorbate, diluted in 80 mM potassium phosphate buffer (pH 7) with 0.1 mM DTPA, and then the absorbance at 290

nm was determined. Controls without H₂O₂ were performed and a calibration curve was set with sodium ascorbate.

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reacting substances (TBARS), as described by Dhindsa et al. (1981). Frozen leaf tissue (0.5 g) was homogenized with 10 mL trichloroacetic acid (TCA) 0.1% (w/v) in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min. Two mL of extract were mixed with 2 mL of either (i) 20% (w/v) TCA solution or (ii) 20% TCA solution containing 0.5% (w/v) TBA. The mixtures were heated at 95°C for 30 min, quickly cooled and centrifuged at 10,000 g and 4°C for 10 min. The absorbance of the supernatant was read at 532 nm with the values for non-specific absorption at 600 nm subtracted. TBARS concentration was calculated using the following formula: $[(A_{532} \times 1000) - (A_{600} \times 1000)] / 155$ being 155 the extinction coefficient in $\text{mM}^{-1} \text{cm}^{-1}$ (Heath and Packer, 1965).

Statistical analyses

One way ANOVA was performed using the SPSS 15.0 statistical package for Windows. When the *F* ratio was significant ($p < 0.05$), differences among groups were tested with Least Significant Difference (LSD) post-hoc test.

3. RESULTS

Gas exchange and chlorophyll fluorescence

The dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ UV-B, applied either for 20 d or 70 d, did not modify significantly the leaf gas exchange and Chl fluorescence parameters (Figures 2 and 3). Short-term exposure (20 d) to $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ UV-B caused a significant decline in A_{net} , g_s , E , C_i , Φ_{PSII} and $\Phi_{\text{exc.}}$, whereas the long-term exposure (75 d) to the same UV irradiance slightly decreased, although not significantly, these parameters (Figures 2 and 3). Dark respiration and photorespiration were not statistically different among leaves exposed and non-exposed

to UV-B (Figure 2). Neither the long- nor the short-term exposure to 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B modified qP, NPQ, Fv/Fm and the ratio ETR/A_{net}+R_D+R_L (Figure 3).

Photosynthetic pigments and MEUVAC

In general, both the short- and the long-term exposure to UV-B reduced total leaf Chl and carotenoid concentration compared with non-irradiated plants (Table 1). There was not significant effect of UV-B on neoxanthin, lutein and violaxanthin concentrations, whereas β-carotene concentration significantly increased in plants exposed to 5.98 kJ m⁻² d⁻¹ for 20 d (Table 1). By contrast, the short-term exposure to 9.66 kJ m⁻² d⁻¹ of UV-B significantly reduced antheraxanthin and zeaxanthin concentrations (Table 1). In general, the depoxidation state of xanthophyll cycle was decreased in UV-B irradiated plants, especially after the short-term irradiation with 9.66 kJ m⁻² d⁻¹ of UV-B (Table 1).

Leaf MEUVAC concentration significantly increased through supplementary UV-B radiation doses (Figure 4). Leaves of plants exposed for 75 d exhibited higher levels of MEUVAC than plants exposed for 20 d. The leaf extract absorbance spectra showed a single maximum at 330-340 nm in the treatments with the low dose of UV-B (5.98 kJ m⁻² d⁻¹), whereas under the high UV-B irradiation (9.66 kJ m⁻² d⁻¹) two maxima absorption were observed at 260 and 330-340 nm (Figure 5).

Total soluble proteins, antioxidant enzymes and lipid peroxidation

Leaves of plants exposed to 9.66 kJ m⁻² d⁻¹ of UV-B for 20 d had significantly lower total soluble protein concentration than non-exposed leaves (Table 2). The concentration of TBARS did not significantly enhance under UV-B radiation, and it even decreased after the long-term treatment (75 d) with the highest dose of UV-B (Table 2). The short-term exposure of plants to 9.66 kJ m⁻² d⁻¹ of UV-B resulted in significantly higher SOD, GPX, CAT and APX activities (Table 2). Nevertheless, after 75 d, UV-B irradiated plants had similar levels of antioxidant activities than those of non-irradiated plants. In the latter case, only GPX maintained significantly higher activity in UV-B exposed plants than in non-exposed ones.

4. DISCUSSION

A recent meta-analysis of more than 140 studies in both indoor controlled environmental and field conditions concluded that UV-B radiation reduced net photosynthesis in herbaceous plants; nevertheless the report displayed conflicting trends for woody species (Li et al., 2010). The photosynthetic response of plants to UV-B radiation depends on crop species and cultivars, environmental conditions, plant acclimation state, UV-B dosage, and the ratio of PAR to UV-B radiation. In the present study, the short-term exposure to high doses of UV-B decreased net photosynthesis, which was mainly associated with stomatal limitations, as reflected by the impaired stomatal conductance, leaf transpiration and sub-stomatal CO₂ concentration (Figure 2). Our study was carried out in plants with a low acclimation state to UV-B and under controlled environment with a square-wave UV-B delivery system and lower PAR irradiance than in an open field, which may have increased the sensitivity of grapevine leaves to UV-B, thus overestimating UV-B effects. Nevertheless, the decrease in A_{net} and stomatal conductance by UV-B was also reported by Dehariya et al. (2012) under natural conditions and by Klem et al. (2012), particularly in low PAR acclimated plants. Regarding the efficiency of PSII, highly sensitive to UV-B (Kakani et al., 2003 and references therein), Fv/Fm ratio was almost unaltered, thus revealing the absence of permanent PSII photoinhibition and the subsequent photodamage in the present study (Pascual et al., 2010). Nevertheless, some down-regulation of PSII activity (Φ_{PSII}) occurred in the short-term exposed plants to high UV-B levels, through a slight decrease in Φ_{exc} . (Figure 3), which may also have contributed to the impaired A_{net} observed in such treatment. In addition, UV-B significantly reduced total soluble protein concentration after 20 d (Table 2), therefore a decrease in the amount of Rubisco protein could not be ruled out (Allen et al., 1997; Jordan, 1996).

Photosynthetic parameters indicate that the short-term exposure to high intensities of UV-B reduced the amount of light energy used in CO₂ fixation. Under such situation, plants can avoid photoinhibition by either decreasing the absorption of light and/or consuming the

excess excitation energy through photochemical (photorespiration and the Mehler reaction) or non-photochemical (thermal dissipation by PSII antenna) mechanisms (Björkman and Demming-Adams, 1994). With respect to decrease of light absorption, the decrease in Chl concentration under solar UV-B radiation has been suggested as a photoprotective mechanism of grapevine leaves to alleviate over-excitation of PSII reaction centers (Núñez-Olivera et al., 2006). In our study, UV-B treated plants for 20 d showed a decrease in total Chl content (Table 1), in agreement with previous studies (Dehariya et al., 2012; Núñez-Olivera et al., 2006; Surabhi et al., 2009; Zhao et al., 2003). However, the extent of the Chl decreases in the present work would lead to minor changes in leaf absorptance and in the amount of light absorbed by leaves (Morales et al., 1991). With regard to non-photochemical protective mechanisms such as thermal dissipation, NPQ and Φ_{exc} did not change markedly in the short-term UV-B exposed plants in the present study (Figure 3). In addition, the A+Z pool and, in turn, the de-epoxidation state of the xanthophyll cycle did not increase under UV-B, and it even decreased in plants treated with $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ for 20 d (Table 1), in agreement with Yang et al. (2007). The concentration of lutein, which also plays a critical role in the thermal dissipation of excess absorbed light energy (Niyogi et al., 1997), was not affected by UV-B either. The results agree with previous studies on grapevine (cv. Viura and Tempranillo) where NPQ was not significantly altered by UV-B under field conditions (Núñez-Olivera et al., 2006). Although the two experiments cannot be compared directly, due to differences in plant material (growth system and age) and PAR conditions (PPFD much lower in our study), both indicate that heat energy dissipation processes are not involved in the early photosynthetic response of grapevine leaves to UV-B radiation under diverse (natural and supplemental UV-B) conditions. However, plants compensated for the decrease in photosynthesis maintaining dark respiration and slightly, non-significantly, increasing photorespiration (Figure 3), both of them being photochemical mechanisms of photoprotection that consume electrons generated in the photosynthetic electron transport chain. Consequently, the ratio of electrons generated to electrons consumed ($ETR/A_{net}+R_D+R_L$) did not increase significantly under UV-B exposure over a 20 d period.

Such result would indicate that in the short-term UV-B treatment, there was not an excess of electrons derived from photosynthetic reactions available to react with oxygen in the Mehler reaction, generating reactive oxygen species (ROS).

UV-B can produce ROS by more than one mechanism. Mackerness et al. (2001) described the activation of NADPH oxidase and/or peroxidases that catalyse the formation of ROS by UV-B. The activities of ROS-scavenging enzymes, such as SOD, GPX, APX, and CAT, have been reported to increase following UV-B treatment, as these enzymes act as antioxidant compounds that help reduce photooxidative damage in plant leaves (references in Jenkins, 2009). Consistent with this, all the antioxidant enzymes analyzed in the present study were enhanced by supplemental radiation with high UV-B doses after 20 d exposure (Table 2). Our results agree with those of Doupis et al. (2011), who described a preferential synthesis/activation of the antioxidant enzymes by grapevine leaves that may counteract the oxidative stress induced by the exposure to supplemental UV-B for 15 days. The results suggest that the antioxidant system plays an important role in protecting grapevine against increasing UV-B during the first days of exposure, thus minimizing a possible UV-B induced damage in leaves. Indeed, grapevine leaves did not show any signs of oxidative damage at the biochemical level, since there was no evidence for enhanced lipid peroxidation, estimated as TBARS concentration (Table 2).

Whereas a reduction in net photosynthesis and an increased antioxidant activity was observed in the plants exposed to high UV-B doses for 20 d, plants seemed able to develop efficient protective mechanisms after the exposure to the same UV-B dose for 75 d. In fact, the plant photosynthetic performance, antioxidant enzymes and leaf protein content were not altered after the long-term exposure to $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B, thus suggesting a high long-term acclimation capacity of grapevine plants to high UV-B levels. The production of secondary metabolites, such as flavonoids and other UV-B absorbing phenolic compounds, which accumulate mostly in the vacuoles of epidermal cells and reduce the penetration of UV wavelengths deeper into leaves, is an important molecular event underlying UV-B

acclimation in plants, including grapevine (Berli et al., 2010; Kakani et al., 2003; Li et al., 2010; Wargent and Jordan, 2013 and references therein). Additionally, phenolic compounds may enhance protection against oxidative stress, as they possess chemical structures capable of scavenging free radicals (Blokhina et al., 2003). In the present study, UV-B radiation increased leaf MEUVAC concentration (Figure 4), especially in the long-term exposed plants, in agreement with previous studies in different cultivars of grapevine under solar and supplemental UV-B radiation (Berli et al., 2010; Doupis et al., 2011; Núñez-Olivera et al., 2006). The results show that the accumulation of UV-B absorbing compounds may be one of the prominent adaptation mechanisms to long-term exposures to UV-B in these plants. Regarding the changes observed in the extracts absorbance spectra, the results suggest not only a quantitative effect of UV-B on the amount of UV-B absorbing compounds, but also a qualitative effect on their composition regardless of the exposure length, as reported by Ryan et al. (1998).

Grapevine in general has been historically cultivated in high-UV environments, and the cultivar Tempranillo, in particular, is considered indigenous to Spain, thus being probably well adapted to relatively high UV-B irradiance in the Mediterranean climate (Núñez-Olivera et al., 2006). The present study was carried out under glasshouse conditions, with lower PAR irradiance than in open field, which would be expected to increase sensitivity to UV-B (Guidi et al., 2011). However, even under these growth conditions, a moderate UV-B dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$, equivalent to those found on summer clear days in the study site, did not have a significant impact on grapevine photosynthesis performance and leaf soluble protein or antioxidant enzymes. When high UV-B irradiance levels were applied, a decrease in photosynthesis was observed after 20 d of exposure. Nevertheless, the long-term exposure to the same UV-B conditions induced acclimation in absence of photosynthetic stress. Although the different response of grapevine to the short- and long-term UV-B exposure may have been conditioned by differences in the developmental stage of the plant during the treatments (the short-term treatment was applied from veraison to maturity, whereas the

long-term was applied from fruit set to maturity), the results show a high adaptation capacity of grapevine to high UV-B irradiance and supporting the general hypotheses that it is unlikely that significant damage to photosynthesis will occur in a natural setting under the current and projected changes in solar UV-B (Allen et al., 1997; Fiscus and Booker, 1995).

5. CONCLUSIONS

To our knowledge, this is the first study evaluating both the short- and long-term physiological response of grapevine to UV-B radiation. The short-term exposure of non-acclimated plants to high UV-B impaired photosynthetic activity of grapevine leaves, which was mainly associated with stomatal factors. The increase in antioxidant enzyme activities seemed to be the preponderant protection mechanism of grapevine against UV-B-induced oxidative burst during the first days of exposure, preserving leaves from oxidative stress. By contrast, long-term exposures to high UV-B can produce acclimation responses in absence of effects on the photosynthetic activity of PSII. The accumulation of MEUVAC, rather than the antioxidant enzyme activities, may be related to the maintenance in the photosynthesis performance of grapevine, and seemed to be an important mechanism of adaptation to UV-B even under low PAR conditions.

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Table 1. Chlorophyll (Chl) and carotenoid concentrations and de-epoxidation state (DES) of xanthophyll cycle in leaves of plants exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B for 20 and 75 d. Values are means (standard error) of 4-6 replicates. Different letters within the same parameter indicate significant differences (p<0.05).

	20 days			75 days	
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹
Chl a+b (μmol m ⁻²)	725.7 (4.8) a	631.4 (2.8) b	645.3 (23.7) ab	598.7 (61.8) b	623.8 (14.8) b
Chla/Chlb	2.24 (0.02) a	2.24 (0.02) ab	2.18 (0.02) b	2.25 (0.03) a	2.24 (0.01) ab
Carotenoids (mmol mol ⁻¹ Chl)	239.1 (7.2) a	216.1 (5.4) ab	213.4 (21.3) ab	203.5 (4.1) b	213.2 (4.6) ab
β-Carotene (mmol mol ⁻¹ Chl)	85.7 (0.7) a	92.2 (0.6) b	86.3 (2.6) a	89.0 (1.8) ab	88.8 (2.4) ab
Neoxanthin (mmol mol ⁻¹ Chl)	38.1 (0.9)	40.3 (1.0)	38.2 (1.0)	39.4 (1.4)	40.1 (0.9)
Lutein (mmol mol ⁻¹ Chl)	136.2 (3.1)	144.3 (2.1)	138.6 (3.8)	141.3 (4.1)	143.9 (1.5)
Violaxanthin (V) (mmol mol ⁻¹ Chl)	45.5 (1.4)	44.7 (1.4)	51.4 (1.2)	51.9 (5.5)	50.4 (1.9)
Antheraxanthin (A) (mmol mol ⁻¹ Chl)	6.09 (0.4) a	6.82 (0.6) a	2.88 (0.2) b	5.56 (0.7) a	6.46 (0.6) a
Zeaxanthin (Z) (mmol mol ⁻¹ Chl)	6.22 (1.0) a	5.08 (0.8) ab	2.67 (0.4) b	2.90 (1.6) b	3.40 (0.6) ab
V+A+Z (mmol mol ⁻¹ Chl)	57.8 (1.0)	56.6 (2.5)	56.9 (1.3)	60.4 (6.2)	60.3 (1.8)
DES (A+Z)/(V+A+Z)	0.21 (0.02) a	0.21 (0.02) a	0.10 (0.01) c	0.14 (0.02) bc	0.16 (0.01) ab

Table 2. Total soluble proteins (TSP), lipid peroxidation, determined as thiobarbituric acid reacting substances (TBARS) concentration, superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) in leaves of plants exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B for 20 and 75 d. Values are means (standard error) of 4-6 replicates. Different letters within the same parameter indicate significant differences (p<0.05).

	20 days			75 days	
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹
TSP (mg g ⁻¹ DW)	6.76 (0.56) a	9.01 (1.70) a	3.94 (0.67) b	8.16 (0.77) a	7.18 (0.51) a
TBARS (nmol MDA g ⁻¹ DW)	43.48 (5.66) a	44.85 (6.03) a	35.06 (2.09) ab	32.09 (3.54) ab	28.71 (2.37) b
SOD (USOD mg ⁻¹ protein min ⁻¹)	6.67 (0.57) a	6.07 (1.63) a	11.81 (2.43) b	5.52 (0.21) a	7.44 (0.74) a
GPX (nmol Guaiacol mg ⁻¹ protein min ⁻¹)	1.12 (0.15) a	1.45 (0.24) ab	2.85 (0.39) c	2.56 (0.35) c	2.16 (0.41) bc
CAT (μmol H ₂ O ₂ mg ⁻¹ protein min ⁻¹)	44.92 (2.43) a	47.83 (3.86) ab	64.95 (11.90) b	36.57 (2.58) a	39.62 (3.00) a
APX (μmol Asc mg ⁻¹ protein min ⁻¹)	3.76 (0.37) a	4.21 (0.46) a	10.90 (3.26) b	3.66 (0.85) a	4.02 (0.39) a

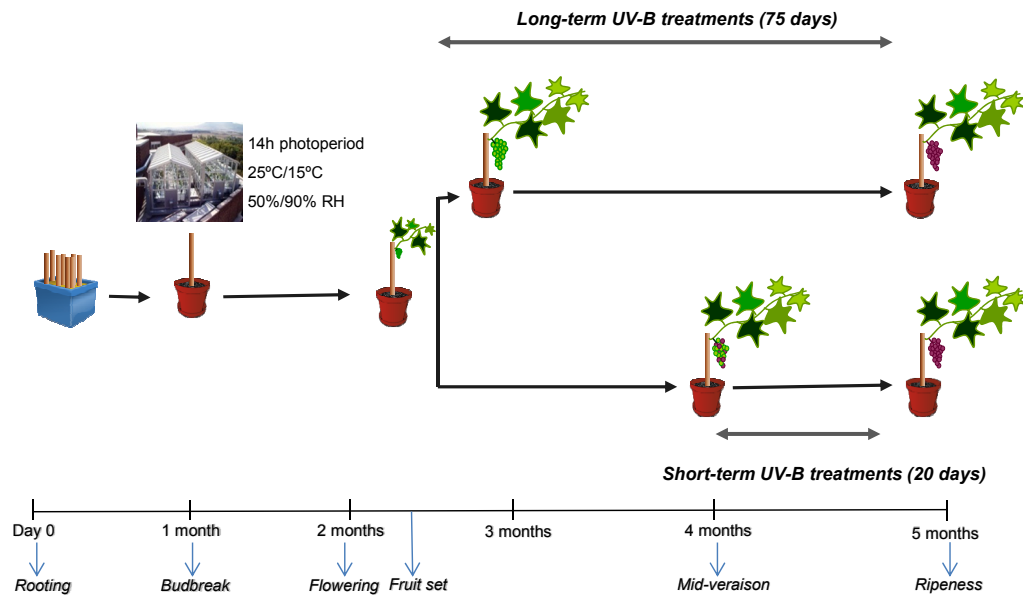


Figure 1. Experimental design. Fruit-bearing cuttings were developed and grown in a glasshouse under controlled conditions. Plants were exposed to two doses of UV-B_{BE}: 5.98 and 9.66 kJ m⁻² d⁻¹. Within each UV-B level, one group of plants was irradiated for 20 d, from mid-veraison to ripeness (short-term UV-B treatments) and another group was irradiated for 75 d, from fruit set to ripeness (long-term UV-B treatments). A 0 kJ m⁻² d⁻¹ UV-B treatment was included as control.

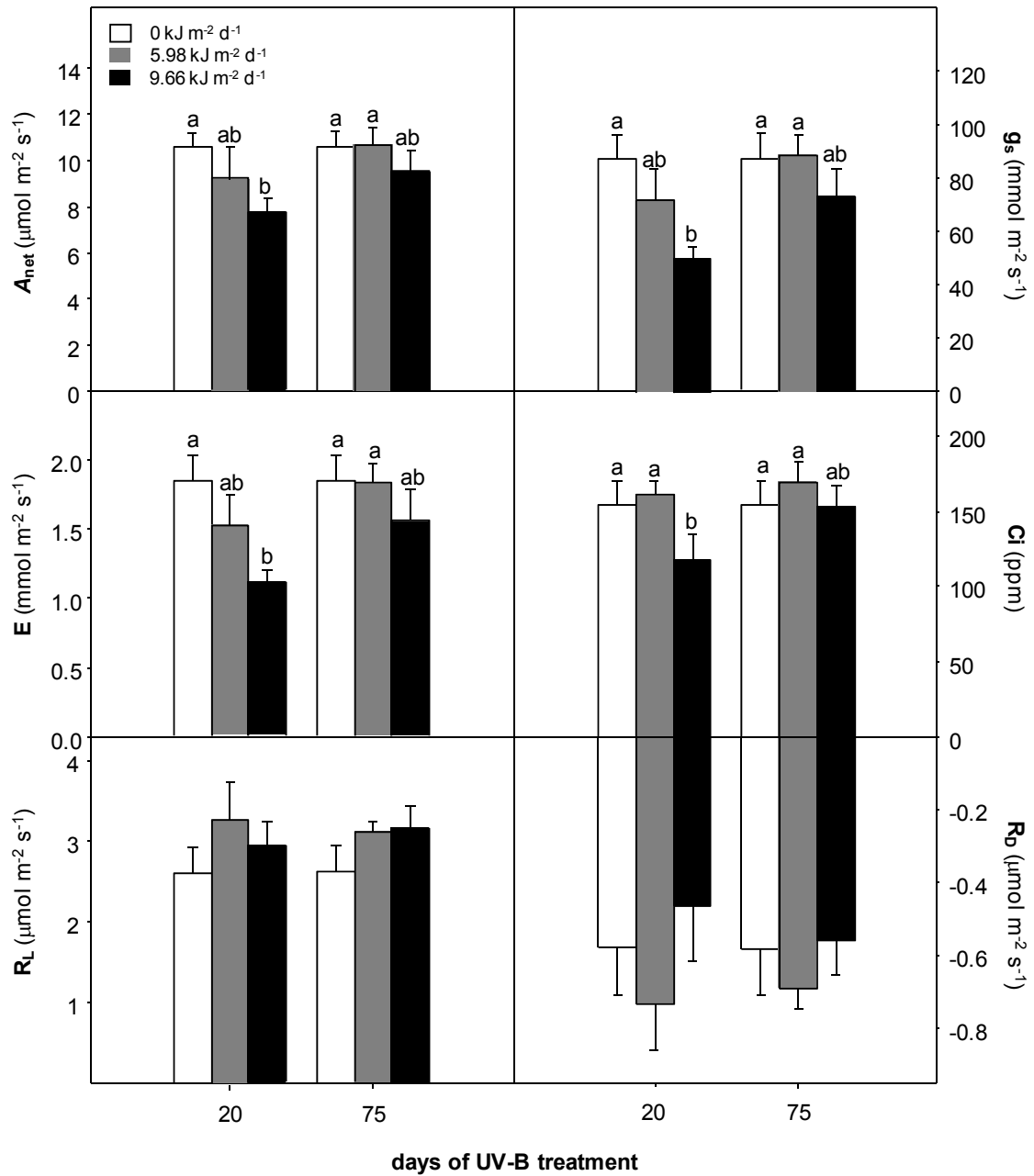


Figure 2. Net photosynthesis (A_{net}), stomatal conductance (g_s), transpiration (E), sub-stomatal CO_2 concentration (C_i), photorespiration (R_L) and dark respiration (R_D) of plants exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B for 20 and 75 d. Columns are means \pm SE (n=4-6). Different letters within the same parameter indicate significant differences (p<0.05).

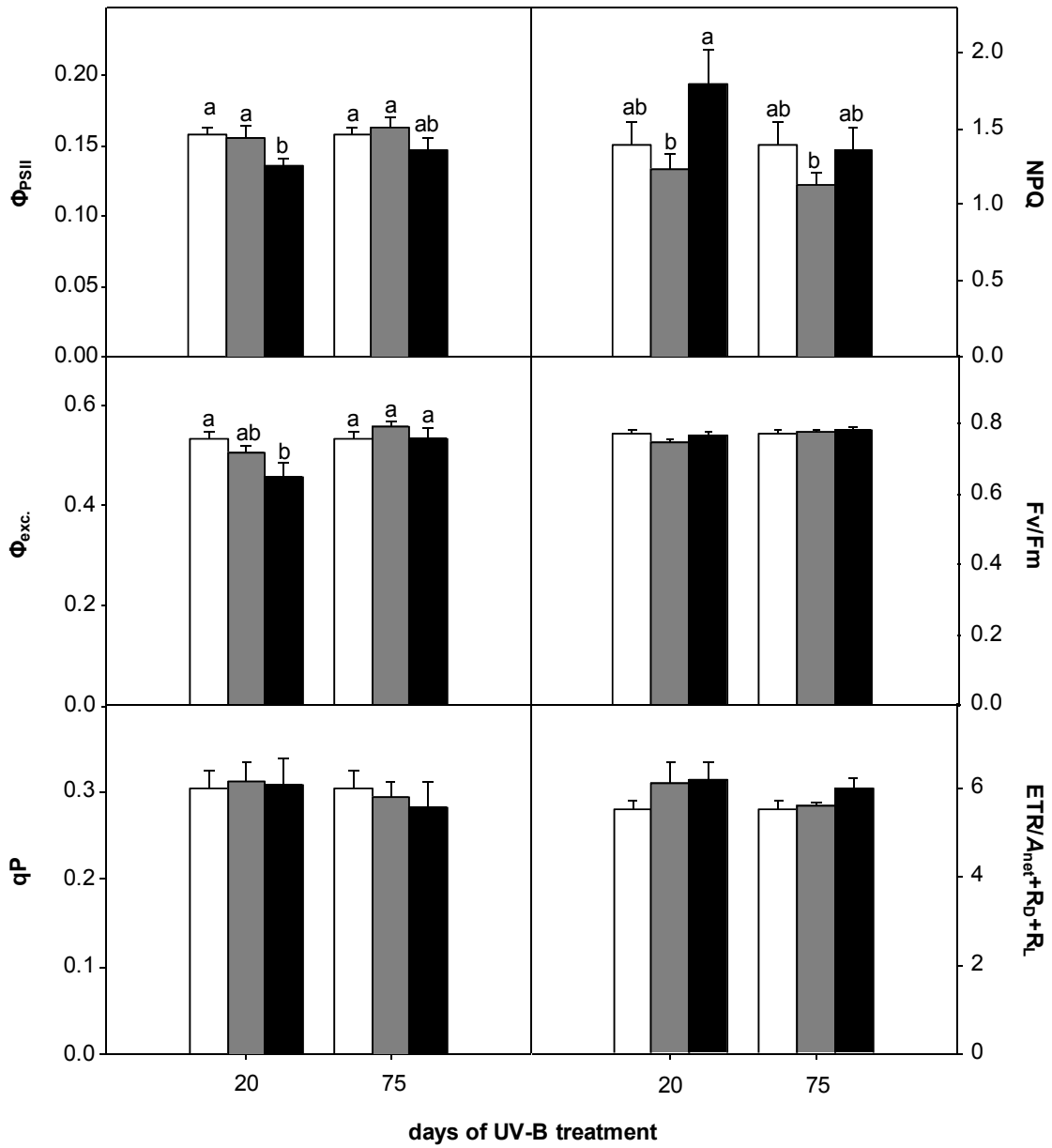


Figure 3. Actual (Φ_{PSII}) and intrinsic ($\Phi_{exc.}$) PSII efficiency, photochemical quenching (qP), non-photochemical quenching (NPQ), maximum potential PSII efficiency (Fv/Fm) and ratio of electrons generated to electrons consumed in A_{net} , R_D and R_L ($ETR/A_{net}+R_D+R_L$) of plants exposed to 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B for 20 and 75 d. Columns are means \pm SE (n=4-6). Different letters within the same parameter indicate significant differences ($p < 0.05$).

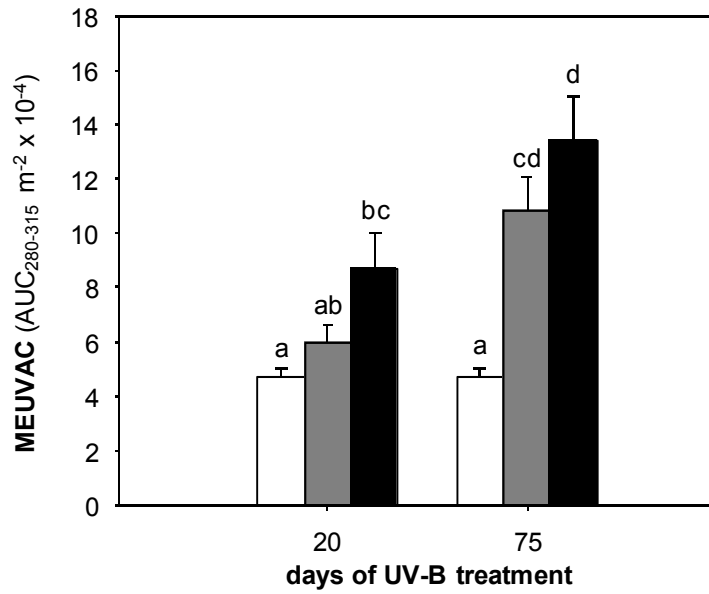


Figure 4. Leaf methanol extractable UV absorbing compounds of plants exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B for 20 and 75 d. Columns are means ± SE (n=4-6). Different letters indicate significant differences (p<0.05).

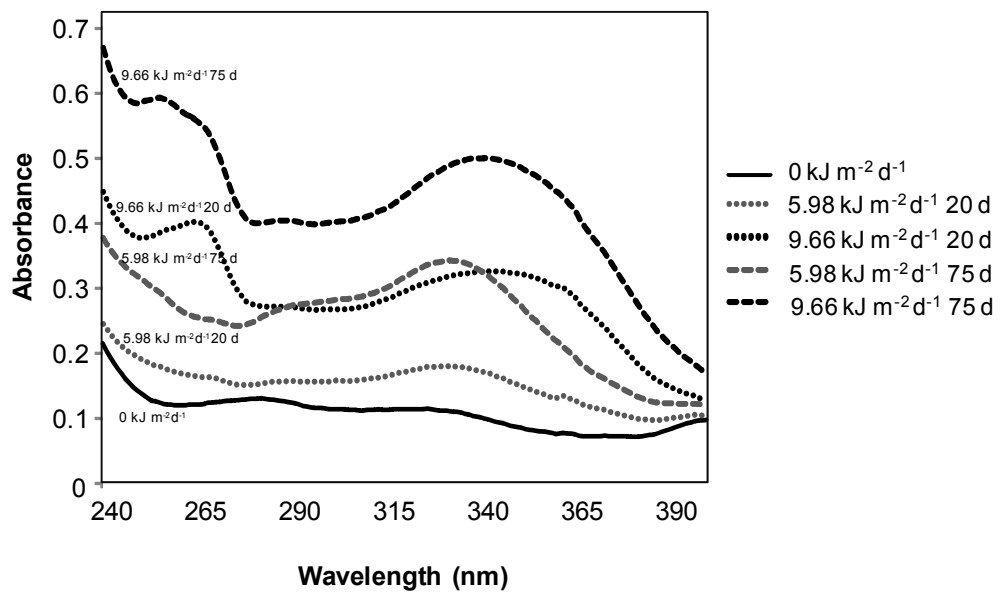


Figure 5. Absorbance spectra of leaf extracts in methanol from plants exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B for 20 and 75 d.

CHAPTER 2

Ultraviolet-B radiation modifies the quantitative and qualitative profile of flavonoids and amino acids in grape berries

Ultraviolet-B radiation modifies the quantitative and qualitative profile of flavonoids and amino acids in grape berries

ABSTRACT

Grapevine cv. Tempranillo fruit-bearing cuttings were exposed to supplemental ultraviolet-B (UV-B) radiation under controlled conditions, in order to study its effect on grape traits, ripening, amino acids and flavonoid profile. The plants were exposed to two doses of UV-B biologically effective (5.98 and $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$), applied either from fruit set to ripeness or from the onset of veraison to ripeness. A $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ treatment was included as a control. UV-B did not significantly modify grape berry size, but increased the relative mass of berry skin. Time to reach ripeness was not affected by UV-B, which may explain the lack of changes in technological maturity. The concentration of must extractable anthocyanins, colour density and skin flavonols were enhanced by UV-B, especially in plants exposed from fruit set. The quantitative and qualitative profile of grape skin flavonols were modified by UV-B radiation. Monosubstituted flavonols relative abundance increased proportionally to the accumulated UV-B doses. Furthermore, trisubstituted forms, which were predominant in non-exposed berries, were less abundant as UV-B exposure increased. Although total free amino acid content remained unaffected by the treatments, the increased levels of gamma-aminobutyric acid (GABA), as well as the decrease in threonine, isoleucine, methionine, serine and glycine, revealed a potential influence of UV-B on the GABA-mediated signalling and amino acid metabolism. UV-B had an overall positive impact on grape berry composition.

Keywords: Grapevine, abiotic stress, metabolites, flavonoids, ultraviolet radiation

1. INTRODUCCION

Due to the anthropic release of ozone depleting compounds, current levels of UV-B irradiation are around their maximum. Models suggest that mid-latitude ozone is expected to return to 1980 levels in the first half of the 21st century (UNEP, 2012). Moreover, factors such as cloudiness, latitude and altitude make UV-B radiation levels vary greatly by location and time of the year (McKenzie et al., 2007). Despite UV-B (280-315 nm) accounts for less than a 0.5% of the total solar electromagnetic radiation reaching the earth surface, it has a great impact on plants biology, acting as a growth modulator (Jansen and Bornman, 2012). UV-B has been historically considered as an environmental stress factor with many negative effects on plants (Kakani et al., 2003). However, environmental levels of UV-B have been reported to have some desirable effects on various crops, including grapevine, enhancing secondary metabolism, reducing vegetative growth and decreasing pathogen incidence (Jug and Rusjan, 2012 and references therein). In addition, many of the compounds involved in the adaptative response of plants to UV-B radiation, such as flavonoids (e.g. anthocyanins and flavonols), may have health promoting effects due to their antioxidant, antitumoral, cardioprotective and anti-inflammatory activities (Nassiri-Asl and Hosseinzadeh, 2009).

Grape quality for winemaking is a concept linked to a good balance of primary metabolites such as sugars and organic acids (e.g. tartaric and malic acid) and secondary metabolites such as aromas and phenolic compounds (e.g. tannins, hydroxycinnamic acids, stilbens, flavanols, flavonols and anthocyanins) that control the organoleptic properties of the wine (Jackson and Lombard, 1993). At the onset of ripening (veraison), berry sugar concentration increases, while organic acids (essentially malic acid) begin to decrease (Conde et al., 2007 and references therein). Most phenolic compounds, such as anthocyanins and flavonols, tend to accumulate in the last part of the development, marked by the onset of veraison, where a change of state in berries, from acid, green and hard to sweet, red and soft, happens (Conde et al., 2007 and references therein).

In the pursuit of grapes of a higher quality, modern viticultural practices aim to enhance sun exposure of the cluster by means of defoliation, leading to a higher flavonoid content (Diago et al., 2012). Hence, leaf removal is generally recommended around the onset of ripening (veraison). However, new approaches recommend a defoliation earlier in development, which may improve berry quality and reduce sun burn damage (Pastore et al., 2013). After veraison, several MYB transcription factors controlling the flavonoid biosynthesis are up-regulated by sun exposure, which leads to an increase in flavonoid content in the berries (Matus et al., 2009). Increased sun exposure implies more UV-B radiation reaching the grapes, but also UV-A, visible and infrared radiation, with a subsequent temperature increase in the berries. While some of the different fractions of solar radiation may up-regulate different genes in flavonoid biosynthesis (Koyama et al., 2012), high temperatures alone can decrease flavonoid content in grape berries, through a combination of both degradation and synthesis inhibition (Mori et al., 2007; Spayd, 2002). Therefore, the effect of sun exposure results of the interaction of several factors that are hardly uncoupled under field conditions.

Anthocyanins and flavonols are the most common flavonoids present in grape berries and wine. Anthocyanins are responsible for the coloration of red grape berries, as they increase in grape berry skins during the ripening phase. Although UV-B may up-regulate some structural genes of the anthocyanin biosynthesis pathway (Park et al., 2007), the process leading to enhance their synthesis has not been associated to an UV-B specific regulatory mechanism (Zhou et al., 2007). Closely related to anthocyanin biosynthesis (Fig. 1), flavonols are important constituents of wines contributing to a great extent to their velvety astringency feeling (Hufnagel and Hofmann, 2008) and colour enhancement and stability, acting as anthocyanin co-pigments (Boulton, 2001). The VvMYBF1 transcription factor has been pointed out as a specific regulator of flavonol biosynthesis in grapevine (Czemmel et al., 2009), and the expression of its homolog in *Arabidopsis thaliana* (AtMYB12; Mehrrens et al. 2005) is regulated by UV-B, through the UV RESISTANCE LOCUS 8 protein (UVR8) (Cloix and Jenkins, 2008; Rizzini et al., 2011). In grape berries, several studies report an

increase in flavonol biosynthesis induced by environmental conditions other than UV-B radiation (Castellarin et al., 2007; Deluc et al., 2009; Koyama et al., 2012). In those studies, the role of an increase in berry sugar concentration in the flavonoid biosynthesis up-regulation observed was discussed. In fact, part of the flavonoid biosynthesis in grape cells is regulated by the sugar levels (Ferri et al., 2011), making it harder to point out the specific effect of environmental stress factors in ripening fruits.

The present study investigates the effect of UV-B radiation, applied from the fruit set to ripeness (FS) or from the onset of veraison to ripeness (OV), on grape berry composition. Since our aim was to work in an environmentally controlled plot, we used a model system for grapevine (the fruit-bearing cuttings technique; Ollat et al., 1998) that allows us to impose in fully controlled greenhouse conditions different UV-B doses, thus limiting the innumerable interactions with other environmental factors that UV-B may have in the field (Caldwell et al., 2007 and references therein). The fruit-bearing cuttings model allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines (Lebon et al., 2005) under controlled environmental conditions. In addition, the chronology of flowering and fructification of the fruit-bearing cuttings is similar to that of the vineyard-grown grapevines (Lebon et al., 2008), thus being a reliable model for studies on grape berry composition (Dai et al., 2013).

2. MATERIAL AND METHODS

Plant material and growth conditions

Dormant cuttings of *Vitis vinifera* cv. Tempranillo were obtained from an experimental vineyard of the Station of Viticulture and Enology of Navarra (Olite, Spain). Cuttings were selected to produce fruit-bearing cuttings according to Ollat et al. (1998) and Santa María (2004). Thereby, indol butyric acid was applied to three-node cuttings, kept for a month in heated rock wool (27°C) to promote rooting, and then transferred to a glasshouse. Growing conditions were set to 25/15°C and 50/90 % RH (day/night) and a photoperiod of 14 h with

natural daylight (an average of $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ during midday) supplemented with high-pressure sodium lamps (SON-T Agro Phillips, Eindhoven, Netherlands), to extend photoperiod to 14h and provide a minimal photosynthetic photon flux density (PPFD) of $350 \mu\text{mol m}^{-2} \text{s}^{-1}$, which corresponded with a minimum of $18 \text{ mol m}^{-2} \text{ day}^{-1}$ PAR during the experiment. When 3 leaves were developed, the cuttings were transplanted to 4 L pots with perlite:peat (2:1 v/v) and watered with the nutrient solution described by Ollat et al. (1998). Vegetation was controlled and conducted horizontally to expose the cluster and achieve a uniform UV-B application along the shoot.

Experimental design

When fruit set was completed in all clusters, plants were divided into 5 homogenous groups corresponding to the following UV-B treatments: i) $0 \text{ kJ m}^{-2} \text{ d}^{-1}$, ii) $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ applied from FS, iii) $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ applied from FS, iv) $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ applied from OV and v) $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ applied from OV (Table 1). UV-B radiation was applied by fluorescent tube lamps TL100W/01 Philips (Philips, Eindhoven, The Netherlands) (Fig. S1), hanging from the top in parallel with the same orientation as the shoots. Spectral distribution of UV-B lamps, as well as UV-B irradiance were measured using a double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd, Livingston, Scotland). UV-B biologically effective irradiance was estimated using the generalized plant damage action spectrum normalized at 300 nm (Caldwell, 1971). The doses of 5.98 and $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$, were obtained through a constant fluent rate and setting the exposition time to 3 h and 4 h 51 min, respectively, around solar noon. These doses are given within the actual climate scenario as $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ accounts for a 86% of the maximum dose under clear sky conditions in the study site (Pamplona, Spain; $42^{\circ}48'14'' \text{ N}$, $1^{\circ}39'54'' \text{ W}$) and the dose of $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ is below to the maximum reachable in vineyards located in the southern hemisphere (Quick TUV calculator; <http://cprm.acd.ucar.edu/Models/TUV/>). The treatment of $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ was obtained through the blockage of UV-B by the glasshouse glasses and UV-B lamps in this treatment were

switched off. UV-B screening clear polyester film (SKC199, Nuenka, Spain) was used to separate plants of the different treatments.

Grape berry composition

Each cluster was followed individually, and samples were collected at TSS concentration of ca. 22°Brix. Then, grape berries were frozen in liquid nitrogen and kept at -80°C until analysis. Samples of 25 berries per cluster were crushed to extract the juice and centrifuged to perform the following determinations: TSS, pH, titratable acidity and L-malic acid as described in Salazar Parra et al. (2010). Another 25 berries were ground in a mortar with liquid nitrogen for the determination of the colour density, hue, total and extractable anthocyanins, % extractability and total polyphenol index (TPI) as described in Salazar Parra et al. (2010). A third group of 20 berries per cluster were separated in the three compartments: skin, pulp and seeds for yield components, flavonol, anthocyanin and amino acid analysis.

Anthocyanin and flavonol profiles

Grape berry skins and pulp were powdered separately in a ball grinder MM200 (Retsch, Haan, Germany). Skin samples were freeze dried and extracted in methanol containing 0.1% HCl (v/v). Extracts were filtered through a 0.45 µm polypropylene syringe filter (Pall Gelman Corp., Ann Arbor, USA) for HPLC analysis of individual anthocyanins and flavonols by means of a summit HPLC System consisting of P680 pump, ASI-100T™ autosampler and UVD 340U UV-Vis detector operating at 520 nm and at 360 nm (Dionex Corporation, Sunnyvale, CA, USA) as described in Acevedo De la Cruz et al. (2012). Malvidin-3-O-glucoside was used as common standard for all the quantified anthocyanins (at 520 nm), and quercetin-3-O-glucoside was used for all the quantified flavonols (at 360 nm) (Extrasynthese, Genay, France).

Formal identification of flavonoids was performed by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). The chromatography apparatus,

Agilent 1200 from Agilent Technologies (Santa Clara, CA, USA) consists of an autosampler module, a degasser, a binary pump, a column heater/selector and a UV-visible-DAD detector from the same supplier. This HPLC was coupled to an Esquire 3000 + ion trap mass spectrometer using an ESI source from Bruker Daltonics (Billerica, MA, USA). The HPLC output flow was split with a passive splitter with an average 1:100 ratio, depending on the flow solvent and viscosity and rate. Anthocyanin and flavonol identifications were carried out from the chromatographic and spectral data and by comparison to literature (Alcalde-Eon et al., 2006; Figueiredo-Gonzalez et al., 2012). Anthocyanin chromatographic profiles were recorded at 520 nm. Anthocyanins corresponding to derivatives of the five common aglycones in *Vitis vinifera* – malvidin (m/z 331), peonidin (m/z 301), petunidin (m/z 317), cyanidin (m/z 287) and delphinidin (m/z 303) – were identified by the extracted ion chromatograms obtained in MS² mode. The glucoside derivatives had similar fragmentation pattern containing two signals, the original M⁺ molecular ion, and the fragments the fragment [M-162]⁺ resulting from the elimination of glucose residue. The acetyl-glucoside and *p*-coumaroyl-glucoside derivatives had similar fragmentation patterns containing three signals, the original M⁺ molecular ion and: (i) the fragments [M-42]⁺ and [M-204]⁺; (ii) the fragments [M-146]⁺ and [M-308]⁺. These resulted from the loss of acetyl-glucoside and *p*-coumaroyl-glucoside residues, respectively. Anthocyanins usually interfere significantly with the chromatographic separation and identification of flavonols. For the identification of flavonols, anthocyanin removal was carried out by the use of a cation exchange resin Dowex 50WX4 (Sigma-Aldrich) according to Decendit et al. (2002). Flavonol chromatographic profiles were recorded at 360 nm. The glucuronide, galactoside and glucoside derivatives of the six common aglycones - myricetin (m/z 319), quercetin (m/z 303), laricitrin (m/z 333), kaempferol (m/z 287), isorhamnetin (m/z 317) and syringetin (m/z 347) were identified by the extracted ion chromatograms obtained in MS² mode.

Amino acid profile

Free amino acids were extracted from pulp samples using decreasing concentrations of ethanol: ethanol 80%, ethanol 50% and 0% (v/v). Samples were then evaporated in a vacuum concentrator and resuspended to 2 ml of distillate water. After derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (Cohen and Michaud, 1993), amino acids were analysed using a Waters 2695 HPLC system equipped with Waters 474 fluorescence detector (Waters, Milford, MA, USA). Separation was performed on a Nova-Pack C18 AccQ-Tag column (Waters, Milford, MA, USA) at 37°C with elution at 1 mL min⁻¹ with a 67 min linear gradient (eluent A, sodium acetate buffer, 140 mM at pH 5.7; eluent B, acetonitrile 60% in water [v/v]). Chromatograms corresponding to excitation at 250 nm and emission at 395 nm were recorded and quantified with chemical standards purchased from Sigma (St Louis, MO, USA). Nineteen amino acids were identified and quantified as described by Pereira et al. (2006), excluding tryptophan.

Statistical analysis

Statistical analysis was performed with SPSS 15.0 software (SPSS inc., Chicago) doing a one-way ANOVA and LSD post-hoc test. Groups marked with no letters in common indicate a significant difference ($p < 0.05$) between them. A linear regression was performed for the most significant results (Pearson correlation coefficient).

3. RESULTS

Grape berry yield and berry characteristics

Regardless the exposure period, UV-B radiation did not affect the number of days needed to reach technological maturity (Total soluble solids [TSS] ca. 22° Brix; Table 2). Berry weight and berry calibre remained unaffected by UV-B treatments ($p > 0.05$). By contrast, the relative mass of skins significantly increased when the 9.66 kJ m⁻² d⁻¹ UV-B dose was applied from OV.

Grape berry composition

The technological maturity parameters measured (TSS, pH, titratable acidity and malic acid) were not significantly affected by the UV-B treatments. UV-B radiation tended to increase colour density, anthocyanin concentrations and extractability, as well as total polyphenol index compared with non-exposed plants ($0 \text{ kJ m}^{-2} \text{ d}^{-1}$) (Table 2). Nevertheless, significant differences were only found in colour density and extractable anthocyanins with the dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ applied from OV.

Anthocyanin and flavonol profile

Liquid chromatography coupled to mass spectrometry led to the identification of 14 anthocyanins and 12 flavonols (Table S1; Fig. S2). All the anthocyanins and all but two flavonols (laricitrin-3-*O*-galactoside and isorhamnetin-3-*O*-galactoside) found were present in concentrations high enough to be quantified accurately by HPLC-DAD. Total skin anthocyanin concentrations measured by HPLC in the 5.98 and $9.66 \text{ kJ m}^{-1} \text{ d}^{-2}$ treatments did not differ from the $0 \text{ kJ m}^{-1} \text{ d}^{-2}$ treatment, whatever the exposure length was (Fig. 2A). The anthocyanin skin concentration was significantly lower in the $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ dose from OV compared to the $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ dose. Non-acylated anthocyanin glucosides were the main compounds accumulated (85% on average), with malvidin-3-*O*-glucoside being the major anthocyanin found (35%) (Table 3). Acylated forms were present in skins in a lower proportion with 3.3 % and 11.7 % of glucosylacylated (acetates) and glucosyl-*p*-coumaric (*p*-coumarates) forms, respectively. The UV-B treatments significantly modified some anthocyanin concentrations (Table 3). However, UV-B did not affect the molar relative abundance of anthocyanin groups (acetates, -*p*- coumarates, trisubstituted or methylated) (Table S2).

Total and almost all the individual flavonol concentrations in the skins were significantly higher in berries treated with UV-B, especially in those berries exposed from FS (Fig. 2B, Table 3). Individual flavonols concentrations were affected in different ways, as the

proportion of mono-, di- and trisubstituted forms changed with UV-B (Fig. 3; Table 3). Thereby, the molar relative abundance of monosubstituted (Fig. 3A) and disubstituted (Fig. 3B) flavonols increased with the UV-B dose, while the relative abundance of trisubstituted flavonols decreased significantly as UV-B dose rose, regardless whether the grapes had been exposed from FS or OV (Fig. 3C).

Correlation analyses

Linear regression tests were performed between the concentration of total and individual flavonols and the accumulated dose of UV-B (Table 4). The results show that quercetin-3-O-glucuronide, isorhamnetin-3-O-glucoside and kaempferol-3-O-glucoside were the flavonols best correlated with the accumulated dose of UV-B received by the clusters. In addition, the molar relative abundance of monosubstituted flavonols and the total accumulated dose of UV-B showed a highly significant correlation (Table 4). When a multivariate correlation was performed, taking into account the accumulated dose of UV-B before and after the onset of veraison, the correlation was improved (Fig 4A). Regardless of the UV-B treatment applied, a highly significant correlation ($p < 0.001$) was found between the total anthocyanins concentration in skin and the total amount of trisubstituted flavonols (Fig. 4B)

Amino acid profile

Total free amino acid concentration remained unaffected by UV-B (Table 5). However, individual amino acids concentrations showed a decrease in threonine, methionine, isoleucine, serine and glycine in berries exposed to UV-B from OV. By contrast, gamma-amino butyric acid (GABA) concentration in berries exposed to $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ from FS was 61% higher than the $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ treatment. The response of proline concentration to UV B depended on the application stage: it tended to decline with the exposure to UV-B from OV and increased in those grapes exposed from FS.

4. DISCUSSION

Sugar accumulation in ripening berries relies on the carbon fixation in mature leaves and their transport and allocation into the grapes (Conde et al., 2007 and references therein). UV-B radiation has been reported to limit stomatal aperture, and, therefore, reduce net carbon fixation (Berli et al., 2013; Martínez-Lüscher et al., 2013). In the present study, the time required to reach ripeness (ca. 22 °Brix) was similar for all of the treatments assayed (Tables 1 and 2), thus suggesting that UV-B did not induce changes in the sugar accumulation rate of berries under the experimental conditions. These results agree with previous studies, where UV radiation did not have an effect on berry TSS (Gregan et al., 2012; Spayd, 2002). Neither pH, acidity nor malic acid concentration in the must were affected by UV-B, probably due to the TSS constraint in the sampling design and the similar maturation times, factors that may affect organic acids contents in the berry (Conde et al., 2007).

Regarding phenolic maturity, UV-B radiation increased the concentration of extractable anthocyanins in the must, and consequently, its colour density (Table 2). By contrast, UV-B did not modify the concentration of total anthocyanins in the skin (Fig 2A). Results suggest that the higher anthocyanins content in the must of berries grown under UV-B was not due to an increase in the concentration of anthocyanins in the skin, but to a higher relative skin mass (Roby et al., 2004). It must be noted that the observed increase in must anthocyanins did not result from a hastening in berry sugar accumulation as in the case of Berli et al. (2011). Kataoka (2003) reported a weak effect of UV-B on the concentration of grape skin anthocyanins compared to UV-A, supporting the idea that UV-B by itself does not strongly impact anthocyanin biosynthesis (Zhou et al., 2007). The relative abundance of the five major anthocyanin glycosides was not affected by the treatments with UV-B regardless the exposure period (Table S2). Our results indicate a weak effect of UV-B radiation on anthocyanin composition, which is usually attributed to changes in the expression levels of the flavonol 3' 5' hydroxylase (*F3'5'H*) and flavonoid O-methyl transferase genes (*FOMT*) (Falginella et al., 2010; Fournier-Level et al., 2011).

Plant tissues accumulate flavonols as UV-B exposure increases (Agati and Tattini, 2010; Jansen et al., 2008), through the up-regulation of flavonol synthase (*FLS*) gene expression by UV-B radiation (Falcone Ferreyra et al., 2010). In the present study, flavonol accumulation was increased by UV-B and the concentration of flavonols was correlated to the accumulated dose of UV-B (Fig. 2B, Tables 3 and 4). However, such effect of UV-B was not uniform for all the individual flavonols studied. For instance, myricetin-3-O-glucuronide was not significantly higher in UV-B treated grape skins and, in general, the content of all the trisubstituted flavonols did not present a good correlation with the UV-B exposure (Tables 3 and 4). This group of flavonols, absent in white cultivars (Castillo-Muñoz et al., 2010; Mattivi et al., 2006), is not synthesized in big quantities until the onset of veraison (Jeong et al., 2006), and its synthesis has been related to the expression levels of the gene encoding the *F3'5'H* (Olsen et al., 2010). The expression of *F3'5'H* is synchronized with the synthesis of anthocyanins in grape berries (Castellarin et al., 2007), through the mediation of the transcription factor MybPA1 (Bogs et al., 2007). The high correlation found between trisubstituted flavonols and total anthocyanin concentration (Fig. 4B) supports that this group of flavonols may share common transcriptional regulatory system and/or enzymatic machinery with anthocyanins that white-skinned cultivars lack (Walker et al., 2007). On the other hand, the flavonols that correlated best to UV-B accumulated exposure, isorhamnetin-3-O-glucoside and quercetin-3-O-glucuronide, belong to the group of disubstituted flavonols. These arise from the hydroxylation in the 3' position of the B-ring of the dihydroflavonols by flavonoid 3' hydroxylase (*F3'H*) (Bogs et al., 2006). The study of *F3'H* deficient mutants in *Petunia* showed a flavonol profile poor in disubstituted flavonols, while wild type plants had higher *F3'H* expression under UV-B exposure, leading to higher concentration of disubstituted flavonols (Ryan et al., 2002). Kaempferol-3-O-glucoside was the third flavonol most highly correlated to UV-B exposure and, together with kaempferol-3-O-galactoside, it was not detected in the $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ UV-B treatment. Among the few UV-B studies performed on grape flavonol profile, Gregan et al. (2012) reported negligible levels of kaempferol glycosylated flavonols in white-skinned grapes when solar UV-B radiation was removed.

The relative abundance of the three groups of flavonols studied revealed important changes in the flavonol profile of grapes under UV-B exposure, increasing the proportion of disubstituted, and, specially, monosubstituted derivatives and reducing the proportion of trisubstituted forms (Fig. 3). The synthesis of monosubstituted flavonols (i.e. kaempferol glycosylates) occurs to a much lesser extent than disubstituted and trisubstituted flavonols in red-skinned cultivars (Mattivi et al., 2006), due to the higher expression levels of *F3'5'H* (Bogs et al., 2006). Such enzyme competes with FLS and F3'H for dihydroflavonol substrates (Fig. 1). Therefore, changes in any of these activities, such as an increase in FLS activity induced by UV-B, may result in a different diversification profile of flavonols (Olsen et al., 2010). In the treatment of $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B, under low apparent FLS activity, naringenin and dihydrokaempferol would have been hydroxylated into myricetin precursors, before being converted into kaempferol glycosylates, thus reducing the amount of Kaempferol derivatives (Fig. 3A). By contrast, under high apparent FLS activity (treatments with 5.98 and $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B), the flux may have been stronger towards the synthesis of quercetin derivatives, but specially kaempferol derivatives, to the expense of myricetin derivatives. This behaviour has been reported in Pastore et al. (2013), where an increase in the biosynthetic flux through FLS (i.e. higher *FLS* transcript levels and flavonol concentration), resulted in a higher proportion of monosubstituted flavonols. The high correlation observed between the relative abundance of monosubstituted flavonols and the ponderated accumulated dose of UV-B supports the linearity of this behaviour.

The increase in flavonoid concentrations observed under UV-B exposure can potentially improve the health-promoting effects of grapes for wine production (Nassiri-Asl and Hosseinzadeh, 2009). Beyond the increase in anthocyanins observed in the must, an increase in flavonol content may also improve wine optical properties due to copigmentation with anthocyanins (Boulton, 2001). In addition, flavonols have been characterized as one of the wine phenolics with the strongest antioxidant activities (De Beer et al., 2003).

UV-B has been reported to affect nitrogen metabolism through alteration of the nitrate and nitrite uptake rates and activities of nitrogen assimilating enzymes (Singh et al., 2012). Schultz et al. (1998) observed a dramatic decrease in the total free amino acid content in grape must, under current levels of UV-B. By contrast, our results, which indicate no effect of UV-B radiation on total amino acid concentration in the pulp (Table 4), are in agreement with the studies of Gregan et al. (2012) and Keller and Torres-Martinez (2004) with three different cultivars of grapevine. Regarding individual amino acids, the levels of arginine and glutamine, the main sources of amino acids for yeast metabolism during wine fermentation (Zoecklein et al., 1999), were maintained in the treatments with UV-B. However, the significant decrease in isoleucine, methionine, threonine, serine and glycine, especially under UV-B exposure from OV, should not be neglected, and reveals a potential influence of UV-B on amino acid metabolism. Finally, GABA was the only amino acid increased by UV-B treatment (Table 4). GABA shunt has been pointed out to be a key mechanism preventing the accumulation of reactive oxygen intermediates and cell death under UV-B (Bouche et al., 2003), therefore the accumulation of GABA in the berries pulp may be a response to UV-B in order to prevent oxidative stress damage.

5. CONCLUSIONS

High levels of UV-B radiation within the range of natural conditions increased must extractable anthocyanins and colour density, as well as flavonol biosynthesis in the skins. UV-B had a clear effect on metabolite profiling, especially in flavonols, leading to a very specific diversification of these. GABA was highlighted as a metabolite enhanced by UV-B, thus suggesting a possible involvement of this amino acid in protection against the UV-B-induced oxidative stress in grapes. These results suggest that UV-B radiation may positively affect grape berry composition, improve must properties for wine production and increase health-promoting compounds content in grape and grape products.

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Table 1. Elapsed time and calculated accumulated dose of UV-B received by Tempranillo grapevine plants exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B radiation from fruit set to ripeness (FS) and from the onset of veraison to ripeness (OV).

Application stage	FS			OV	
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹
Elapsed time (days)					
Fruit set to onset of veraison	55.5±0.9	52.5±0.7	54.5±2.2	53.8±2.0	56.8±4.2
Onset of veraison to ripeness	28.8±0.7	30.0±0.0	29.5±0.7	30.0±0.0	28.8±0.7
UV-B accumulated dose (kJ m⁻²)					
Fruit set to onset of veraison	0.0±0.0	367.5±4.9	599.5±24.2	0.0±0.0	0.0±0.0
Onset of veraison to ripeness	0.0±0.0	210.0±0.0	324.5±7.8	210.0±0.0	316.3±7.4

Table 2. Berry traits, technological and phenolic maturity parameters performed in must of Tempranillo grapes exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B radiation from fruit set to ripeness (FS) and from the onset of veraison to ripeness (OV) (mean ± SE; n = 4). Means within the same line followed by different letters indicate significant differences (p<0.05)

Application stage	UV-B Dose	FS			OV	
		0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹
Berry traits						
Berry FW	(g berry ⁻¹)	1.38±0.4	1.53±0.2	1.30±0.2	1.23±0.2	1.29±0.2
Berry calibre	(mm)	12.6±0.1	12.9±0.1	12.5±0.0	12.9±0.1	12.5±0.1
Relative skin mass	(% of fr wt)	18.2±0.7 b	18.8±0.4 b	20.2±0.6 ab	18.2±0.4 b	23.9±0.9 a
Technological maturity						
TSS	(°Brix)	22.0±0.5	21.9±0.6	23.3±0.4	22.9±0.7	22.2±0.6
pH		3.99±0.0	3.91±0.1	3.91±0.1	3.87±0.0	3.98±0.1
Acidity	(g L ⁻¹)	6.92±0.2	6.78±0.3	7.25±0.5	6.75±0.2	7.26±0.6
Malic Acid	(g L ⁻¹)	4.94±0.2	5.11±0.5	4.74±0.4	4.21±0.1	4.80±0.2
Phenolic maturity						
Colour density	(OD _{420nm+520nm+620nm})	12.4±2.6 b	17.53±1.9 ab	17.6±2.4 ab	20.8±1.0 a	17.4±1.2 ab
Hue	(OD _{420nm/520nm})	0.40±0.01	0.41±0.02	0.41±0.02	0.43±0.04	0.40±0.02
Total anthocyanins	(mg L ⁻¹)	828±99	1119±155	1142±193	1253±72	977±100
Extractable anthocyanins	(mg L ⁻¹)	338±26 b	469±59 ab	518±79 a	508±35 a	474±22 ab
Anthocyanin extractability	(%)	58.5±2.3	57.3±2.6	54.0±3.7	59.6±3.5	50.3±4.2
Total polyphenol index	(OD _{280nm})	39.5±1.9	40.3±6.0	48.2±4.1	47.8±3.2	44.3±2.5

Table 3. Individual anthocyanin and flavonol content measured by HPLC in Tempranillo grape skins exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B from fruit set to ripeness (FS) and from the onset of veraison to ripeness (OV) determined by HPLC-DAD (mean ± SE; n = 4). Means within the same line followed by different letters indicate significant differences (p<0.05)

Berry Skin Anthocyanins (mg g ⁻¹ dry wt)					
Application stage	FS			OV	
UV-B dose	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹
delphinidin-3-O-glucoside	2.45±0.195	3.07±0.209	2.73±0.194	3.02±0.123	2.11±0.052
cyanidin-3-O-glucoside	1.01±0.147	1.24±0.093	1.35±0.195	1.43±0.147	0.82±0.048
petunidin-3-O-glucoside	1.97±0.111	2.49±0.160	2.07±0.113	2.40±0.068	1.77±0.038
peonidin-3-O-glucoside	1.88±0.203	2.50±0.147	2.35±0.237	2.46±0.136	1.63±0.066
malvidin-3-O-glucoside	5.77±0.089ab	6.31±0.312ab	5.34±0.135ab	5.97±0.120a	4.85±0.125b
sum of acetates	0.50±0.012ab	0.58±0.031a	0.50±0.018ab	0.57±0.011a	0.45±0.008b
sum of <i>p</i> coumarates	1.80±0.098	1.76±0.078	1.58±0.044	1.76±0.087	1.53±0.045

Berry Skin Flavonols (mg g ⁻¹ dry wt)					
Application stage	-	FS		OV	
UV-B dose	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹
Monosubstituted					
kaempferol-3-O-galactoside	0.000±0.000 c	0.005±0.000 ab	0.008±0.001 a	0.005±0.000 b	0.005±0.000 b
kaempferol-3-O-glucoside	0.000±0.000 c	0.019±0.001 b	0.044±0.004 a	0.012±0.001 bc	0.020±0.002 b
Disubstituted					
quercetin-3-O-galactoside	0.018±0.002 c	0.047±0.003 bc	0.087±0.009 a	0.043±0.005 bc	0.057±0.006 ab
quercetin-3-O-glucuronide	0.070±0.006 c	0.331±0.018 a	0.451±0.033 a	0.117±0.013 b	0.114±0.010 b
quercetin-3-O-glucoside	0.168±0.017 b	0.368±0.026 ab	0.635±0.068 a	0.401±0.043 ab	0.433±0.040 a
isorhamnetin-3-O-glucoside	0.001±0.000 c	0.016±0.001 b	0.031±0.004 a	0.005±0.001 c	0.006±0.001 bc
Trisubstituted					
myricetin-3-O-glucuronide	0.120±0.010	0.139±0.011	0.137±0.006	0.165±0.013	0.125±0.003
myricetin-3-O-glucoside	0.633±0.038 b	0.844±0.066 ab	0.813±0.039 ab	0.925±0.044 a	0.743±0.012 ab
laricitrin-3-O-glucoside	0.048±0.002 b	0.062±0.005 ab	0.064±0.003 ab	0.070±0.004 a	0.059±0.002 ab
syringetin-3-O-glucoside	0.202±0.015 b	0.344±0.025 a	0.407±0.034 a	0.356±0.008 a	0.272±0.013 ab

Table 4. Correlation coefficients (*r*) and significance values (*p*) between the total accumulated dose of UV-B and the individual flavonol concentrations.

Compound total concentration	<i>r</i>	<i>p</i>
Monosubstituted		
kaempferol-3- <i>O</i> -galactoside	0.797	<0.001
kaempferol-3- <i>O</i> -glucoside	0.853	<0.001
Disubstituted		
quercetin-3- <i>O</i> -galactoside	0.706	<0.001
quercetin-3- <i>O</i> -glucuronide	0.879	<0.001
quercetin-3- <i>O</i> -glucoside	0.634	0.002
isorhamnetin-3- <i>O</i> -glucoside	0.855	<0.001
Trisubstituted		
myricetin-3- <i>O</i> -glucuronide	0.088	0.698
myricetin-3- <i>O</i> -glucoside	0.260	0.242
laricitrin-3- <i>O</i> -glucoside	0.284	0.284
syringetin-3- <i>O</i> -glucoside	0.574	0.005
Total flavonols	0.652	0.001
Relative abundance		
% Monosubstituted	0.904	<0.001
% Disubstituted	0.827	<0.001
% Trisubstituted	-0.839	<0.001

Table 5. Free ammonia, individual and total free amino acid (FAA) content ($\mu\text{mol g}^{-1}$ fr wt) measured by HPLC in Tempranillo grapes exposed to 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B from fruit set to ripeness (FS) and from the onset of veraison to ripeness (OV) determined by HPLC-DAD (mean \pm SE; n = 4). Means within the same line followed by different letters indicate significant differences ($p < 0.05$)

Application stage UV-B dose	OV			FS	
	0 $\text{kJ m}^{-2} \text{d}^{-1}$	5.98 $\text{kJ m}^{-2} \text{d}^{-1}$	9.66 $\text{kJ m}^{-2} \text{d}^{-1}$	5.98 $\text{kJ m}^{-2} \text{d}^{-1}$	9.66 $\text{kJ m}^{-2} \text{d}^{-1}$
Free ammonia	8.59 \pm 1.29	8.46 \pm 1.27	7.62 \pm 0.88	9.98 \pm 0.81	9.61 \pm 0.51
α-ketoglutarate					
Proline	8.02 \pm 0.64 abc	6.87 \pm 0.90 bc	6.36 \pm 0.42 c	8.56 \pm 0.40 ab	9.31 \pm 0.93 a
Arginine	4.58 \pm 0.84	3.60 \pm 0.55	3.83 \pm 0.22	3.10 \pm 0.21	4.16 \pm 0.71
Glutamine	5.22 \pm 1.95	3.31 \pm 0.36	3.04 \pm 0.95	2.80 \pm 0.34	3.86 \pm 0.55
GABA	1.42 \pm 0.24 b	1.45 \pm 0.37 b	1.57 \pm 0.22 ab	1.90 \pm 0.13 ab	2.30 \pm 0.23 a
Glutamic Acid	1.14 \pm 0.32	1.01 \pm 0.12	0.85 \pm 0.14	0.92 \pm 0.04	1.25 \pm 0.09
Histidine	0.30 \pm 0.05	0.22 \pm 0.03	0.23 \pm 0.02	0.22 \pm 0.01	0.29 \pm 0.04
Pyruvate					
Alanine	2.26 \pm 0.37 ab	1.92 \pm 0.25 ab	1.82 \pm 0.27 ab	1.51 \pm 0.13 b	2.32 \pm 0.19 a
Valine	0.20 \pm 0.03	0.16 \pm 0.01	0.15 \pm 0.01	0.16 \pm 0.01	0.21 \pm 0.02
Leucine	0.09 \pm 0.02	0.07 \pm 0.01	0.07 \pm 0.01	0.08 \pm 0.00	0.09 \pm 0.01
Aspartate					
Threonine	1.38 \pm 0.20 a	1.01 \pm 0.05 b	0.91 \pm 0.01 b	1.07 \pm 0.03 b	1.21 \pm 0.09 ab
Aspartic Acid	0.61 \pm 0.10	0.45 \pm 0.10	0.50 \pm 0.09	0.51 \pm 0.03	0.58 \pm 0.11
Asparagine	0.31 \pm 0.07	0.23 \pm 0.02	0.19 \pm 0.03	0.20 \pm 0.05	0.27 \pm 0.03
Isoleucine	0.05 \pm 0.01 a	0.03 \pm 0.00 b	0.03 \pm 0.00 b	0.04 \pm 0.00 ab	0.04 \pm 0.00 ab
Methionine	0.05 \pm 0.01 a	0.03 \pm 0.00 ab	0.03 \pm 0.00 b	0.03 \pm 0.00 ab	0.04 \pm 0.01 ab
Lysine	0.02 \pm 0.01	0.02 \pm 0.00	0.02 \pm 0.00	0.02 \pm 0.00	0.02 \pm 0.00
Shikimate					
Tyrosine	0.13 \pm 0.02	0.09 \pm 0.01	0.11 \pm 0.01	0.11 \pm 0.01	0.13 \pm 0.02
Phenylalanine	0.06 \pm 0.02	0.05 \pm 0.01	0.04 \pm 0.01	0.05 \pm 0.01	0.06 \pm 0.02
3-phosphoglycerate					
Serine	1.20 \pm 0.24 a	0.77 \pm 0.06 b	0.58 \pm 0.04 b	0.82 \pm 0.07 b	0.94 \pm 0.10 ab
Glycine	0.06 \pm 0.02 a	0.01 \pm 0.01 b	0.01 \pm 0.01 b	0.04 \pm 0.01 ab	0.03 \pm 0.01 ab
Total FAA	27.11 \pm 4.56 a	21.31 \pm 1.23 a	20.33 \pm 1.35 a	22.16 \pm 0.77 a	27.12 \pm 2.80 a

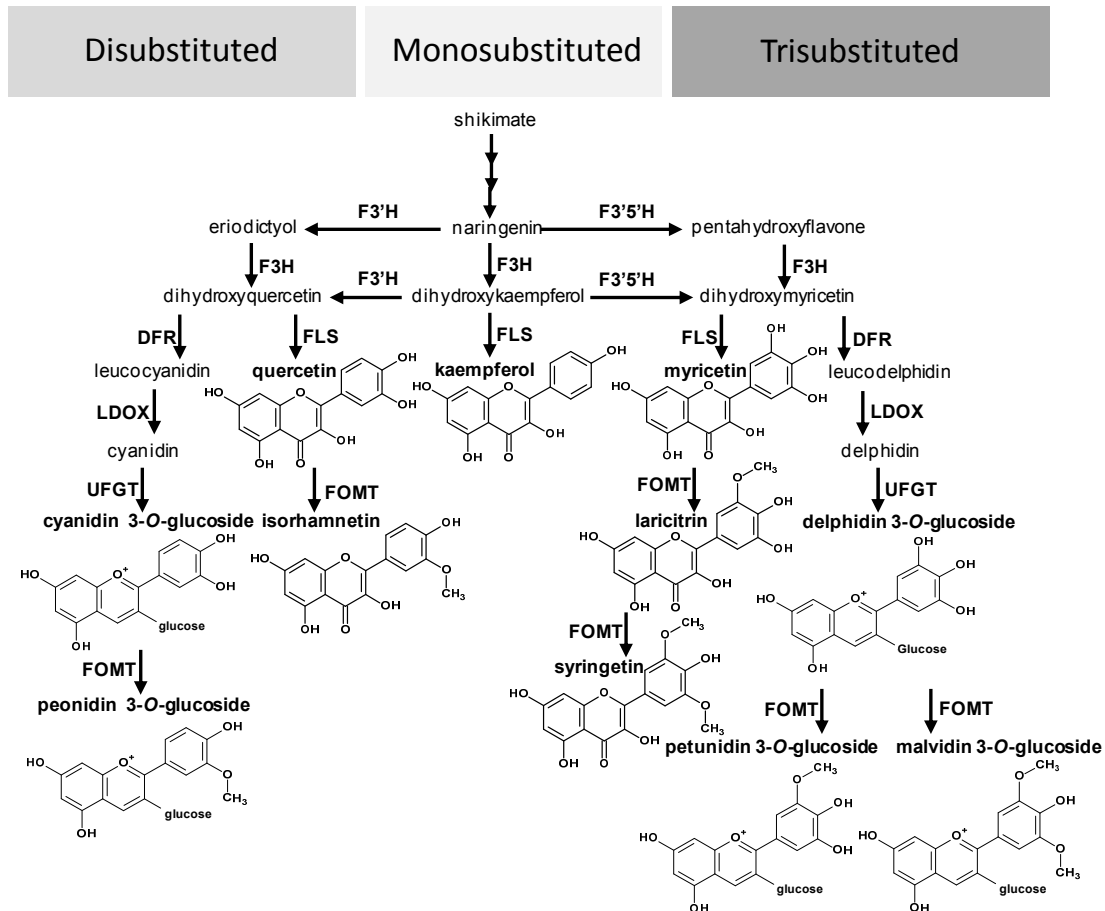


Figure 1. Schematic representation of biosynthetic pathway of flavonoids. Abbreviations: F3H, flavonoid 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; LDOX, leucocyanidin dioxygenase; UFGT, UDP: glucose: flavonoid 3-glucosyltransferase; FOMT, Flavonoid O-methyltransferase. Adapted from Bogs et al. (2006).

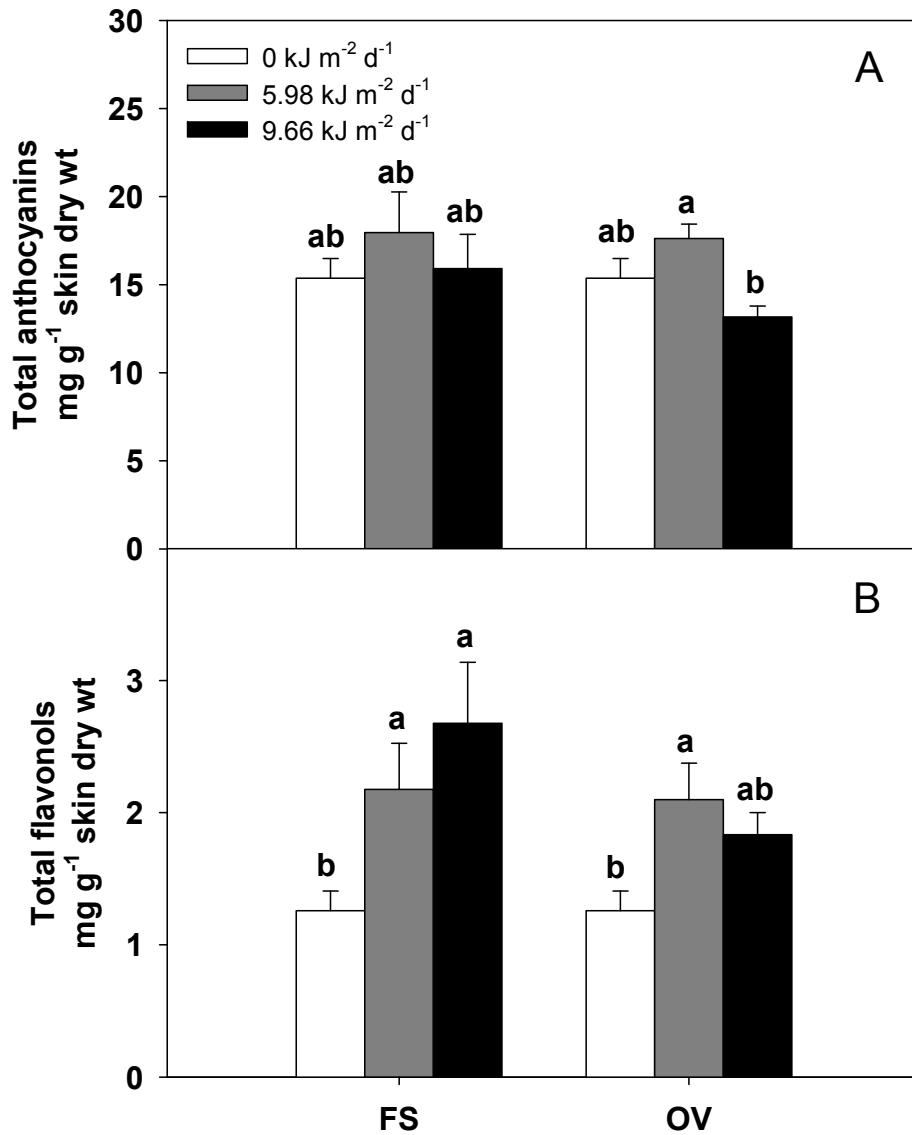


Figure 2. Total anthocyanin (A) and flavonol (B) contents of Tempranillo grape skins exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B from fruit set to ripeness (FS) and from the onset of veraison to ripeness (OV) determined by HPLC-DAD (mean ± SE; n = 4). Groups marked with no letters in common indicate a significant difference (p<0.05) between them.

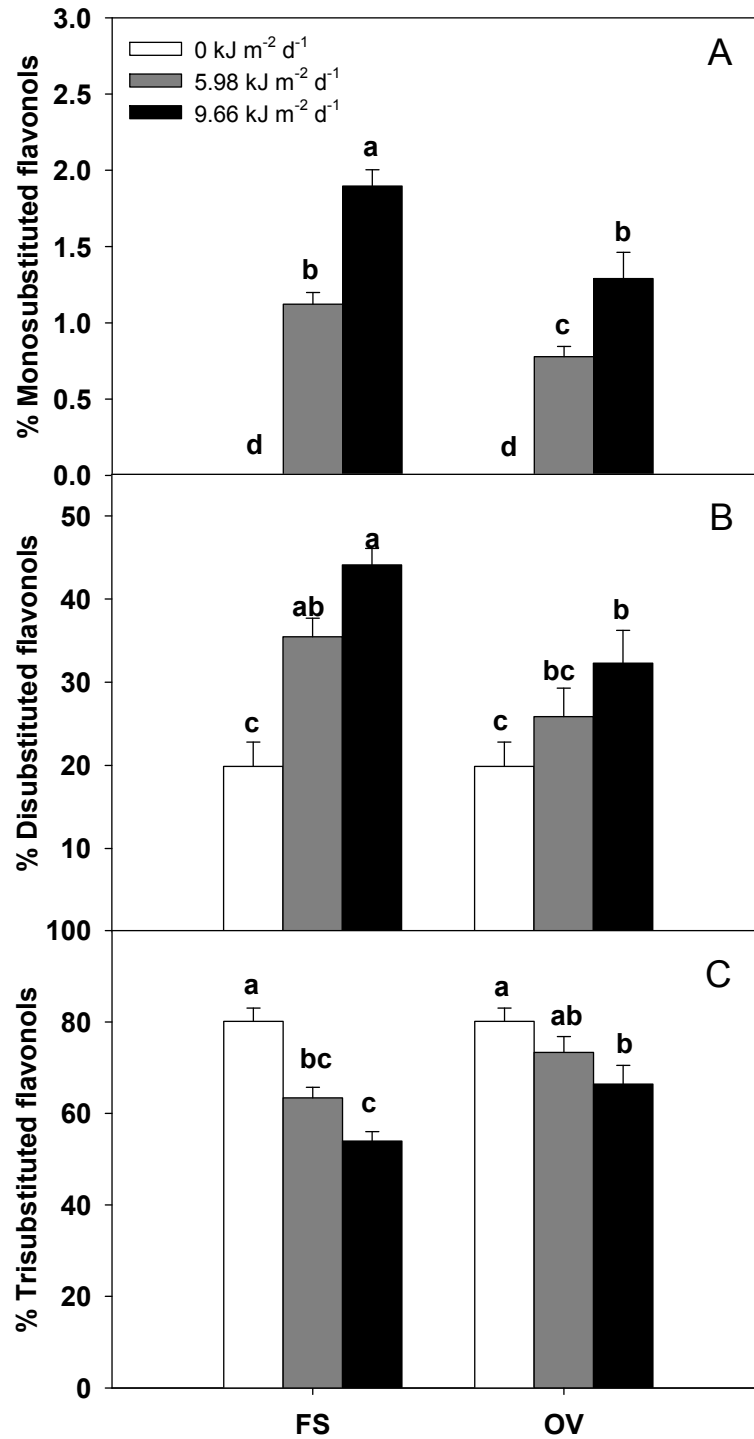


Figure 3. Molar relative abundance of monosubstituted(A), disubstituted (B) and trisubstituted (C) flavonols in Tempranillo grape skins exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B from fruit set to ripeness (FS) and from the onset of veraison to ripeness (OV) determined by HPLC-DAD (mean ± SE; n = 4). Groups marked with no letters in common indicate a significant difference (p<0.05) between them.

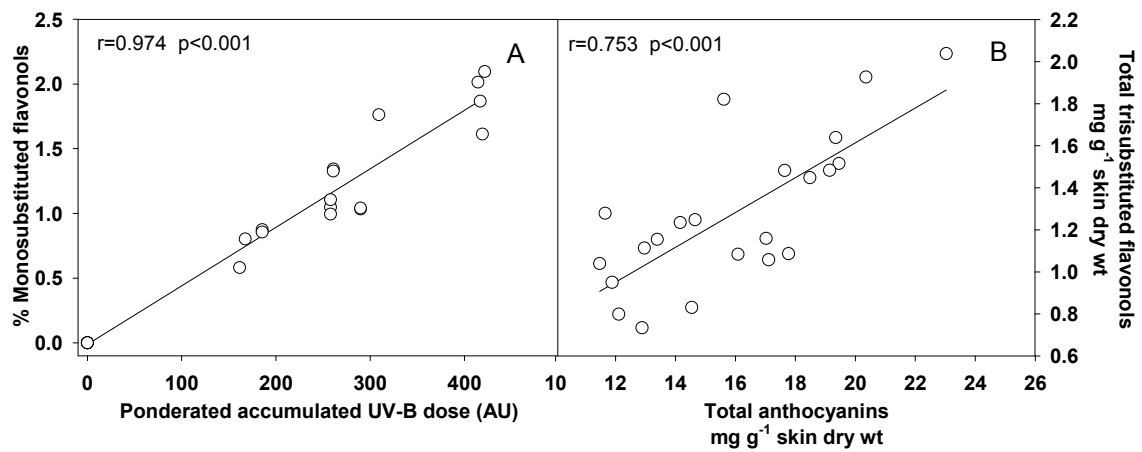


Figure 4. Correlation between (A) the accumulated UV-B biologically effective dose of UV-B and the molar relative abundance of monosubstituted flavonols by the equation: % monosubstituted flavonols = $-0.0108 + 0.00115 \cdot [\text{UV-B before veraison}] + 0.00451 \cdot [\text{UV-B after veraison}]$ and (B) total anthocyanin content with myricetin forms (myricetin-3-O-glucuronide, myricetin-3-O-glucoside and myricetin) concentration in Tempranillo grape skins.

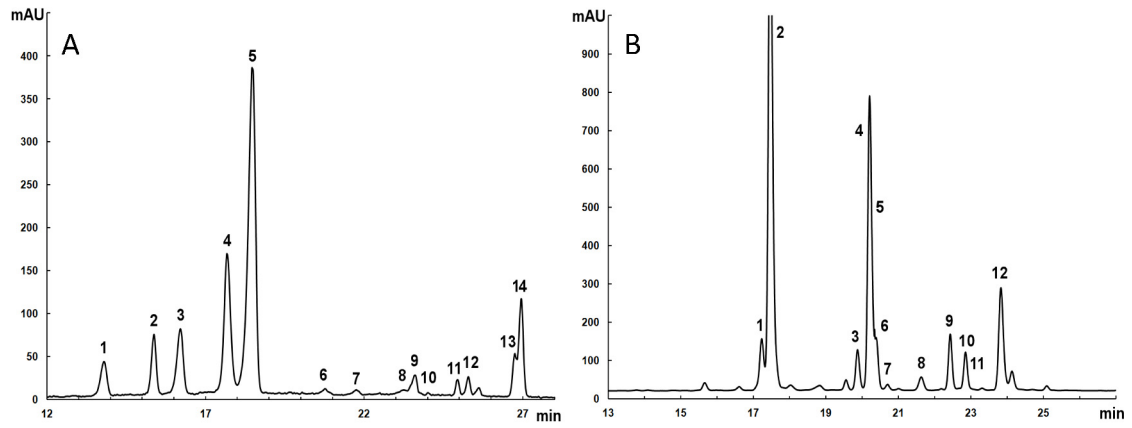


Figure S1. Chromatographic anthocyanins pattern (detection at 520 nm) (A) and flavonols pattern (detection at 360 nm) (B) of Tempranillo grape skins.

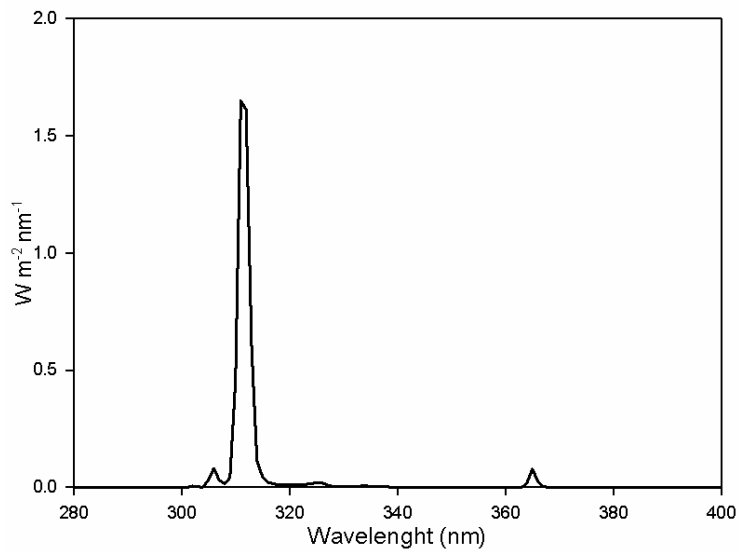


Figure S2. Spectral irradiance of the lamps used as UV-B source

Table S1. Chromatographic data (peak number and retention time) and MS² *m/z* values (molecular and fragment ions) of the anthocyanins and flavonols detected in Tempranillo grape skins.

Peak	Anthocyanin	<i>t_R</i> (min)	MH ⁺ (<i>m/z</i>)	Fragments (<i>m/z</i>)
1	delphinidin 3-O-glucoside	13.8	465	303
2	cyanidin 3-O-glucoside	15.3	449	287
3	petunidin 3-O-glucoside	16.1	479	317
4	peonidin 3-O-glucoside	17.8	463	301
5	malvidin 3-O-glucoside	18.5	493	331
6	cyanidin 3-O-acetyl-glucoside	20.8	491	449, 287
7	petunidin 3-O-acetyl-glucoside	21.8	521	479, 317
8	peonidin 3-O-acetyl-glucoside	23.4	505	463, 301
9	malvidin 3-O-acetyl-glucoside	23.6	535	493, 331
10	delphinidin 3-O-coumaroyl-glucoside	24.0	611	465, 303
11	cyanidin 3-O-coumaroyl-glucoside	24.9	595	449, 287
12	petunidin 3-O-coumaroyl-glucoside	25.3	625	479, 317
13	peonidin 3-O-coumaroyl-glucoside	26.7	609	463, 301
14	malvidin 3-O-coumaroyl-glucoside	26.9	639	493, 331
Peak	Flavonol	<i>t_R</i> (min)	MH ⁺ (<i>m/z</i>)	Fragments (<i>m/z</i>)
1	myricetin 3-O-glucuronide	17.2	495	319
2	myricetin 3-O-glucoside	17.5	479	319
3	quercetin 3-O-galactoside	19.9	465	303
4	quercetin 3-O-glucuronide	20.2	479	303
5	quercetin 3-O-glucoside	20.3	465	303
6	laricitrin 3-O-galactoside	20.6	495	333
7	laricitrin 3-O-glucoside	20.8	495	333
8	kaempferol 3-O-galactoside	21.5	449	287
9	kaempferol 3-O-glucoside	22.5	449	287
10	isorhamnetin 3-O-glucoside	22.8	479	317
11	syringetin 3-O-galactoside	23.0	509	347
12	syringetin 3-O-glucoside	23.9	509	347

Table S2. Relative abundance (%) of delphinidins, cyanidins, petunidins, peonidins, malvidins, anthocyanin acetates, anthocyanin *-p* coumarates, methylated anthocyanins and trisubstituted anthocyanins in Tempranillo grapes exposed to 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B from fruit set to ripeness (FS) and the onset of veraison to ripeness (OV) (mean ± SE; n = 4).

Application stage	OV			FS	
	UV-B dose	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹
% delphinidins	16.93 ± 0.64	18.12 ± 0.30	17.24 ± 0.13	17.90 ± 0.30	17.90 ± 0.32
% cyanidins	6.89 ± 0.76	8.97 ± 0.68	7.17 ± 0.35	7.71 ± 0.28	8.89 ± 0.77
% petunidins	14.87 ± 0.23	15.76 ± 0.07	15.77 ± 0.02	15.86 ± 0.16	15.09 ± 0.20
% peonidins	13.42 ± 0.87	15.68 ± 0.44	14.40 ± 0.39	15.74 ± 0.23	16.05 ± 0.68
% malvidins	47.74 ± 2.33	41.34 ± 1.35	45.27 ± 0.87	42.70 ± 0.75	41.97 ± 1.60
% acetates	3.30 ± 0.07	3.30 ± 0.12	3.44 ± 0.03	3.25 ± 0.06	3.25 ± 0.17
% coumarates	12.23 ± 1.08	10.09 ± 0.69	11.51 ± 0.30	10.07 ± 0.52	10.43 ± 0.72
% methylated	76.03 ± 1.35	72.78 ± 0.94	75.45 ± 0.48	74.30 ± 0.52	73.11 ± 1.03
% trisubstituted	79.54 ± 1.59	75.22 ± 1.10	78.28 ± 0.73	76.46 ± 0.49	74.97 ± 1.42

CHAPTER 3

Characterization of the adaptive response of grapevine (cv. Tempranillo) to UV-B radiation under water deficit conditions

Article in preparation

Characterization of the adaptive response of grapevine (cv. Tempranillo) to UV-B radiation under water deficit conditions

ABSTRACT

The aim of this work was to characterize the physiological response of grapevine (*Vitis vinifera* L.) cv. Tempranillo to UV-B radiation under water deficit conditions. Grapevine fruit-bearing cuttings were exposed to three levels of supplemental biologically effective UV-B radiation (0, 5.98 and 9.66 kJ m⁻² day⁻¹) and two water regimes (well watered and water deficit), in a factorial design, from fruit-set to maturity under glasshouse-controlled conditions. UV-B induced a transient decrease in net photosynthesis (A_{net}), actual and maximum potential efficiency of photosystem II, which was more evident in well watered plants, recovering afterwards in absence of lipid peroxidation. The concentration of methanol extractable UV-B absorbing compounds (MEUVAC) and superoxide dismutase activity increased with UV-B, irrespective of water availability. Water deficit decreased A_{net} and stomatal conductance, and did not change non-photochemical quenching and the de-epoxidation state of xanthophyll cycle, dark respiration and photorespiration being alternative ways to dissipate the excess of energy. Water deficit did not induced changes in antioxidant enzymes and lipid peroxidation. Little interactive effects between UV-B and water deficit were detected on photosynthesis performance, where the impact of UV-B was overshadowed by the effects of water deficit. Grape berry ripening was strongly delayed when UV-B and water deficit were applied in combination. In summary, deficit irrigation did not modify the adaptive response of grapevine to UV-B, through the accumulation of MEUVAC. However, combined treatments caused additive effects on the phenological development of grape berries.

Key words: photosynthesis, phenology, UV-B radiation, UV-B absorbing compounds, *Vitis vinifera* L., water deficit

1. INTRODUCTION

Mediterranean vegetation is often exposed to high fluence rates of UV-B radiation (280-315 nm), because of cloudless summer sky. In addition, the absence of precipitation is considered as a major limiting factor for plant growth and development during the summer months in this area (Patakas et al., 2005). In many regions, reduced water availability is frequently accompanied by increased UV-B radiation levels. Predicted scenarios of climate change over the next decades include a pronounced decrease in precipitation, especially in the warm season, thus increasing the probability of extreme drought events in the Mediterranean area (Trenberth and Fasullo, 2009). Besides, changes in mean cloudiness may affect the levels of solar radiation, including UV-B, reaching Mediterranean ecosystems in the near future (Giorgi and Lionello, 2008).

The effects of UV-B radiation on leaf physiology have been extensively studied. They include changes on leaf ultrastructure and anatomy, reduction in the concentration of photosynthetic pigments and photosynthetic capacity, altered plant phenology and reduction of plant biomass production (Ballaré et al., 2011; Kakani et al., 2003; Zlatev et al., 2012). In addition, when exposed to UV-B, plant cells usually produce reactive oxygen species (ROS), regardless of the dose applied, as a result of the disruption of metabolic activities (Lidon et al., 2012) or by the activation of membrane-localized NADPH-oxidase (Kalbina and Strid, 2006). In order to cope with oxidative damage, plants activate their antioxidant metabolism. Increases in superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities have been found in many UV-B exposure studies (Hideg et al., 2013 and references therein). Another of the direct UV-B adaptive mechanisms best studied is the biosynthesis of UV-B absorbing compounds, mainly flavonoids. UV-B responsive flavonoids located in the vacuoles of epidermal cells have the potential to attenuate the penetration of UV-B radiation (Taylor et al., 1997). In addition, dihydroxy flavonoids located in the chloroplasts of mesophyll cells may have a central role in the antioxidant defence system, inhibiting the generation of ROS and reducing ROS once they

have formed (Agati et al., 2013), thus avoiding oxidative damage to DNA, structural proteins, lipids and other cellular compounds. The chloroplast-located flavonols may have a very peculiar location which is of increasing significance when excess of radiant energy-induced depletion of key components of the antioxidant network system allows the diffusion of ROS out of the chloroplast (Agati et al., 2013).

Evidence of interaction between UV-B exposure and drought stress in plants has emerged in recent years. A series of similar defence mechanisms, as well as adverse changes, leading to disruption of physiological processes, growth inhibition and yield reduction, take place in plants exposed to single action of water deficit and UV-B radiation (Bandurska et al., 2013 and references therein). Therefore, combined action of water deficit and UV-B radiation can modify the response patterns. Previous studies show that UV-B radiation can interact with water deficit to induce protective mechanisms, i.e. increased levels of UV-B absorbing compounds (flavonoids) and antioxidant enzymes (SOD, CAT and APX), osmolyte accumulation (i.e. proline), increased leaf cuticle thickness or reduced stomatal conductance. These responses may substantially decrease UV-B and/or drought sensitivity when both factors are applied in combination (Alexieva et al., 2001; Drilias et al., 1997; Hofmann et al., 2003; Lu et al., 2007; Poulson et al., 2006). Furthermore, both UV-B and water deficit cause the generation of ROS, nitric oxide, and induce the synthesis of abscisic acid, ethylene, jasmonic and salicylic acid. These molecules, controlling plant reactions to both water deficit and UV-B applied separately, may also be involved in cross-talk under their combined actions (Bandurska et al., 2013; Kovacs et al., 2014). Nevertheless, responses are not always consistent between studies, thus leading in some cases to contradictory results. This is the case of Nogués and Baker (2000), Sullivan and Teramura (1990), Duan et al. (2008) and Bernal et al. (2013) who found few significant interactions between UV-B and water stress on plant growth and photosynthetic activity. On the other hand, when UV-B or water deficit, applied alone, causes damages, their combined action usually became more detrimental. For instance, combined stresses treatment led to the inhibition of soluble sugars

accumulation, restricting the capacity for osmotic adjustment, which caused negative additive effect on rosette growth of *Arabidopsis thaliana* (Comont et al., 2012). Additive negative effects on the growth of grapevine and willows have also been reported (Doupis et al., 2011; Turtola et al., 2006). Thus, the coincidence of water deficit and UV-B radiation, depending on their intensity, duration and sequence of occurrence, may activate mechanisms allowing plants to cope under stress conditions or enhance harmful effects. Such contrasting responses highlight the capacity for interaction between water deficit and UV-B, but clearly demonstrate the need for further studies of these interactive responses.

The aim of this work was to characterize the response of grapevine cv. Tempranillo to UV-B radiation under water deficit conditions, focusing on plant development, photosynthetic performance, antioxidant enzymes and UV-B absorbing compounds. Although the cultivar Tempranillo, in particular, is probably well adapted to relatively high UV-B irradiance (Martínez-Lüscher et al., 2013; Núñez-Olivera et al., 2006), the study of the interactive effects of UV-B and water deficit may help to understand the acclimation response of grapevine to UV-B within a climate change context, where the influence of other co-occurring abiotic stress factors can modulate grapevine responses to UV-B radiation (Caldwell et al., 2007). To this end, the fruit-bearing cutting model system for grapevine (fruiting plants developed from rooted cuttings) was used, providing a feasible system to study the impact of stress factors on grapevine physiology, without other confounding environmental influences. This model allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines, under fully controlled environmental conditions. Fruit-bearing cuttings respond like vineyard plants to different cultural factors (Dai et al., 2013; Lebon et al., 2005; Ollat et al., 1998). Indeed, the fruit-bearing cuttings model has been previously useful in the evaluation of the physiological response of grapevine, as well as berry ripening, to environmental or developmental factors (Antolín et al., 2010; Carbonell-Bejerano et al., 2013; Dai et al., 2013; Martínez-Lüscher et al., 2013; Niculcea et al., 2014; Salazar-Parra et al., 2012a, b; Salazar Parra et al., 2010).

2. MATERIAL AND METHODS

Plant material and growth conditions

Fruit-bearing cuttings of *Vitis vinifera* L. cv. Tempranillo were produced as described in Martínez-Lüscher et al. (2013). Briefly, three-node segments of grapevine cv. Tempranillo were collected in January 2011, from Station of Viticulture and Enology of Navarra (Olite, Spain). Rooting was induced using indol butiric acid in a heat-bed (27°C) kept in a cool room (5°C) in darkness. When cuttings developed enough roots, they were transplanted to 6.5 L pots containing 1:1 soil:peat (v/v). Only one flowering stem was allowed to develop on each plant. Shoots were fixed to horizontal rods compelling leaves to face upwards. Vegetative growth was controlled by pruning, in order to not-exceed an optimal leaf area to grape mass ratio (ca. 12 leaves per plant) (Jackson and Lombard, 1993). The experiment was carried out in glasshouses (Morales et al., 2014) at the University of Navarra (Pamplona, Spain, 42°48'14" N, 1°39'54" W) from April to September 2011. Throughout all the experiment, growth conditions in the glasshouses were 25/15°C, 50/90% relative humidity (day/night) and natural light. Glasshouse walls and structure filtered up to 99.9% of UV-B, 35% of UV-A and 15% of the photosynthetically active radiation (PAR) coming from the sun. A supplemental system of high-pressure sodium lamps (SON-T Agro Philips, Eindhoven, Netherlands) was triggered when PAR dropped below a photon flux density of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (14h photoperiod) (Morales et al., 2014).

Experimental design

When fruit-set was complete for all plants, three levels of biologically effective UV-B (0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$), calculated using the generalized plant action spectrum of Caldwell (1971) as formulated by Green et al. (1974), and two water availabilities (well watered and water deficit) were applied in a factorial design (12 plants per treatment) until maturity.

UV-B was applied by means of narrow band lamps TL100W/01 (311-313 spectrum peaking; Philips, Netherlands) hanging above the canopy. Spectral scans were performed with a

double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd., Livingstone, Scotland) (Fig. S1). UV-A radiation emitted by the lamps accounted for less than 1% of the solar UV-A irradiance (W m^{-2}) reaching the plants during the irradiation period. In the treatment of $0 \text{ kJ m}^{-2} \text{ d}^{-1}$, lamps on top were unlit. The UV-B doses of 5.98 and $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ were reached after 3h and 4h 51m of irradiation (0.55 W m^{-2}), respectively. The applied biologically effective dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ corresponded to 86% of the maximum daily dose at the study site, whereas $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ is only reached in viticulture suitable sites of the southern hemisphere, such as Argentina, South Africa or Australia (Quick TUV Calculator; <http://cprm.acd.ucar.edu/Models/TUV/>).

Soil water sensors (Watermark™ soil moisture sensors, Irrrometer, USA) placed in every pot, were used to control irrigation. Well watered plants were maintained at around 80% of substrate field capacity. Water deficit plants received only 50% of the water received by well-watered plants. Irrigation was performed with the nutritive solution described by Ollat et al. (1998). Nutrient solution concentrations were adjusted to provide the same amount of nutrients to each treatment batch.

Water status, gas exchange, chlorophyll fluorescence and MEUVAC were measured 7, 30 and 60 days after the beginning of the treatments. Maximum potential efficiency of photosystem II (Fv/Fm) was measured on days 1 to 7, 15, 30 and 60 after the beginning of the treatments. Plant growth, leaf photosynthetic pigments, antioxidant enzymes and lipid peroxidation were measured after 60 days of treatment. Given the importance of comparing leaves of the same physiological age in UV studies (Majer and Hideg, 2012), young fully expanded leaves of the same age (around four week old) were chosen. On day 7, these leaves had been fully developed before the beginning of the treatments, under the glasshouse conditions described previously. On day 30 and 60, young fully expanded leaves developed during the treatments were chosen, as far as possible.

Plant growth parameters and phenological development

At maturity, leaf area was measured immediately after harvest with a leaf area meter (LI-300 model; Li-Cor, Lincoln, USA). Leaf dry matter (DM) was determined after drying at 80°C for 2 days. Distance between shoot nodes was also determined. Three events (fruit-set, onset of veraison and maturity) were selected for the study of phenological development, determined as the elapsed time (days) between fruit-set to the onset of veraison, between the onset of veraison to maturity and between fruit-set and maturity.

Leaf relative water content (RWC)

Relative water content (RWC) was estimated by a modification of the method of Wheatherley (1950), using leaf discs of 1 cm², and calculated as $RWC = 100 \times (FW - DW) / (TW - DM)$, where FW, DW and TW denote fresh, dry and turgid weight, respectively. TW was calculated after fully hydrating fresh leaf discs in darkness at 4°C for 24h. DW was determined after drying at 80°C for 2 days in an oven.

Gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll (Chl) fluorescence measurements were conducted using a portable photosynthesis system (GFS-3000, Walz, Germany) with a 3 cm² cuvette. Dark respiration (R_D) measurements were performed 2 h before the beginning of the light period, with the CO₂ concentration set at 375 ppm, the temperature in the measurement chamber at 25°C, and 50% RH. Gas exchange characteristics in illuminated leaves were measured 3 h after the beginning of the light period, under a photon flux density of 1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 375 ppm CO₂, 25°C, and 50% RH. Dark respiration, net photosynthesis (A_{net}), stomatal conductance (g_s) and sub-stomatal CO₂ concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). Chl fluorescence was measured immediately after gas exchange measurements (in the dark and in the light) with a fluorescence module (PAM-fluorometer 3055-FL, Walz, Germany) attached to the photosynthesis equipment. The minimal and maximal fluorescence (F_o and F_m , respectively) were measured in dark-adapted leaves, whereas F_o' and F_m' were measured in the same leaves, with a photon flux

density of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, also measuring steady-state fluorescence signal (F_s). The maximum potential PSII efficiency was calculated as F_v/F_m , where F_v is $F_m - F_o$ (Morales et al., 1991). Actual PSII efficiency (Φ_{PSII}) was calculated as $(F_m' - F_s)/F_m'$. Photochemical quenching (qP) was calculated as $(F_m' - F_s)/F_v'$ (where F_v' is $F_m' - F_o'$), and non-photochemical quenching (NPQ) as $(F_m/F_m') - 1$ (Larbi et al., 2004). Electron transport rate (ETR) was calculated according to Krall and Edwards (1992) as $\Phi_{\text{PSII}} \times \text{PPFD} \times 0.5 \times 0.84$, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII (Ögren and Evans, 1993) and 0.84 is the fractional leaf absorptance, considered the most common absorptance coefficient for *Vitis vinifera* leaves under a wide range of environmental conditions and ages (Schultz, 1996). Multiplying 0.84×0.5 gives a value of 0.42, a value very similar to the α term used by other researchers to calculate ETR, which includes the product of leaf absorptance and the partitioning of absorbed quanta between PSI and PSII, and determined as the slope of the relationship between Φ_{PSII} and Φ_{CO_2} (i.e. the quantum efficiency of gross CO_2 fixation), obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing 1% O_2 (Valentini et al., 1995). For grapevine cv. Tempranillo, α was reported to be 0.425 (Bota et al., 2009). Photorespiration (R_L) was estimated as $1/12(\text{ETR} - 4 \times (A_{\text{net}} + R_D))$, according to Valentini et al. (1995).

Sampling for biochemical assays

The same leaves used for photosynthesis and Chl fluorescence measurements were used for the biochemical analysis. Two leaf disks of 1 cm^2 were harvested immediately after gas exchange and Chl fluorescence measurements, cut with a calibrated cork borer, wrapped in aluminum foil, immediately plunged into liquid N_2 and stored at -80°C until photosynthetic pigments and MEUVAC analyses. Then, the leaf was detached and frozen in liquid N_2 for lipid peroxidation (thiobarbituric acid reacting substances, TBARS), antioxidant enzyme activities and protein determinations.

Methanol extractable UV-B absorbing compounds (MEUVAC) and photosynthetic pigments

For extraction of UV-absorbing compounds, frozen leaf disks were extracted with 10 mL of methanol:water:7M HCl, equivalent to 70:29:1 (v/v/v). After 15 min at 85°C in the dark, the extracts were centrifuged for 15 min at 2,000 g. The absorbance spectrum was measured in a spectrophotometer UV/Vis (UVMini 1240, Shimadzu, Kyoto, Japan). The amount of MEUVAC was expressed in arbitrary units as the area under the absorbance curve in the UV-B interval ($AUC_{280-315}$) calculated per unit of leaf area (Dai et al., 2004).

Leaf photosynthetic pigments were extracted with 100% acetone in the presence of Na ascorbate, filtered through a 0.45- μ m filter and quantified by HPLC (Larbi et al., 2004). Two steps were used: mobile phase A (acetonitrile:methanol, 7:1, v/v) was pumped for 3.5 min, and then mobile phase B (acetonitrile:methanol:water:ethyl acetate, 7:0.96:0.04:8 by volume) was pumped for 4.5 min. To both solvents, 0.7% (volume) of the modified triethylamine (TEA) was added to improve pigment stability during separation (Hill and Kind, 1993). De-epoxidation state of the xanthophyll cycle (DES) was calculated as the sum of antheraxanthin (A) and zeaxanthin (Z) concentrations divided by the sum of violaxanthin (V), A and Z concentrations.

Determination of total soluble protein and antioxidant enzymes

Frozen leaf tissue (1 g) was homogenized with 10 mL of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA- Na_2 , 0.5 mM ascorbate and 1% PVPP (polyvinyl polypyrrolidone) in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min. The supernatant was used for determinations of protein content and antioxidant enzyme activity. Total soluble protein concentration was determined as described by Bradford (1976) using bovine serum albumin as standard. Superoxide dismutase (SOD, EC 1.15.1.1) was determined by the nitroblue tetrazolium (NBT) method (Dhindsa et al., 1981) with some modifications. Guaiacol peroxidase (GPX, EC 1.11.1.7) assay was performed using the method described by Pütter (1974). Catalase (CAT, EC 1.11.1.6) activity

was assayed by measuring the rate of H₂O₂ disappearance at 260 nm as described by Aebi (1984) with some modifications. Ascorbate peroxidase (APX, CE 1.11.1.11) activity was determined as described by Nakano and Asada (1981) with some modifications. See Martínez-Lüscher et al. (2013) for more details on enzyme activity determination and methods modification.

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reacting substances (TBARS), as described by Dhindsa et al. (1981), in leaf samples taken 60 days after the beginning of the experiments. Frozen leaf tissue (0.5 g) was homogenized with 10 mL trichloroacetic acid (TCA) 0.1% (w/v) in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min. Two mL of extract were mixed with 2 mL of either (i) 20% (w/v) TCA solution or (ii) 20% TCA solution containing 0.5% (w/v) TBA. The mixtures were heated at 95°C for 30 min, quickly cooled and centrifuged at 10,000 g and 4°C for 10 min. The absorbance of the supernatant was read at 532 nm with the values for non-specific absorption at 600 nm subtracted. TBARS concentration was calculated using the following formula: $[(A_{532} \times 1000) - (A_{600} \times 1000)] / 155$ being 155 the extinction coefficient in $\text{mM}^{-1} \text{cm}^{-1}$ (Heath and Packer, 1965).

Statistical analyses

Statistical analysis was performed using XLstat-Pro (Addissoft). The data were subjected to a three-factor analysis (ANOVA 3x3x2) to partition the variance into the main effects (stage, UV-B and water deficit) and the interaction among them. In case of significant interaction among factors, treatments were compared using the least significant difference (LSD) post-hoc test ($p < 0.05$).

3. RESULTS

Phenological development and plant growth parameters

UV-B radiation applied alone significantly delayed (up to 11 days) the phenological development of grapevine plants compared with those non-exposed, particularly after veraison (Table 1). Water deficit significantly shortened (up to three days) the period between fruit-set and the onset of veraison, but clearly delayed maturity, especially in the plants exposed to $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B compared with those not exposed to UV-B and well irrigated (27 days). UV-B radiation significantly reduced shoot growth, measured as internodal length, irrespective of water regime applied, whereas water deficit reduced leaf area, leaf dry matter and shoot internodal length (Table 1).

Leaf water status

Leaf stomatal conductance decreased as substrate water potential decreased, regardless of the UV-B dose (Fig. 1A). Both UV-B and water deficit led to small reductions in the relative water content of plants (between 2-5%, Fig. 1A and 1B). However, the effect of UV-B was more evident in well irrigated plants compared with those subjected to water deficit on day 7 and 60 after the beginning of the treatments (Fig. 1).

Gas exchange and chlorophyll fluorescence

The treatment with $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B transiently (day 7) reduced A_{net} , g_s , Φ_{PSII} and qP under well-watered and water deficit conditions (differences not significant in the last situation), and increased R_L and R_D only in the plants subjected to water deficit (Fig. 2 and 3). Water deficit, in general, significantly reduced A_{net} , g_s , E , C_i , Φ_{PSII} and qP values throughout the experiment, with respect to well watered conditions (Fig. 2 and 3). By contrast, deficit irrigation increased R_L and the ratio $\text{ETR}/A_{\text{net}}+R_D+R_L$, regardless of the dose of UV-B applied. NPQ was not significantly modified by UV-B or water deficit (Fig. 3). UV-B transiently decreased F_v/F_m during the first eight days of exposure, recovering afterwards to control

values (Fig. 4). There was no significant interaction between UV-B dose, water availability and developmental stage for most of the photosynthetic parameters measured, except for R_D (Fig. 2).

Photosynthetic pigments and methanol extractable UV-B absorbing compounds (MEUVAC)

Plants treated with UV-B radiation had higher concentrations of MEUVAC in leaves than those non-irradiated, after 30 and 60 days of exposure and under both water availabilities (Fig. 5). Water deficit did not affect the concentration of MEUVAC of plants irradiated with UV-B. Only in non-irradiated plants, the concentration of UV-B absorbing compounds under water deficit was significantly lower compared with well watered conditions on day 30 (Fig. 5).

UV-B radiation applied alone did not significantly modify the concentration of Chl and carotenoids after 60 days of treatment (Table 2). Water deficit significantly increased Chl concentration, especially when combined with UV-B ($P_{(UV \times WA)}=0.05$). When UV-B was applied in combination with water deficit, it increased neoxanthin and decreased zeaxanthin concentrations and the de-epoxidation stage of xanthophyll cycle, with respect to plants non-irradiated. Significant interactions between UV-B and water availability were observed for β -carotene, lutein, neoxanthin, zeaxanthin and DES.

Total soluble proteins, antioxidant enzymes and lipid peroxidation

UV-B radiation did not significantly modify the concentration of leaf soluble proteins, whereas water deficit increased TSP after 60 days of exposure (Table 3). Neither UV-B nor water deficit significantly modified the levels of TBARS (Table 3). Plants irradiated with UV-B had higher SOD activity, but similar CAT, GPX and APX activities, compared with those non-irradiated, regardless of water availability (Table 3). Water deficit did not modify, in general, the activity of antioxidant enzymes, and only significantly decreased CAT activity in plants exposed to $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$. There were no significant interactions between UV-B and water availability for any of these biochemical parameters (Table 3)

4. DISCUSSION

Grapevine acclimation to UV-B

The main effects of UV-B radiation reducing A_{net} were observed after 7 days of exposure on well-watered plants (Fig. 2). Contrarily to previous studies on grapevine (Berli et al., 2013; Martínez-Lüscher et al., 2013), such decrease was not accompanied by a reduction in the concentration of sub-stomatal CO_2 concentration. Therefore, reduction in CO_2 availability did not seem to be the main factor limiting CO_2 fixation in the plants exposed to UV-B. The slight down-regulation of PSII activity (Φ_{PSII}) observed on day 7, through a decrease in the proportion of the reaction centers that remained open (qP), may have contributed to the impaired A_{net} of UV-B exposed plants (Fig. 3). In addition, it has been recently reported that the short-term exposure to similar doses of UV-B reduced the concentration of total soluble proteins in grapevine leaves (Martínez-Lüscher et al., 2013), therefore a decrease in the amount of Rubisco protein could not be ruled out (Allen et al., 1997; Jordan, 1996). Along with the decrease observed in A_{net} , UV-B transiently induced a significant decrease in the maximum potential efficiency of PSII (Fv/Fm) during the first seven days of exposure (Fig. 4), thus indicating that grapevine leaves experienced a certain degree of stress during this period, recovering afterwards in absence of permanent PSII photoinhibition. The Fv/Fm is also a direct measurement of the PSII efficiency that excludes the indirect effect of UV-B reducing stomatal aperture and thereby limiting gas exchange (Berli et al., 2013).

One of the most important molecular events underlying UV-B protection and acclimation in plants, including grapevine, is the accumulation of phenolic compounds, mainly dihydroxyl flavonoids (Martínez-Lüscher et al., 2014), in the vacuoles of epidermal cells that reduces the penetration of UV wavelengths deeper into leaves (Berli et al., 2013). These compounds are also accumulated in the chloroplasts and vacuoles of mesophyll cells, where they may have a functional role in photoprotection, as H_2O_2 and singlet oxygen scavengers (Agati et al., 2013). In the present study, the levels of MEUVAC in the plants exposed to UV-B for 7 days

were not significantly higher than the levels of non-exposed plants (Fig. 5). These results suggest that during the first days of UV-B exposure, grapevine leaves, which had been fully developed without UV-B, were not able to accumulate enough amounts of phenolic compounds to protect the photosynthetic apparatus from UV-B radiation, thus showing a transient impairment of the photosynthetic performance (Fig. 2 to 4). Comont et al. (2012) reported that whereas this protective response builds up gradually in the field, plants grown in zero UV-B glasshouse conditions (as was this case) do not build such protection and/or the rapid capacity to respond to UV-B. Nevertheless, after 30 days of exposure to UV-B, grapevine leaves doubled their concentration of MEUVAC. This provided not only an effective protection of the photosynthetic apparatus, but also protected the leaves against a potential oxidative damage induced by UV-B, as suggests the low levels of TBARS (Table 3). All of these changes are indicative of UV-B acclimation occurring following long-term exposures (Martínez-Lüscher et al., 2013).

Effects of water deficit and its interaction with UV-B on the photosynthetic performance of grapevine

Grapevine plants significantly decreased A_{net} after 7, 30 and 60 days of water deficit, regardless of the UV-B dose applied (Fig. 2). Stomatal closure seemed to be the main limitation to photosynthesis, as deduced from the impaired g_s , E and C_i values. Unlike that observed for UV-B, the decrease of Φ_{PSII} induced by water deficit was smaller than that of A_{net} (Fig. 2 and 3), thus reflecting an unbalance between photochemistry and CO_2 fixation, which may lead to an excess of electrons generated in the photosynthetic electron transport chain. However, plants compensated for the decrease in photosynthesis maintaining R_D and significantly increasing R_L under water deficit conditions ($P_{(WA)} < 0.0001$) (Fig. 2). R_L and R_D are important mechanisms of photoprotection on grapevine, particularly under conditions of low A_{net} , as it occurs under water stress (Flexas et al., 2002). Therefore, the ratio of electrons generated to electrons consumed ($\text{ETR}/A_{\text{net}} + R_D + R_L$) only increased from ca. 5 to 6-7 (Fig. 3), which suggests that under water deficit conditions alternative sink for the excess of electrons,

such as the Mehler reaction, and the risk of water stress-mediated oxidative damage were low. In fact, there were not marked changes in the concentration of proteins and Chl, main targets of ROS, as well as in the levels of TBARS and the antioxidant enzyme activities (SOD, GPX, CAT and APX), after 60 days of water deficit. Similarly, Doupis et al. (2011) observed that the activity of antioxidant enzymes in grapevine leaves did not significantly increase under water-stress. More recently, Król et al. (2014) have reported that the antioxidant activity of grapevine leaves can even decrease under long-term drought stress, as happened with CAT activity in the present study (Table 3).

Regarding the combined effect of UV-B and water deficit, the results indicate that UV-B radiation significantly affected leaf water status (RWC) and photosynthetic activity (A_{net} , Φ_{PSII} and Fv/Fm, during the first days of exposure), primarily when water availability was high, and these effects less evident under water deficit conditions, when plant water content and photosynthetic performance was already reduced (Fig. 1 to 4). In addition, the combination of UV-B and deficit irrigation did not cause pronounced additive effects, with respect to the exposure to UV-B alone, on the activity of antioxidant enzymes and the accumulation of UV-B protecting pigments, two major acclimation responses of plants to UV-B radiation (Table 3 and Fig. 5). The results suggest that the adaptive response of grapevine to UV-B radiation was not modified by water deficit, and agree with previous studies, in which few interactive effects of UV-B radiation and water availability on water status, photosynthetic capacity and enzymatic antioxidant system of different plant species were observed (Duan et al., 2011; Kovacs et al., 2014; Nogués and Baker, 2000; Qaderi et al., 2010; Sullivan and Teramura, 1990). There was only one key functional trait, R_D , which exhibited relatively strong synergistic effect from the combined application of UV-B and water deficit on day 7. The increase in R_D , only observed in the plants exposed to UV-B and deficit irrigation, coincided with the beginning of the period of greater synthesis of UV-B protecting compounds (between day 7 and 30, Fig. 5). An increase in cell respiration has been related to the greater need for energy to protect against UV-B and for the repair of UV-B damage (Germ et al., 2004). In

addition, R_L increased especially in those plants grown under UV-B and water deficit (not significant interaction in this case) (Fig. 2), in which the decrease in the rates of carbon fixation with respect to Φ_{PSII} was more evident. The results suggest that when UV-B and water deficit were applied simultaneously, R_L and R_D were the two major photoprotective processes, whereas thermal energy dissipation was not involved in the adaptation of grapevine leaves to the combination of these factors, as indicated by the absence of changes in NPQ and DES (Fig. 3 and Table 2).

Phenological response of grapevine to UV-B and water deficit

One of the less studied aspects of the response of plants to the combination of UV-B and drought is their role as regulators of plant phenology and reproductive success (Comont et al., 2012). In the present study, plants grown under a single environmental factor, UV-B radiation or water deficit, took up to 11 and 13 days longer, respectively, to reach berry ripeness (ca. 22 °Brix) (Table 1). A delay of plant developmental stages (flowering and ripening) induced by UV-B has been reported for other species such as *A. thaliana* and wheat (Comont et al., 2012; Yuan et al., 1998). Schultz (2000) also reported that grapevine phenology may be affected by UV-B radiation. With respect to the effect of water deficit, moderate drought, in general, promotes sugar accumulation, thus accelerating grape ripening in the field (Castellarin et al., 2007; Chaves et al., 2010). Nevertheless, the effect varies depending on the timing and intensity of water stress imposition (Chaves et al., 2010) and a delay in fruit maturity has been observed after pre-veraison irrigation cut off in Cabernet Sauvignon (Sipiora and Granda, 1998). In the present work, the onset of veraison was triggered earlier (and with a lower total soluble solids content, data not shown) in berries under water deficit conditions, compared to well watered plants. Under water deficit, abscisic acid (ABA) biosynthesis is promoted (Stoll et al., 2000), which may have mediated the early veraison induction. Several studies have pinpointed ABA as the signal triggering berry ripening (Kuhn et al., 2014). Even though they started the ripening process sooner, sugar accumulation process was clearly slower in plants under water deficit, thus delaying maturity.

Since berry growth and ripening greatly depend on the import of photoassimilates (Davies et al., 2012), the strong reduction in leaf area and photosynthetic activity observed in the plants subjected to water deficit, may explain the slow-down in ripening.

Interestingly, the effect of water deficit delaying plant development was enhanced by UV-B radiation, and ripeness was delayed up to 28 days, when UV-B and water deficit were combined, with respect to the non-irradiated well-watered plants (Table 1). This delay was mainly associated with a slow-down of the stage III of berry growth comprised between the onset of veraison to maturity. Similarly, Comont et al. (2012) reported that UV-B and drought acting simultaneously caused a delay in phenology of *A. thaliana*, compared with drought alone. In the present study, the treatment with $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B slightly reduced A_{net} in plants grown under water deficit conditions on day 7 and 30 (stages of fruit-set and veraison, respectively). Such additive effect of UV-B, although not statistically significant, represented an additional decrease in A_{net} per unit of leaf area of between 10% and 23% (day 7 and 30, respectively), with respect to the photosynthetic rates of non-irradiated water deficit plants. Taken together with the decrease in total leaf area (19%, not significant differences), this may have reduced the total amount of carbon fixed per plant, and consequently allocated to grape ripening. However, the decreases in A_{net} and leaf area may not be enough to explain such phenological delay. Additionally, the effect of UV-B and water deficit increasing respiratory and photorespiratory activities should be also considered. Plants use up to 50% of recently formed C in respiration processes, which means that an important fraction of photoassimilates could have been wasted through R_L and R_D , thus contributing to an impaired carbon balance. Finally, the secondary metabolism activation cost, associated to the use of photoassimilates to produce phenolics and other UV-B screening compounds, cannot be ruled out in order to explain the delayed phenological development in the combined treatment.

5. CONCLUSIONS

The results suggest that under environmental controlled conditions, water deficit had larger effects on grapevine growth and photosynthetic activity than UV-B, which only exhibited a transient detrimental effect on the photosynthesis of plants. Little interaction effects between UV-B and water deficit were detected on photosynthesis performance and UV-B radiation did not strongly aggravate the impact of deficit irrigation on the photosynthetic activity of grapevine. In general, the acclimation process of grapevine leaves to a long-term exposure to UV-B, through the accumulation of UV-B absorbing compounds, was not altered by water deficit. However, the combined exposure to UV-B and water deficit led to additive effects on plant phenological development, slowing-down grape ripening, which was associated with an impaired carbon balance under such conditions.

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Table 1. Phenological development and growth parameters of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹, well watered and water deficit, at maturity. Values are means (standard error). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B and water availability ($P_{(UV-B \times WA)}$). In case of significant interaction between factors, different letters within the same parameter indicate significant differences ($p < 0.05$) according to LSD test.

	Well Watered			Water Deficit			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV-WA)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
Elapsed time from fruit set to the onset of veraison (days)	41.2(1.3)	41.7 (0.7)	42.9(0.9)	40.0(0.8)	39.8(0.9)	40.1(1.0)	0.574	0.012	0.666
Elapsed time from the onset of veraison to maturity (days)	23.2(0.4)	34.6(5.4)	27.7(3.7)	37.7(2.9)	42.6(2.7)	51.3(3.0)	0.015	<0.001	0.074
Elapsed time from fruit set to maturity (days)	64.5(1.0)	76.0(5.7)	70.6(3.5)	77.7(3.4)	82.4(2.4)	91.6(2.8)	0.010	<0.001	0.113
Leaf area (dm ² plant ⁻¹)	13.5(0.7)	13.5(1.2)	13.5(0.5)	5.4(0.6)	4.6(0.5)	4.4(0.2)	0.748	<0.001	0.739
Leaf dry matter (g plant ⁻¹)	8.8(0.8)	10.1(1.1)	10.2(0.5)	3.7(0.4)	3.6(0.4)	3.5(0.2)	0.556	<0.001	0.348
Internodal length (cm)	8.0(0.3)	7.0(0.6)	6.6(0.3)	3.9(0.3)	2.8(0.2)	2.9(0.2)	0.001	<0.001	0.723

Table 2. Chlorophyll (Chl) and carotenoid concentrations and de-epoxidation state (DES) of xanthophyll cycle in leaves of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹, well watered and water deficit, at maturity. Values are means (standard error). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B and water availability ($P_{(UV-B \times WA)}$). In case of significant interaction between factors, different letters within the same parameter indicate significant differences ($p < 0.05$) according to LSD test.

	Well Watered			Water Deficit			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV \times WA)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
Chl a+b (μmol m ⁻²)	602.7(25.8)	581.3(24.1)	501.8(34.1)	557.3(45.5)	720.8(34.8)	638.0(64.6)	0.114	0.029	0.050
Chla/Chlb	2.96(0.04)	3.04(0.03)	3.12(0.06)	3.09(0.03)	3.02(0.05)	3.01(0.07)	0.686	0.986	0.056
β-Carotene (mmol mol ⁻¹ Chl)	88.9(1.2) ab	86.5(1.0) b	91.5(1.3) a	85.8(1.3) b	89.9(1.1) a	88.6(1.2) ab	0.092	0.539	0.012
Neoxanthin (mmol mol ⁻¹ Chl)	43.1(0.03) a	41.2(0.9) ab	41.8(0.6) ab	40.1(0.8) b	42.6(1.0) a	42.7(0.7) a	0.742	0.838	0.026
Lutein (mmol mol ⁻¹ Chl)	138.9(1.0) ab	130.7(3.3) b	135.5(1.9) ab	132.9(4.6) ab	139.5(1.6) a	134.6(3.3) ab	0.953	0.791	0.042
Violaxanthin (V) (mmol mol ⁻¹ Chl)	57.1(3.5)	53.1(1.2)	55.2(3.2)	52.3(5.7)	59.9(2.1)	56.9(2.4)	0.845	0.544	0.208
Antheraxanthin (A) (mmol mol ⁻¹ Chl)	7.0(0.4)	6.4(1.3)	8.5(1.2)	10.1(2.5)	6.8(0.6)	6.0(0.5)	0.293	0.751	0.121
Zeaxanthin (Z) (mmol mol ⁻¹ Chl)	5.6(0.5) ab	6.0(1.4) ab	9.7(2.2) ab	10.1(2.7) a	6.8(0.8) ab	5.2(1.1) b	0.628	0.831	0.032
V+A+Z (mmol mol ⁻¹ Chl)	69.6(3.6)	67.3(1.6)	73.4(2.2)	72.5(7.0)	73.0(1.9)	68.0(2.9)	0.970	0.721	0.311
DES (A+Z)/(V+A+Z)	0.18(0.01) ab	0.18(0.03) ab	0.25(0.04) ab	0.28(0.06) a	0.18(0.02) ab	0.16(0.02) b	0.405	0.901	0.048

Table 3. Total soluble proteins (TSP), lipid peroxidation, determined as thiobarbituric acid reacting substances (TBARS) concentration, superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) activities in leaves of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered and water deficit, at maturity. Values are means (standard error). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B and water availability ($P_{(UV-B \times WA)}$). In case of significant interaction between factors, different letters within the same parameter indicate significant differences ($p < 0.05$) according to LSD test.

	Well Watered			Water Deficit			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV-WA)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
TSP (mg g ⁻¹ DW)	12.0(0.18)	13.4(0.85)	15.1 (1.60)	18.9(2.24)	16.2 (1.22)	19.8(2.18)	0.232	0.001	0.445
TBARS (nmol MDA g ⁻¹ DW)	130.1(36.9)	98.3(11.2)	87.3(5.1)	94.7 (8.8)	92.6(14.6)	106.4(34.1)	0.412	0.692	0.485
SOD (USOD mg ⁻¹ g ⁻¹ DW min ⁻¹)	380.9(43.9)	840.6(52.2)	850.1(42.6)	526.8(64.3)	842.9(70.6)	943.1(39.2)	< 0.001	0.080	0.413
GPX (nmol guaiacol g ⁻¹ DW min ⁻¹)	31.3(8.3)	28.6(6.8)	30.1(9.4)	23.7(6.1)	32.3(6.4)	41.3(11.2)	0.577	0.683	0.538
CAT (mmol H ₂ O ₂ g ⁻¹ DW min ⁻¹)	2.48(0.52)	2.59(0.16)	2.37(0.37)	2.33(1.08)	2.07(0.46)	2.28(1.02)	0.583	0.002	0.051
APX (μmol asc g ⁻¹ DW min ⁻¹)	15.0(1.62)	16.9(3.4)	14.8(1.96)	13.8(1.1)	12.7(1.2)	12.8(1.6)	0.889	0.131	0.742

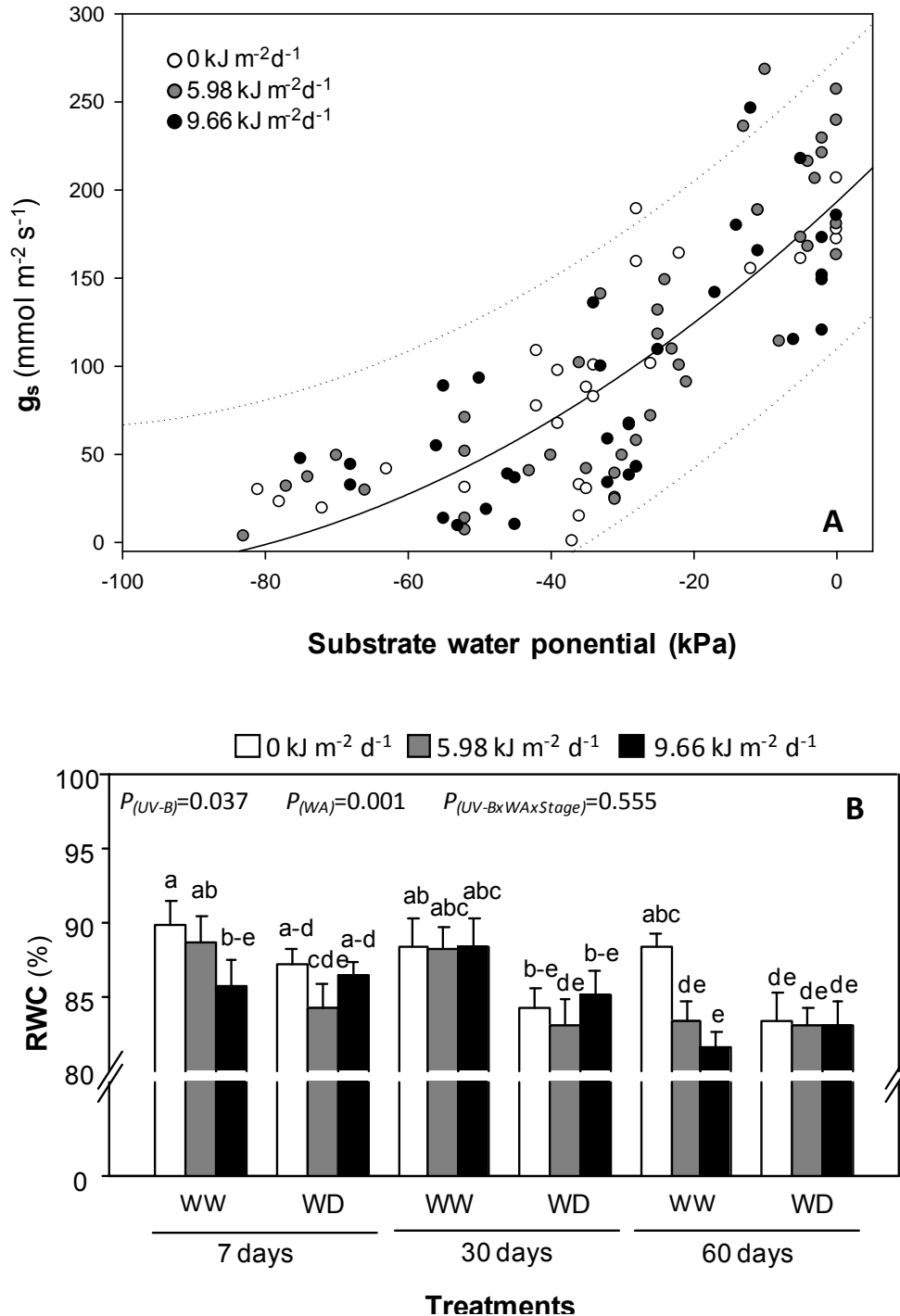


Figure 1. Relationship between stomatal conductance (g_s , A) and substrate water potential and relative water content (RWC, B) of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B, well watered and water deficit. In Fig. B, columns are means \pm SE. Different letters within the same parameter indicate significant differences ($p < 0.05$).

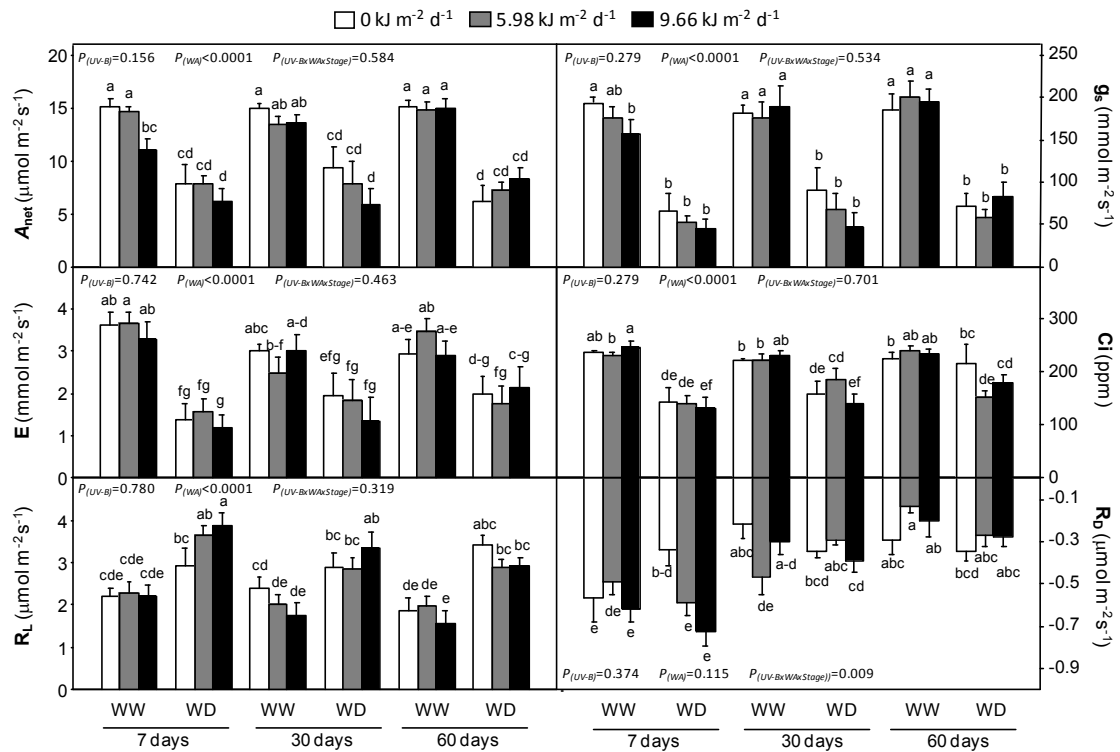


Figure 2. Net photosynthesis (A_{net}), stomatal conductance (g_s), transpiration (E), sub-stomatal CO₂ concentration (C_i), photorespiration (R_L) and dark respiration (R_D) of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered (WW) and water deficit (WD), after 7, 30 and 60 days of treatment. Columns are means \pm SE. Different letters within the same parameter indicate significant differences (p < 0.05). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$).

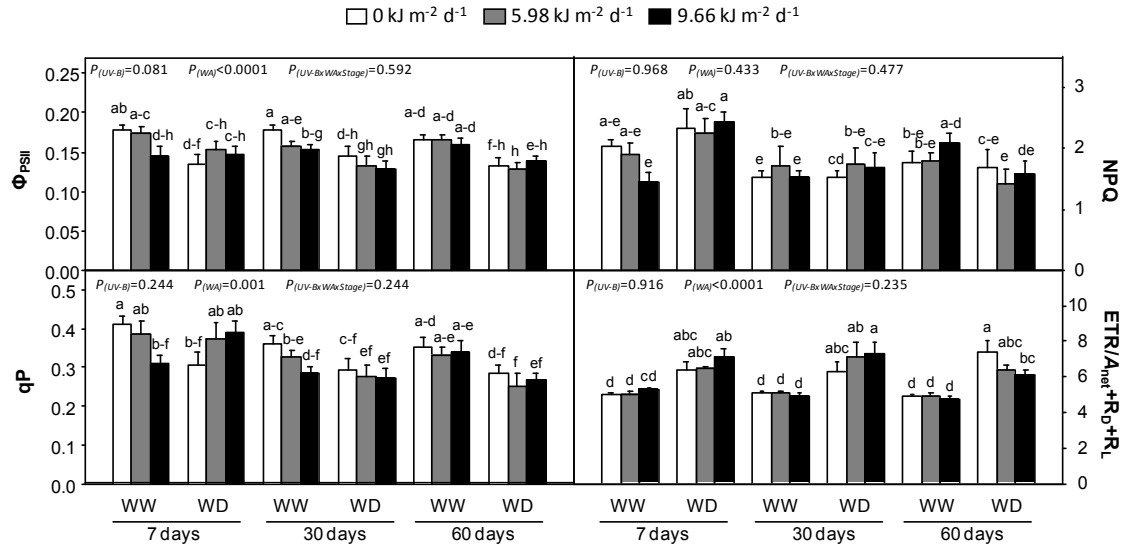


Figure 3. Actual PSII efficiency (Φ_{PSII}), non-photochemical quenching (NPQ) and photochemical quenching (qP), and ratio of electrons generated to electrons consumed in A_{net} , R_D and R_L ($ETR/A_{net}+R_D+R_L$) of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered (WW) and water deficit (WD), after 7, 30 and 60 days of treatment. Columns are means \pm SE. Different letters within the same parameter indicate significant differences ($p < 0.05$). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$).

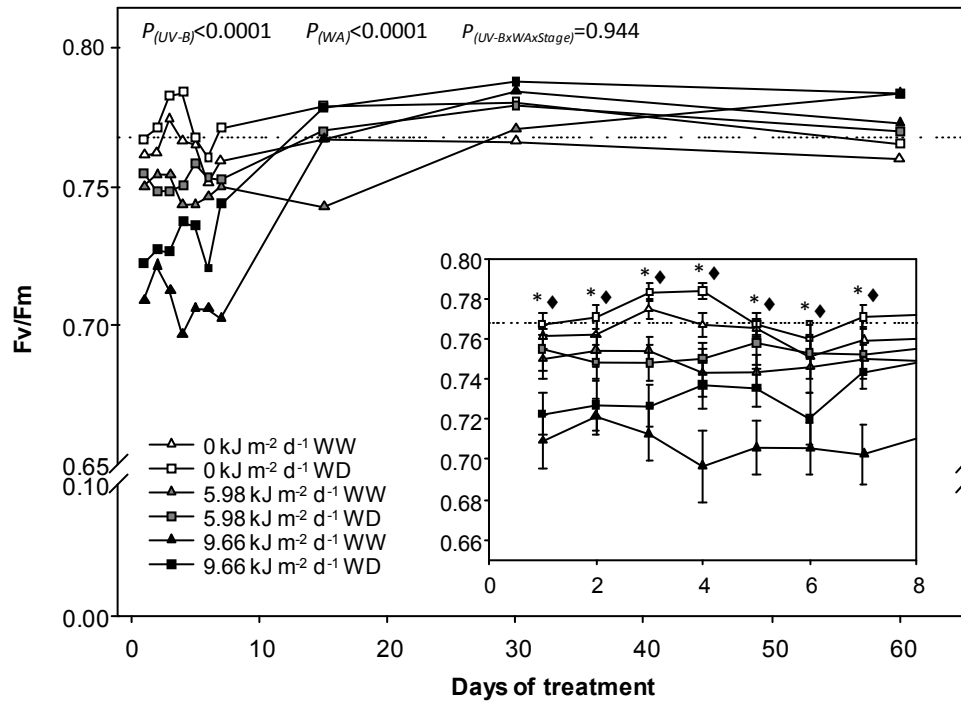


Figure 4. Maximum potential PSII efficiency (Fv/Fm) of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered (WW) and water deficit (WD), throughout the experiment. Points are means \pm SE. Significant differences ($p < 0.05$) between UV-B doses and water regimes within the same date are indicated as * and \blacklozenge , respectively. Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$). Inset is a zoom of the 8 first days of treatment.

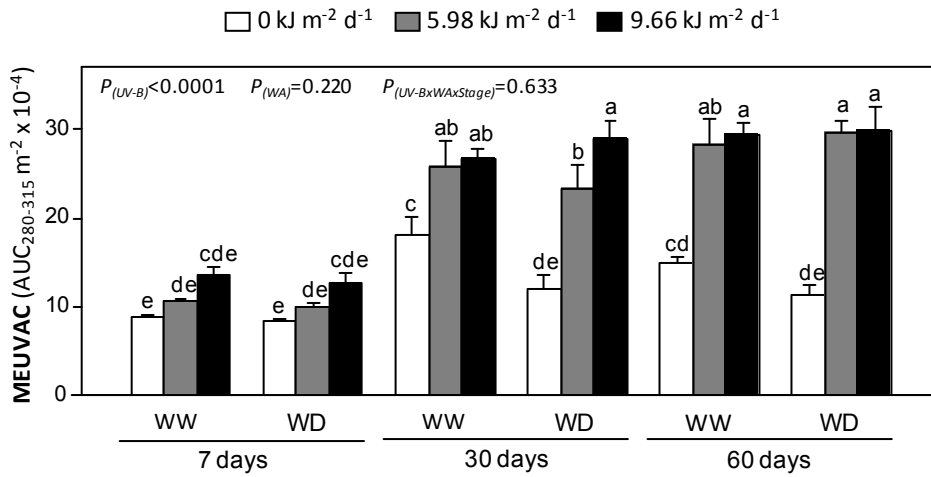


Figure 5. Leaf methanol extractable UV-B absorbing compounds (MEUVAC) of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B, well watered (WW) and water deficit (WD), after 7, 30 and 60 days of treatment. Columns are means \pm SE. Different letters within the same parameter indicate significant differences ($p < 0.05$). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$).

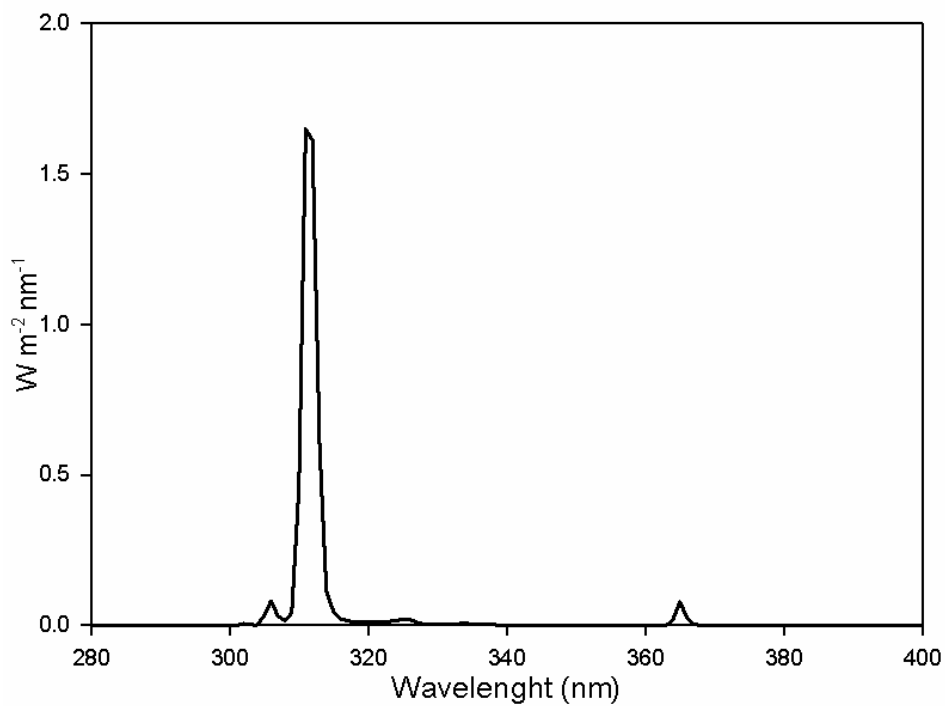


Figure S1. Spectral irradiance of the UV-B lamps.

CHAPTER 4

Ultraviolet-B radiation and water deficit interact to alter flavonol and anthocyanin profile in grapevine berries through transcriptomic regulation

Ultraviolet-B radiation and water deficit interact to alter flavonol and anthocyanin profile in grapevine berries through transcriptomic regulation

ABSTRACT

UV-B radiation triggers flavonol and anthocyanin biosynthesis in plant tissues. Previous research has showed strong quantitative effects on grape skin flavonol and anthocyanin profiles in response to UV-B and water deficit. The aim of this study was to elucidate the mechanisms leading to changes in quality-related metabolites, such as flavonols and anthocyanins, in response to UV-B and water deficit from fruit set to maturity. Grapevines (*Vitis vinifera* L. cv. Tempranillo) were exposed to three levels of UV-B radiation (0, 5.98 and 9.66 kJ m⁻² day⁻¹) and subjected to two water regimes. A strong effect of UV-B on flavonol and anthocyanin biosynthesis was found, resulting in an increased concentration and a change in their profile. Concomitantly, two key biosynthetic genes (*FLS1* and *UFGT*) were up-regulated by UV-B. Changes in flavonol and anthocyanin composition were explained to a large extent by the transcript levels of *F3'H*, *F3'5'H* and *OMT2*. A significant interaction between UV-B and water deficit was found in the relative abundance of 3'4' and 3'4'5' substituted flavonols, but not in their anthocyanin homologues. The ratio between 3'4'5' and 3'4' substituted flavonols was linearly related to the ratios of *F3'5'H* and *FLS1* transcription, two steps up-regulated independently by water deficit and UV-B radiation respectively. The results suggest that changes in flavonol profile in response to environmental conditions are, not only a consequence of changes in the expression of flavonoid hydroxylases; but also, the result of the competition of FLS, F3'5'H and F3'H for the same flavonol substrates.

Keywords: Climate change, flavonoid biosynthesis, fruit quality, grapevine, UV-B radiation water deficit.

1. INTRODUCTION

Moderate water deficit is widely utilized in improving wine grape quality. The influence of low water availability on berry composition can be attributed to several factors, including the hydraulic restriction of growth and chemical signalling (e.g. abscisic acid) from drying roots (Chaves et al., 2010). Water deficit may reduce berry size, but it may also accelerate ripening and increase the biosynthesis of specific secondary metabolites such as anthocyanins, proanthocyanidins and tannins (Bucchetti et al., 2011; Castellarin et al., 2007a; Deluc et al., 2009; Olle et al., 2011). Transcriptional analyses also reveal a potential influence of water deficit on cell-wall, sugar and hormone metabolism (Deluc et al., 2007), and important flavour and quality traits (Deluc et al., 2009). Within the predicted climate change scenario (IPCC, 2013), meteorological conditions are expected to change. For places such as Mediterranean Europe and almost viticultural regions in the world, this change may lead towards a situation with less cloud coverage during summer, meaning fewer precipitations during this period, as well as enhanced levels of incident UV-B radiation.

UV-B radiation is considered as an environmental stress factor and the adverse effects of increasing levels of UV-B on plants, aiming to simulate ozone layer reductions, has been widely reported (reviewed by Kakani et al., 2003) . However, current research is mainly driven to investigate the regulatory effects of UV-B radiation within the natural light environment (Jansen and Bornman, 2012). In spite of accounting for less than 0.5 % of the solar electromagnetic radiation reaching the earth's surface, UV-B radiation (280-315 nm) has been proposed as a useful factor to enhance the quality and health-promoting molecules of vegetables and fruits (Jansen et al., 2008). The positive effect of UV-B on grape berry ripening has also been reported, increasing flavonoid content in the berries and affecting flavonoid profiles (Berli et al., 2011; Gregan et al., 2012; Martínez-Lüscher et al., 2014). The transcriptional regulation of flavonoid metabolism in grape berry development is one of the most extensively studied, with 14 flavonoid related genes of the R2R3 MYB family described (Fournier-Level et al., 2010; Matus et al., 2008). Flavonol and anthocyanin biosynthesis is

mediated by the expression of encoding MybF1 and MybA1 transcription factors, which trans-activate flavonol synthase (*FLS1*) and UDP-glucose flavonoid glucosyl transferase (*UFGT*). FLS and UFGT enzymes are involved in the last step of flavonol and anthocyanin biosynthesis, respectively (Czemmel et al., 2009; Walker et al., 2007). Nevertheless, the regulation of flavonoid biosynthesis in response to environmental factors needs further research (Czemmel et al., 2012).

Recent research has been focused on unravelling the mechanisms leading to flavonoid diversification (Nishihara and Nakatsuka, 2011; Saito et al., 2013; Tan et al., 2013; Zhu et al., 2012). The general mechanism of diversification of grape flavonoids has been described by Bogs et al. (2006) through the identification of flavonoid hydroxylases. Thus, the constitutive expression of the genes encoding for these enzymes determines, to a great extent, berry colour (Falginella et al., 2010). However, when comparing the diversification of anthocyanins and flavonols, great dissimilarities may be observed (Castillo-Muñoz et al., 2009; Mattivi et al., 2006). Martínez-Lüscher et al. (2014) have recently reported that B-ring hydroxylation of flavonol profile of grape berry was linearly affected by UV-B exposure under controlled conditions. Differences in the hydroxylation profile of anthocyanins have been reported for different grapevine cultivars (Castellarin and Di Gaspero, 2007) and water availabilities (Castellarin et al., 2007b). These differences have been explained by transcript levels of *flavonoid 3'5'hydroxylase (F3'5'H)*, which has an expression peak one week after the onset of veraison, concomitant to the configuration of the hydroxylation profile of anthocyanins (Castellarin et al., 2006). In addition, variation in the methylated anthocyanin accumulation has been explained by the expression level of *O-methyltransferase 2 (OMT2)* (Fournier-Level et al., 2011). However, the knowledge of the diversification of anthocyanins may not be always applicable to the flavonol profile (Mattivi et al., 2006).

Despite the almost inseparable coexistence of UV-B radiation and water deficit in the predicted future climate no study has combined UV-B radiation and water deficit treatments to investigate their interactive effects on fruit composition. The aim of this study was to

investigate this interaction especially with regard to changes in the composition of flavonols and anthocyanins. Fruit-bearing cuttings model (Ollat et al., 1998) was chosen to analyse the impact of UV-B radiation and water availability with precision. This model allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines under controlled environmental conditions. In addition, the chronology of flowering and fructification of the fruit-bearing cuttings is similar to that of the vineyard-grown grapevines (Lebon et al., 2008). Hence, fruit-bearing cuttings have been successfully used as a model for studies on grape berry composition (Dai et al., 2013) and the environmental regulation of the transcript profile (Carbonell-Bejerano et al., 2013).

2. MATERIAL AND METHODS

Growing conditions and experimental design

Grapevine (*Vitis vinifera* L. cv. Tempranillo) fruit-bearing cuttings were produced as in Martínez-Lüscher et al. (2013). Briefly, three-node segments of grapevine shoots were collected in January 2011, from Station of Viticulture and Enology of Navarra (Olite, Spain). Rooting was induced using indol butiric acid in a heat-bed (27°C) kept in a cool room (5°C) in darkness. When cuttings developed enough roots, they were transplanted to 6.5 L pots containing 1:1 soil:peat (v/v). Only one flowering stem was allowed to develop on each plant. Shoots were fixed to horizontal rods compelling leaves to face upwards. Vegetative growth was controlled by pruning, so as not to exceed an optimal leaf area to fruit mass ratio (ca. 12 leaves per plant) (Jackson and Lombard, 1993). The experiment was carried out in glasshouses at the University of Navarra (Pamplona, Spain; 42°48'14" N, 1°39'54" W) from April to September 2011. Throughout all the experiment, growth conditions in the glasshouses were 25/15°C and 50/90% relative humidity (day/night). Glasshouse walls and structure filtered up to 99.9% of UV-B, 35% of UV-A and 15% of the photosynthetically active radiation (PAR) coming from the sun. A supplemental system of high-pressure sodium lamps (SON-T Agro Philips, Eindhoven, Netherlands) was triggered when PAR dropped below a

photon flux density of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (14 h photoperiod). When fruit set was complete for all plants, three levels of biologically effective UV-B (0, 5.98 and $9.66 \text{ kJ m}^{-2} \text{d}^{-1}$), calculated using the generalized plant action spectrum of Caldwell (1971); and two water availabilities (well watered and drought) were applied in a factorial design (12 plants per treatment) until maturity.

UV-B was applied by means of narrow band lamps TL100W/01 (311-313 spectrum peaking; Philips, Netherlands) hanging above the canopy. Spectral scans were performed with a double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd., Livingstone, Scotland; Fig. S1). UV-A radiation emitted by the lamps accounted for less than 1 % of the solar UV-A irradiance (W m^{-2}) reaching the plants during the irradiation periods. In the treatments of $0 \text{ kJ m}^{-2} \text{d}^{-1}$, UV-B lamps were unlit. The UV-B doses of 5.98 and $9.66 \text{ kJ m}^{-2} \text{d}^{-1}$ were reached after 3h and 4h 51m of irradiation (0.55 W m^{-2}) around midday, respectively. The applied biologically effective dose of $5.98 \text{ kJ m}^{-2} \text{d}^{-1}$ corresponded to 86% of the maximum daily dose at the study site, whereas $9.66 \text{ kJ m}^{-2} \text{d}^{-1}$ is only reached in viticulture suitable sites of the southern hemisphere, such as Argentina, South Africa or Australia (Quick TUV Calculator; <http://cprm.acd.ucar.edu/Models/TUV/>).

Soil water content was measured daily with granular matrix sensors (Irrometer, USA) placed in every pot. Well watered plants were maintained at around 80 % of substrate field capacity (Fig S2) which corresponded to a water stem potential of $0.71 \pm 0.03 \text{ MPa}$. In the water deficit treatments, plants were subjected to cyclic drought, which consisted in withholding irrigation until water content reached 27-35% of substrate field capacity (Fig. S2), which corresponded to a water stem potential of $1.45 \pm 0.04 \text{ MPa}$. Drought plants were only considered for samplings when substrate water content was below 40% of substrate field capacity, which resulted in a reduction in stomatal conductance of 65%. Irrigation was performed with the nutritive solution described by Ollat et al. (1998). The number of water deficit cycles was not statistically different between the UV-B treatments. Nutrient solution concentrations were adjusted to provide the same amount of nutrients to each treatment batch. Grape berry

samplings were performed at 4 developmental stages: i) pea size (after 14 days of treatments); ii) onset of veraison, when berries started to turn colour (after 41.4 days of treatments on average); iii) one week after the onset of veraison (48.4 days of treatments on average), when transcripts levels of the target genes are around their maximum (Castellarin et al., 2007b); and iv) maturity, when the grapes in the cluster of each plant reached a TSS content of ca. 22°Brix (after 77.7 days of treatments on average). All samples were collected right after the daily UV-B irradiation period, frozen immediately in liquid nitrogen and preserved at -80°C until analysis.

Grape technological and phenolic maturity

At maturity clusters were detached and berry traits and yield were determined. Samples of at least 25 berries from each cluster were pressed and centrifuged to obtain the must. Titratable acidity, pH, malic acid and TSS were analysed in this must as in Salazar Parra et al. (2010). Another 25 berries were ground in liquid nitrogen with a mortar and 6 g of homogenate was used to perform the determination of extractable and total anthocyanins, tonality, colour density and total polyphenol index (TPI) as in Salazar Parra et al. (2010).

Flavonol and anthocyanin profiling

Grape skins from samples collected at pea size, at the onset of veraison and at maturity were freeze-dried, ground and extracted in methanol containing 0.1% HCl (v/v). Extracts were filtered through a 0.45 µm polypropylene syringe filter (Pall Gelman Corp., Ann Arbor, USA) for HPLC routine analysis of individual anthocyanins and flavonols by means of a summit HPLC system consisting of P680 pump, ASI-100T™ autosampler and UVD 340U UV-Vis diode array detector operating at 520 nm and at 360 nm (Dionex Corporation, Sunnyvale, CA, USA) as described in Acevedo De la Cruz et al. (2012). Malvidin-3-O-glucoside was used as common external standard for all the quantified anthocyanins (at 520 nm), and quercetin-3-O-glucoside was used for all the quantified flavonols (at 360 nm) (Extrasynthese, Genay, France).

Formal identification of flavonoids was carried out by using high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). The chromatography apparatus, Agilent 1200 from Agilent Technologies (Santa Clara, CA, USA) consists of an autosampler module, a degasser, a binary pump, a column heater/selector and a UV-visible diode array detector (DAD) from the same supplier. This HPLC was coupled to an Esquire 3000+ ion trap mass spectrometer using an ESI source from Bruker Daltonics (Billerica, MA, USA). The HPLC output flow was split with a passive splitter with an average 1:100 ratio, depending on the solvent flow, viscosity and rate. Anthocyanins usually interfere significantly with the chromatographic separation and MS identification of flavonols. For the identification of flavonols, anthocyanin removal was, therefore, carried out by the use of a cation exchange resin Dowex 50WX4 (Sigma-Aldrich) according to Decendit et al. (2002). More details are provided in (Martínez-Lüscher et al., 2014).

According to the substituents in the B ring, flavonols and anthocyanins were grouped as 4' substituted flavonols (sum of kaempferols), 3'4' substituted flavonols (sum of quercetins and isorhamnetins), 3'4'5' substituted flavonols (sum of myricetins, laricitrins and syringetins), methylated flavonols (sum of isorhamnetins, laricitrins and syringetins), 3'4' substituted anthocyanins (sum of cyanidins and peonidins), 3'4'5' substituted anthocyanins (sum of delphinidins, petunidins and malvidins) and methylated anthocyanins (sum of peonidins, petunidins and malvidins). Anthocyanins presented additional substituents such as *-p* coumaroyl (sum of *-p* coumaroyl acylated anthocyanins) and acetate (sum of acetyl acylated anthocyanins) (See Chapter 2: Table S1). Molar relative abundances (molar percentages) were calculated as the percentage of molar concentration of each of the groups, of the total molar concentration of flavonols or anthocyanins.

Quantitative real-time RT-PCR

Four independent pools of berry samples, collected from the exposed part of the clusters one week after the onset of veraison, were powdered in a ball grinder MM200 (Retsch, Haan,

Germany). Total RNA was extracted according to the method described in Lecourieux et al. (2010). RNA isolation was followed by DNase I treatment. Reverse transcription was performed from 2 µg of purified RNA using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. Resulting cDNA was diluted 1:10 in ultrapure water. Primer sequences were obtained from the literature and amplification efficiencies were tested for the cv. Tempranillo samples (Table S5). Quantitative real-time RT-PCR (qRT-PCR) expression analysis was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad). Reaction mixes (10 µL) were prepared in triplicate, including 5 µL of iQ™SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer, and 2 µL of diluted (1:10) cDNA. Four candidate reference genes proposed by Reid et al. (2006) were evaluated for expression stability by means of GeNorm M value within the qBase PLUS software (Biogazelle), and as a result glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen for normalization. Thereby, gene transcripts were quantified upon normalization to GAPDH by comparing the cycle threshold of the target gene with that of GAPDH.

Statistical treatment

Statistical analysis was performed with XLstat-Pro (Addinsoft). Technological and phenolic maturity parameters, as well as the relative abundance of each group of flavonols and anthocyanins (molar percentage), were analysed using a principal component analysis PCA. Total flavonols and flavonol groups concentrations were subjected to a three-factor analysis (ANOVA 3x3x2) in order to partition the variance into the main effects (stage, UV-B and water deficit) and the interaction among them. Relative abundances, total anthocyanins and anthocyanin groups at maturity; as well as transcript levels were subjected to a two-way ANOVA (3X2) in order to partition the variance into the main effects of UV-B and water deficit, and the interaction between them. A linear regression was performed on variables linked in the biological context.

3. RESULTS

Principal component analysis of grape berry technological and phenolic maturity and flavonol and anthocyanin profiles

Principal component analysis (PCA) of grape must analyses separated samples according to their water availability; however, UV-B treatments only were well separated under drought conditions (Fig. 1 A, Table S1). The first two principal components (PC) explained about 56% of the total variability. UV-B treatments under drought conditions were well separated along PC1 due to their higher colour density and extractable anthocyanins. PC2 discriminated according to their water availability based on titratable acidity, pH and tonality (Fig. 1 C). Regarding flavonol and anthocyanin profile, PCA readily separated the 0 kJ m⁻² d⁻¹ treatments from the UV-B supplemented treatments, both under well watered and drought conditions with no overlaps (Fig. 1 B). PC1 and PC2 explained about 82% of the total variance. PC1 discriminated UV-B doses (Fig. 1 D), based on the total concentration of anthocyanins and flavonols, as well as flavonol hydroxylation patterns (Figs. 2 and 3). PC2 best separated samples according to their water availability based on hydroxylation pattern of anthocyanins, and their higher relative abundance of methylated, acetyl acylated and *-p* coumaroyl acylated anthocyanins (Fig. 1 D; Table 1).

Effect of UV-B and water availability on flavonol and anthocyanin profiles

Total skin flavonol concentration increased 4.5 fold under UV-B and this took place mostly after the onset of veraison (Fig. 2 A), while total anthocyanin increase was about 16% (Fig. 3 A). The 3'4' substituted were the most abundant flavonols until veraison (Fig. 2 C) whereas 3'4'5' substituted were the major flavonols at maturity (Fig. 2 D). UV-B and water deficit altered to some extent these developmental changes, leading to profiles richer in 4' and 3'4' substituted flavonols in the case of UV-B treated plants (Fig. 2 B) and richer in 3'4'5' substituted flavonols in plants subjected to water deficit (Fig. 2 D). A significant interaction between UV-B and water availability was found in the relative abundance of 3'4' and 3'4'5'

substituted flavonols (Table 1). The 4' substituted flavonols were detected in small concentrations in all of the treatments at any stage, but especially in the treatment without UV-B. Indeed, in the $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ treatments 4' substituted flavonols were only detected at pea size stage but not in the next samplings (onset of veraison and maturity stages). Methylated flavonols (Fig. 2 E) were at low concentrations in all the treatments at the pea size stage and the onset of veraison samplings, and in fact, they were not detected under $0 \text{ kJ m}^{-2} \text{ d}^{-1}$ treatments at those stages. The methylated flavonols increased at maturity, but they were not further increased by water deficit. Hydroxylation of the B ring of anthocyanins was affected by both UV-B and water deficit. An increase in 3'4' substituted anthocyanins was observed with UV-B (Fig. 3 B). However, this did not result in an increase in their relative abundance (Table 1). Water deficit increased the concentration of 3'4'5' substituted anthocyanins (Fig 3 C), in detriment of 3'4' substituted anthocyanins (Fig. 3 B; Table 1). Methylated anthocyanins were higher in UV-B and water deficit treated plants (Fig. 3 D). However, only in the case of water deficit this resulted in increased relative abundance (Table 1). The concentration of *-p* coumaroyl and acetyl acylated anthocyanins increased under UV-B and water deficit (Fig. 3 E and F)

Transcriptional regulation of flavonol and anthocyanin biosynthesis by UV-B and water deficit in the grape berry

UV-B strongly increased the transcript levels of *FLS1* (10 fold induction), *UFGT*, and flavonoid 3' hydroxylase (*F3'H*) (Fig. 4). Water deficit significantly increased the expression levels of *F3'5'H*. The expression levels of the flavonoid O-methyl transferase 2 (*OMT2*), significantly increased with UV-B doses and water deficit conditions. *MybF1* was the only regulatory gene affected by UV-B, with a significant increase (4.9 fold induction) regardless of water availability (Fig. 4). Expression levels of *MybA1* were not significantly affected by the treatments.

The ratio 3'4'5' to 3'4' substituted anthocyanins was affected by both UV-B and water deficit. The ratio 3'4'5' to 3'4' substituted flavonols was significantly affected by UV-B and water deficit, but also by the combination of both factors (Fig. 5 C). The expression levels of *F3'5'H* were correlated to the 3'4'5' to 3'4' substituted anthocyanins ratio at maturity (Fig 5 D), whereas, the ratio 3'4'5' to 3'4' substituted flavonols exhibited a better correlation with the *F3'5'H* to *FLS1* expression level ratio (Fig. 5 B).

4. DISCUSSION

Metabolite profiling reveals a strong effect of UV-B radiation and water deficit on flavonol and anthocyanin biosynthesis, leading to clear-cut differentiation by PCA

Moderate water deficit is considered to determine grape composition by reducing berry size, and, therefore, increasing skin to pulp ratio, which helps to concentrate berry metabolites in the must (McCarthy, 1997). In the present study, a berry size reduction and an increase in the relative skin mass were observed (Table S1). Grape technological and phenolic maturity analyses performed in whole berries helped to discriminate samples under different water availabilities (Fig. 1). However, the maturity analyses were not sufficient to separate berry samples developed according to their UV-B exposure. In contrast, determination of berry skin flavonol and anthocyanin profiles by liquid chromatography proved to be a highly valuable tool to unambiguously discriminate samples according to their UV-B exposure and water availability. This discrimination was based on a higher concentration of anthocyanins and flavonols, but mainly on the diversification profiles that constituted a clear-cut metabolite signature. Metabolite profiling has proven useful in discriminating vintages with different environmental conditions which produce different wine qualities (Lee et al., 2009). Wine ratings have also been associated with specific metabolite signatures in Ali et al. (2011), where flavonols like quercetin, amino acids like proline and organic acids were a common characteristic of higher quality wines. In the present work, the concentration of these metabolites was significantly affected by the treatments. Flavonoid profiles have also been

proposed as a way to determine the authenticity of wine appellations (Hermosín-Gutiérrez et al., 2010), based on the assumption that anthocyanin and flavonol profiles are basically cultivar-dependent (Mattivi et al., 2006). Our results show that flavonol and anthocyanin profiles are also highly responsive to environmental conditions. Therefore, the specific metabolite signatures of appellations may be resulting from the combination of both varietal (genotypic) and environmental (phenotypic) factors.

The metabolites contributing to the specific signatures of UV-B (i.e. higher flavonol and anthocyanin concentration) and water deficit (i. e. higher 3'4'5' substituted and methylated anthocyanins), in the present work, may also increase the health promoting effects of grapes and wine. Flavonols and anthocyanins in general have been reported to exhibit antioxidant and anti-cancer activities and to prevent cardiovascular diseases (reviewed by Ali et al., 2010). Moreover, highly hydroxylated and methylated profiles may favour anthocyanin absorption and assimilation (Passamonti et al., 2002).

Flavonol and anthocyanin biosynthesis regulation by UV-B radiation: beyond berry sugar levels

Part of the flavonoid biosynthesis in grape cells is regulated by sugar levels (Ferri et al., 2011), and in the case of anthocyanins, increased sugar levels may be enough to trigger their biosynthesis (Dai et al., 2014). In the present study, both anthocyanin and flavonol skin concentrations were increased by UV-B regardless of TSS (Table S1 and Fig. 2 and 3). Our results are similar to those found by Berli et al. (2011), where solar UV-B increased flavonoid concentration (anthocyanins, flavonols and proanthocyanidins). However, in that case, the process was mediated by a hastening in sugar accumulation. Nevertheless, solar radiation may up-regulate this pathway regardless of sugar accumulation (Matus et al., 2009). The expression of *UFGT*, responsible for anthocyanin stabilisation, was up-regulated by UV-B leading to an increase in anthocyanins in the skin (Figs. 3 and 4). *UFGT* promoter is activated by *MYBA1/2* regulatory genes (Walker et al., 2007). However, *MYBA1* transcript

level was not significantly higher under UV-B. The expression of the genes coding for enzymes in the early steps of this pathway, such as phenylalanine ammonia lyase (data not shown) and chalcone synthase (CHS), were not affected in any case. Castellarin et al. (2007b) showed strong correlations of both *UFGT* and *CHS* expression levels with total anthocyanin content, but these authors acknowledged the prime importance of UFGT due to its anthocyanin specificity. *MYBF1* and flavonol synthase 1 up-regulation was triggered by UV-B (Figs. 4), leading to a strong increase in flavonol concentration (Fig. 2). The VvMYBF1 transcription factor is a flavonol biosynthesis specific regulator in grapevine (Czemmel et al., 2009), and the up-regulation of its homolog in *Arabidopsis thaliana* (*AtMYB12*; Mehrtens et al., 2005) is the consequence of UV-B radiation interception by the UV-B photoreceptor UV RESISTANCE LOCUS 8 (*UVR8*; Tilbrook et al., 2013). Thus, *UVR8* associates with chromatin containing the *AtMYB12* promoter, which in turn trans-activates the expression of *CHS* and flavonol biosynthetic genes (Cloix and Jenkins, 2008). In the present study, *FLS1* was clearly up-regulated under UV-B, but not *CHS* (Fig. 4), thus supporting the previously reported idea that MYBF1 has a higher specificity for *FLS1* than *CHS* promoter in grapevines (Czemmel et al., 2012). The results also confirm that *FLS1* (also referred to as *FLS4*, Fujita et al., 2006) is activated mainly by UV-B radiation in ripening grapes, leading to flavonol accumulation reported in previous research (Gregan et al., 2012; Matus et al., 2009).

Increase in anthocyanin accumulation in response to water deficit has been reported in several ways. Water deficit may increase sugar accumulation, leading to a higher concentration of anthocyanins and flavonols per berry weight (Castellarin et al., 2007a; Deluc et al., 2009). A second case has been reported by Olle et al. (2011), who showed that anthocyanin content per berry increased with water deficit regardless of sugar levels. Water deficit may also increase relative skin mass, which may result in higher anthocyanin concentration per berry weight (Roby et al., 2004), or higher must anthocyanin concentration, as in the present study. Contrasting results may be related to differences in timing of application and severity of water deficit among studies. In the present case, cyclic drought

may find resemblance with cases in which irrigation is used to prevent excessive canopy temperature, maintain yield and, in more extreme cases, guarantee plant survival (Chaves et al., 2010), rather than seasonal moderate water deficit.

UV-B and water deficit led to distinct changes in flavonol and anthocyanin profiles

Flavonol and anthocyanin profiles were affected by both UV-B and water deficit in very different ways (Fig. 6). Transcript levels for *F3'5'H*, the gene encoding the enzyme responsible for the hydroxylation in the 3' and 5' position of flavonoid precursors, were increased under water deficit as was the expression of the *OMT2* gene (Fig. 4), leading to anthocyanin profiles richer in methylated anthocyanins (Fig. 3). Castellarin et al. (2007b) reported a similar effect of water deficit on anthocyanin profiles, which was explained by higher *F3'5'H* and *OMT2* expression levels. Methylation of anthocyanins depends, among others, on the transcript levels of flavonoid *OMTs* (Fournier-Level et al., 2011). However, these were not enough to explain the methylation of flavonols in the present study. Substrate affinity of flavonoid *OMTs* is quite variable among anthocyanins and flavonols, which could explain the accumulation of methylated flavonols and anthocyanins in addition to transcript levels (Fournier-Level et al., 2011; Lucker et al., 2010). For instance, the *OMT* reported in Lucker et al. (2010), showed a higher affinity for 3'4'5' substituted anthocyanin substrates, but in the case of flavonols, *OMT* presented higher affinities for 3'4' substituted substrates, which concentrations were lower under water deficit conditions.

Even though flavonol profiles were affected by the up-regulation of *F3'5'H* in the same way as anthocyanins, UV-B exposure clearly controlled the flavonol spectrum, concomitantly with higher transcript levels of *FLS1*, and *F3'H*. The trans-activation of *FLS* promoter by *AtMYB12* has been unambiguously demonstrated, however, *AtMYB12* may not be able to activate the *F3'H* promoter (Mehrtens et al., 2005). The expression of *F3'H* and *F3'5'H* is often coordinated in flower petals such as in the *Petunia*, while in grape berries, these two enzymes can show cases of uncoupled patterns of expression (Bogs et al., 2006). In the

present study, the up-regulation of *F3'5'H* by water deficit, and *F3'H*, in the case of UV-B, contributed to shape the flavonol and anthocyanin profiles, confirming that these two enzymes can be regulated independently in grapevines. However, due to the contrasting effects found on anthocyanin and flavonol accumulation profiles (Fig. 5; Table 1), *F3'H* and *F3'5'H* expression may not fully explain the hydroxylation patterns of flavonoids. For instance, UV-B increased the relative abundance of 3'4' hydroxylated flavonols, whereas it did not increase the relative abundance of 3'4' hydroxylated anthocyanins. As suggested in Martínez-Lüscher et al. (2014), FLS most certainly competes with *F3'H* and *F3'5'H* for dihydrokaempferol (Fig. 6), and FLS also competes with *F3'5'H* for dihydroquercetin (Kaltenbach et al., 1999). Consequently, changes in any of their activities will lead to different hydroxylation profiles of flavonols. In this work, high expression levels of *FLS1* under UV-B clearly contributed to produce less hydroxylated profiles of flavonols (i.e. richer in 4' and 3'4' substituted flavonols). In addition, when UV-B radiation and water deficit were combined, the interactions found in flavonol profile were explained by the competition of *FLS1* and *F3'5'H* for the same substrates. This constitutes a new finding, aside from the imbalance between *F3'H*/*F3'5'H* created by UV-B or water deficit, which has been used to explain flavonoid profiles in previous research (Bogs et al., 2006; Castellarin et al., 2007b).

5. CONCLUSIONS

UV-B radiation was able to up-regulate gene expression which resulted in a higher flavonol and anthocyanin concentration in the skins regardless of sugar levels. MYB genes regulating flavonol and anthocyanin biosynthesis were less sensitive than the structural genes that they regulate. The up-regulation of *FLS*, *UFGT*, *OMT2* and *F3'H* by UV-B radiation, and *F3'5'H* and *OMT2* by water deficit led to strong changes in the metabolites they are committed to synthesize. Strong interactions between UV-B and water deficit were observed in the flavonol profile hydroxylation, as a result of the competition among *FLS*, *F3'5'H* and *F3'H* for the same flavonol substrates. This constitutes a new mechanism of flavonol profile

diversification, aside from the changes in the expression of flavonoid hydroxylases already reported.

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Table 1. Molar relative abundance of flavonol and anthocyanin groups (according to their B-ring substituents) of berries at maturity grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B; and two water availabilities (WA): well watered and drought. Means ± SE (n=4). Main effects of UV-B, $P_{(UV-B)}$; and WA, $P_{(WA)}$; and interaction between UV-B and WA, $P_{(UV-B \times WA)}$.

Rel. abundance (%)	Well watered			Drought			Main factor		
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV-B \times WA)}$
Flavonols									
4' Substituted	0.0±0.0	1.3±0.2	1.9±0.3	0.0 ±0.0	1.4±0.2	1.6±0.1	<0.001	0.611	0.584
3' 4' Substituted	16.3±1.1	45.9±2.0	48.6±2.2	10.7 ±0.4	34.0±1.8	32.5±1.4	<0.001	<0.001	0.012
3' 4' 5' Substituted	83.8±1.1	52.7±2.1	49.5±2.5	89.3 ±0.4	64.6±2.0	65.9±1.5	<0.001	<0.001	0.019
methylated	30.8±1.0	18.7±0.5	17.7±0.8	26.2 ±0.6	16.7±0.4	17.1±0.3	<0.001	0.0002	0.029
Anthocyanins									
3' 4' Substituted	21.6±1.2	22.2±2.1	21.7±1.6	8.5 ±0.6	10.5±1.2	9.5±0.1	0.423	<0.001	0.779
3' 4' 5' Substituted	78.4±1.2	77.8±2.1	78.3±1.6	91.5 ±0.6	89.5±1.2	90.5±0.1	0.423	<0.001	0.779
methylated	69.9±0.6	69.7±1.0	68.7±0.5	81.7 ±0.6	79.9±1.3	80.0±0.1	0.140	<0.001	0.55
p coumaroyl acylated	9.0±0.6	11.2±1.1	10.7±0.7	18.9 ±0.9	19.4±1.8	20.6±0.4	0.022	<0.001	0.306
acetyl acylated	3.3±0.4	3.8±0.2	3.8±0.4	4.0 ±0.4	4.3±0.2	4.7±0.2	0.001	<0.001	0.652

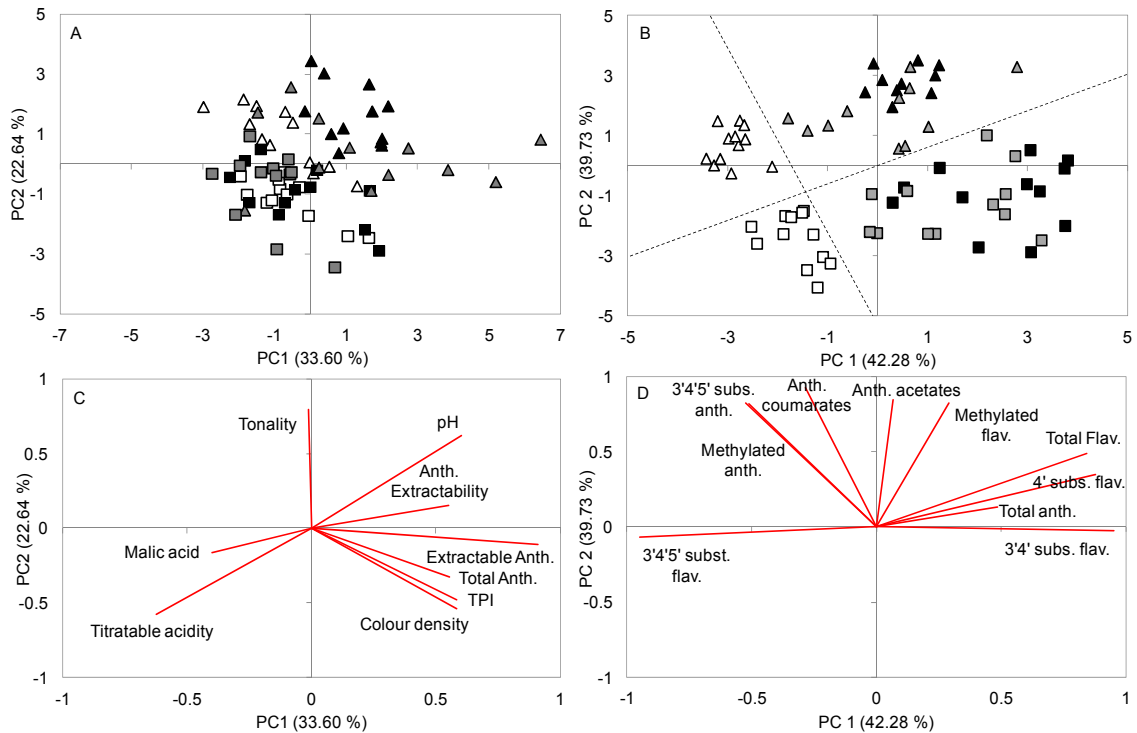


Figure 1. PCA of grape technological and phenolic maturity (A) and berry skin flavonol and anthocyanin profiles (B). Loading plots of grape technological and phenolic maturity (C) and berry skin flavonoid profiles (D) for the two principal components. Squares represent well watered plants and triangles plants under water deficit; open, grey and black symbols represent 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B radiation treatments respectively (n=10-12). Subs, substituted; Flav, flavonols and Anth, anthocyanins.

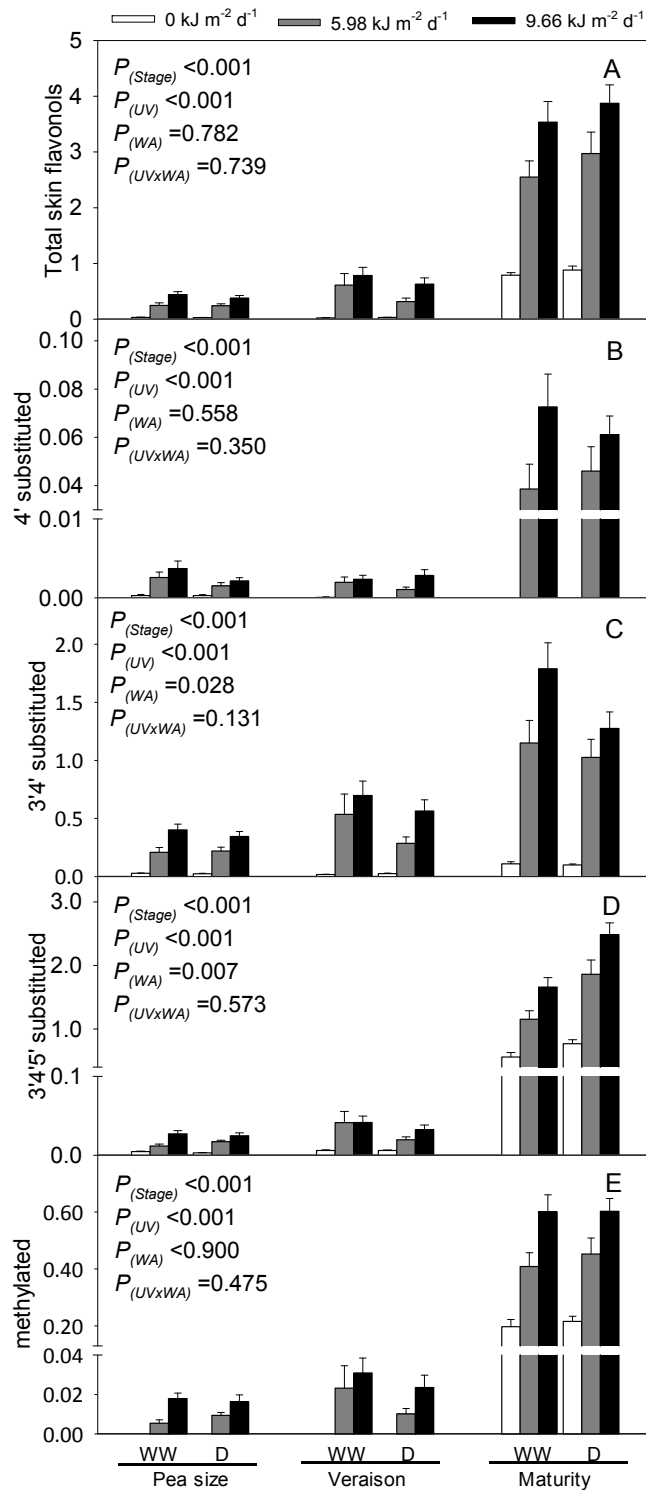


Figure 2. Total berry skin flavonols (A), 4' substituted (B), 3'4' substituted (C), 3'4'5' substituted (D), 3' methylated (E) and 3'5' methylated (F) flavonols concentrations (mg g⁻¹ skin dry wt) of berries at pea size stage, onset of veraison and maturity grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered (WW) and drought (D). Columns are means ± SE (n=4). Main effects of developmental stage, $P_{(Stage)}$; UV-B, $P_{(UV-B)}$; and water availability, $P_{(WA)}$; and interaction between UV-B and water availability, $P_{(UV-B \times WA)}$.

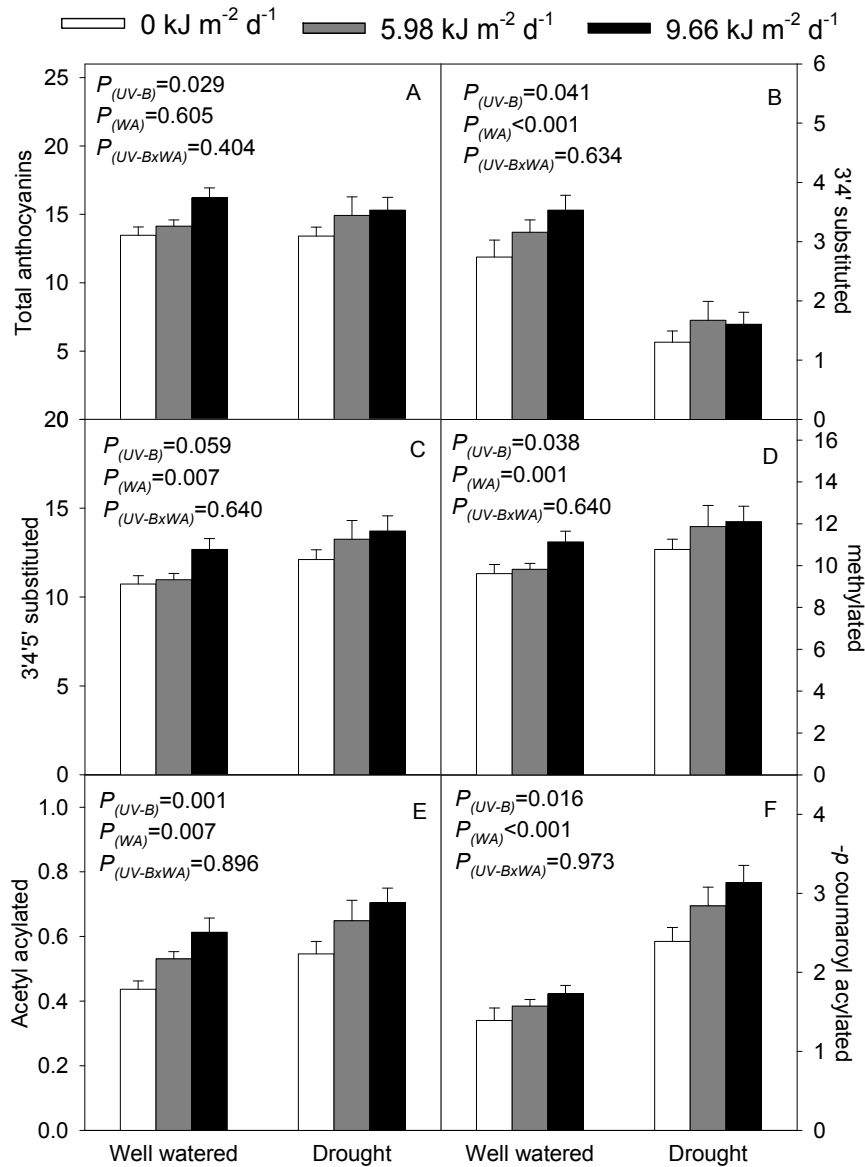


Figure 3. Total berry skin anthocyanins (A), 3',4',5' substituted (B), 3' methylated (C), 3',5' methylated (D), acetyl acylated (E) and -p coumaroyl acylated (F) anthocyanins concentrations (mg g^{-1} skin dry wt) of berries at maturity grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{ d}^{-1}$ of UV-B, well watered and drought. Columns are means \pm SE (n=4). Main effects of UV-B; $P_{(UV-B)}$ and water availability; $P_{(WA)}$, and interaction between UV-B and water availability; $P_{(UV-B \times WA)}$.

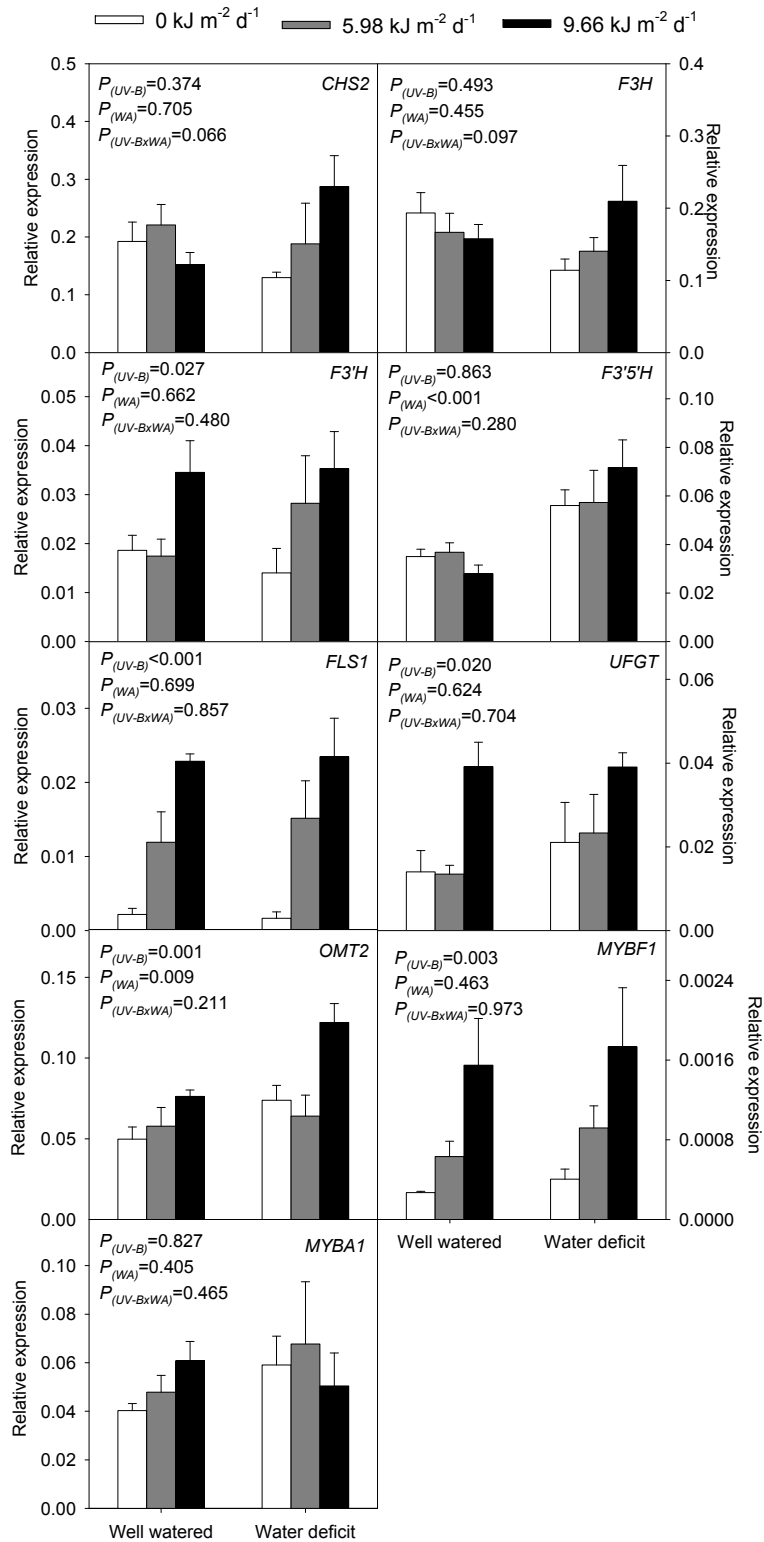


Figure 4. Transcript profiling of structural and regulatory genes of the flavonol and anthocyanin pathway in grape berries grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered and drought, one week after the onset of veraison. Columns are means ± SE (n=4). Main effects of UV-B; $P_{(UV-B)}$ and water availability; $P_{(WA)}$, and interaction between UV-B and water availability; $P_{(UV-B \times WA)}$.

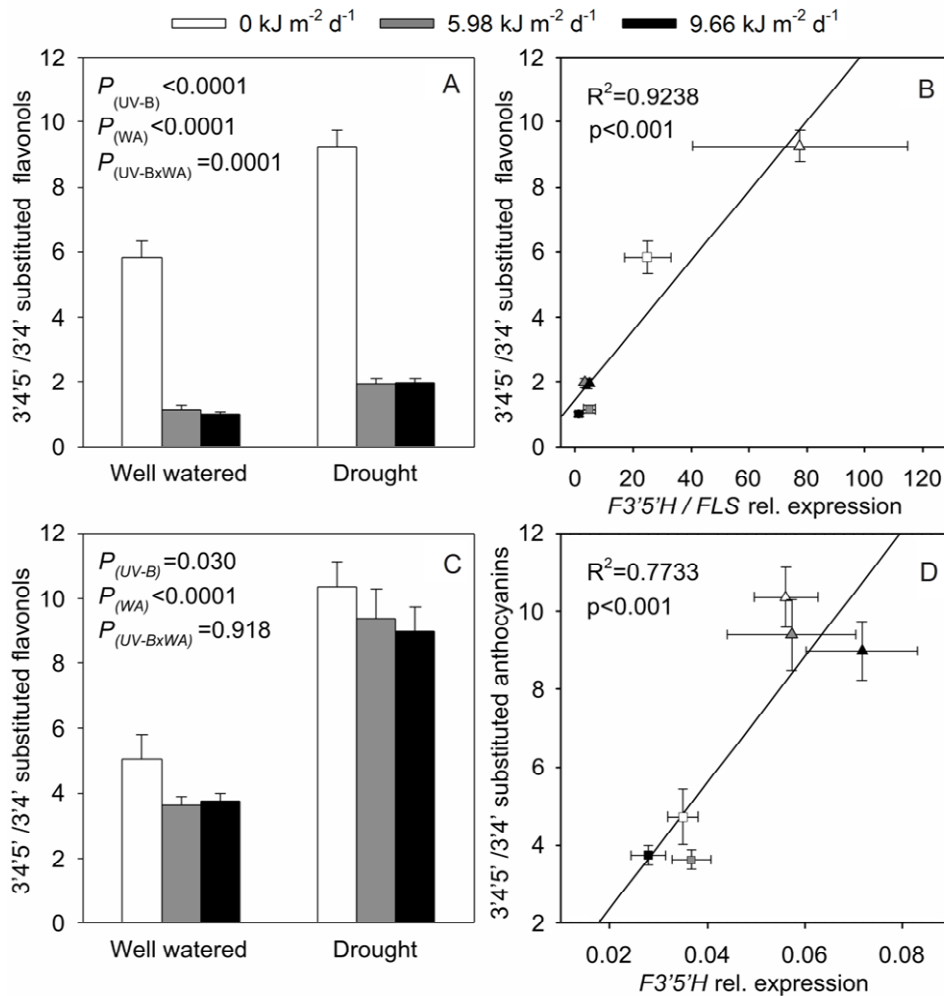


Figure 5. Hydroxylation of the flavonol profile (A) and its correlation with the ratio between the relative expression of flavonoid 3'5' hydroxylase and flavonol synthase ($F3'5'H / FLS$; B) and the hydroxylation of the anthocyanin profile (C) and its correlation with the relative expression of $F3'5'H$ (D). Squares represent well watered plants and triangles plants under drought; open, grey and black symbols represent 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B radiation treatments respectively. Columns and symbols are means \pm SE (n=4). Main effects of UV-B; $P_{(UV-B)}$ and water availability; $P_{(WA)}$, and interaction between UV-B and water availability; $P_{(UV-B \times WA)}$.

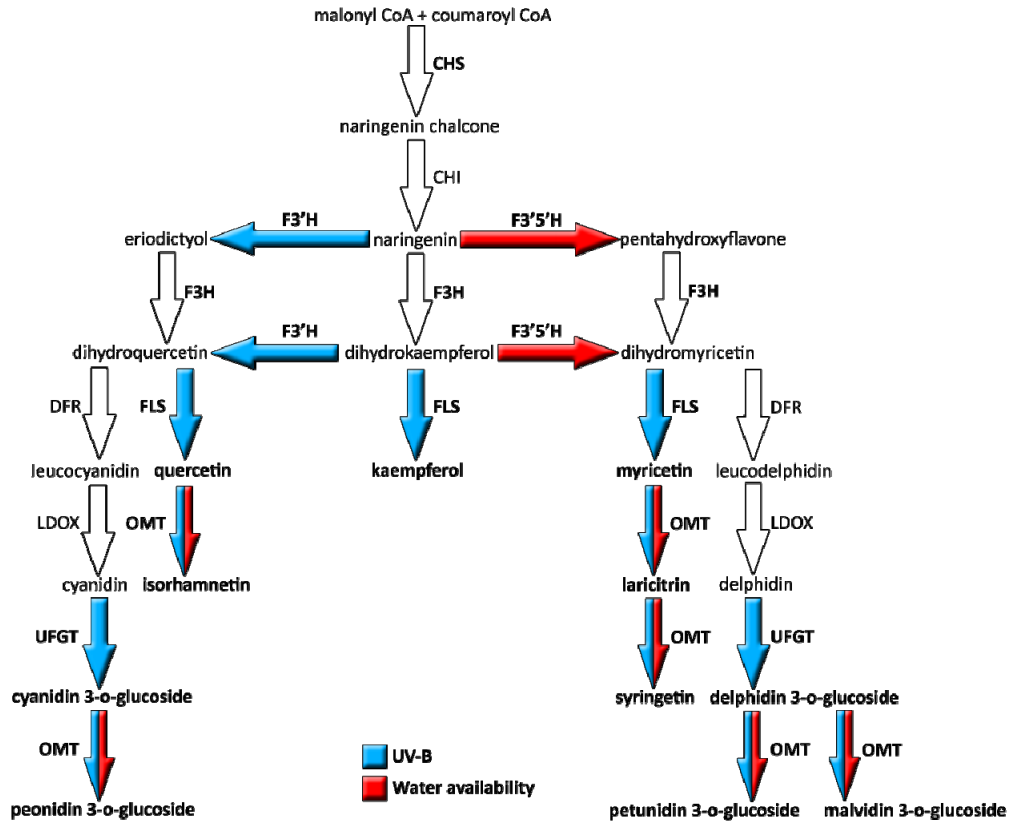


Figure 6. Schematic representation of the biosynthetic pathway of flavonol and anthocyanins with gene expression results represented by coloured arrows: two-way ANOVA significantly up-regulated genes by UV-B in blue and by water deficit in red. Abbreviations: F3H: flavonoid 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; F3'5'H: flavonoid 3'5'-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; LDOX: leucocyanidin dioxygenase; UFGT: UDP-glucose flavonoid 3-O-glucosyltransferase; OMT: O-methyltransferase.

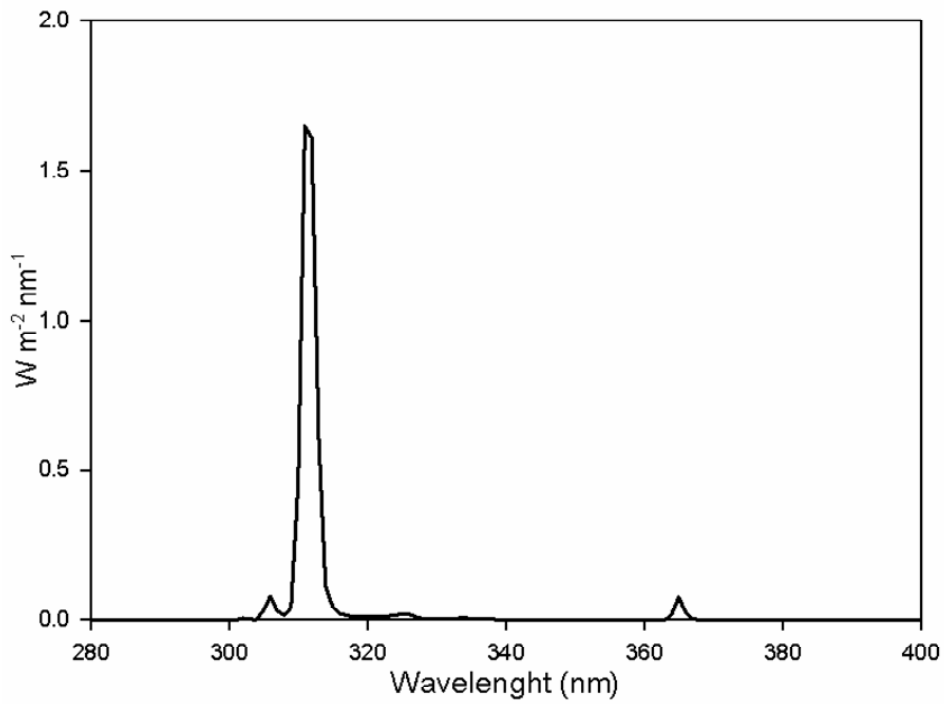


Figure S1. Spectral irradiance of the lamps used as UV-B source

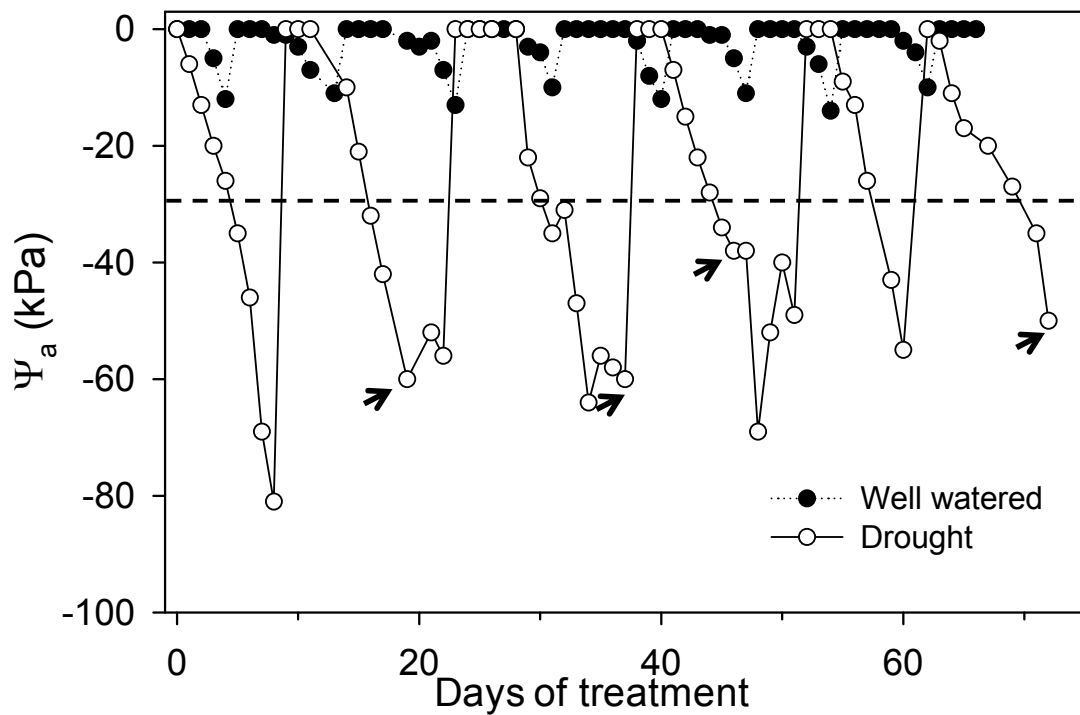


Figure S2. Example of substrate water potential (kPa) in pots recorded by granular matrix sensor Watermark™. Dash line represents the minimum value of the sensor for considering grape sampling in drought treatments, which corresponds to a 40% of the water content at field capacity. Arrows indicates approximately the sampling dates.

Table S1. Basic characterization of grape berries at maturity, grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B; and two water availabilities (WA): well watered and drought. Main effects of UV-B, $P_{(UV-B)}$; and WA, $P_{(WA)}$; and interaction between UV-B and WA, $P_{(UV-B \times WA)}$.

	Well watered			Drought			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV-B \times WA)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
Total soluble solids (°Brix)	21.98±0.13	21.90±0.14	21.81±0.10	21.57±0.09	22.10±0.12	21.62±0.15	0.244	0.287	0.251
Berry weight (fr wt)	1.67±0.05	1.56±0.06	1.63±0.06	1.34±0.04	1.27±0.05	1.36±0.05	0.034	< 0.001	0.618
Relative skin mass (% fr wt)	14.29±0.29	14.89±0.41	14.93±0.35	17.31±0.47	18.35±0.68	18.22±0.73	0.065	< 0.001	0.918
Calibre (mm)	14.10±0.16	13.68±0.20	13.95±0.17	13.09±0.14	12.75±0.17	13.01±0.14	0.007	< 0.001	0.751
pH	3.38±0.03	3.48±0.06	3.59±0.07	3.65±0.04	3.89±0.07	4.05±0.06	< 0.001	< 0.001	0.231
Malic acid (g L ⁻¹)	4.76±0.32	3.55±0.47	4.84±0.45	4.42±0.41	4.24±0.51	3.25±0.27	0.246	0.296	0.036
Titrateable acidity (g L ⁻¹)	10.13±0.26	10.92±0.47	10.13±0.26	8.65±0.32	6.60±0.60	6.89±0.46	< 0.001	0.021	0.003

Table S2. Accession ID, sequences and efficiency of primers user for real-time RT qPCR analyses

Gene name	GEN ID (GenBank Accession)	Source	Primers sequence	Primer efficiency
CHS2	AB066275	Jeong et al., 2004	F-.GAAGATGGGAATGGCTGCTG R-.AAGGCACAGGGACACAAAAG	86.3 %
F3H	X75965	Ali et al., 2011	F-. GCAGACTGTCCATAGCAACATTCC R-. CACTGCCTTCTCTCCCTCTCTTATC	77.5 %
F3'H	AB213603.1	Castellarin et al., 2006	F-.CAACAAGAGCTGGACGCAGT R-. AGCCGTTGATCTCACAGCTC	81.9 %
F3'5'H	AB213606	Jeong et al., 2006	F-. AAACCGCTCAGACCAAACC R-. ACTAAGCCACAGGAACTAA	88.9 %
FLS1	AB092591	Fujita et al., 2006	F-. AAACCACCTACTTACAGAGC R-. ACC TAA CCC CAG TGA CAG AC	87.6 %
UFGT	AF000372	Jeong et al., 2004	F-. GGGATGGTAATGGCTGTGG R-. ACATGGGTGGAGAGTGAGTT	81.2%
OMT2	HQ702997	Fournier-Level et al., 2011	F-. TGTCCAGCTCAAGTCATAGGG R-. AGCTTCTAATAGAGGCGCCTGCAGAG	92.5 %
MYBF1	FJ948477	Czemmel et al., 2009	F-. GGAGGTTGAGGGTTGTG R-.AAGTTGGGGAAGAGCAGGAG	87.6 %
MYBA1	AB097923	Jeong et al., 2004	F-. TAGTCACCACTTCAAAAAGG R-.GAATGTGTTTGGGGTTTATC	86.8 %
GAPDH	CB973647	Reid et al., 2006	F-. TTCTCGTTGAGGGCTATTCCA R-. CCACAGACTTCATCGGTGACA	98.7 %

CHAPTER 5

Climate change conditions (elevated CO₂ and temperature) and UV-B radiation affect grapevine (*Vitis vinifera* cv. Tempranillo) leaf carbon metabolism, altering fruit ripening rates

Article in preparation

Climate change conditions (elevated CO₂ and temperature) and UV-B radiation affect grapevine (*Vitis vinifera* cv. Tempranillo) leaf carbon metabolism, altering fruit ripening rates

ABSTRACT

The increase in grape berry sugar accumulation under climate change conditions is a growing concern in the last decades. Since ripening berries relies on leaf photoassimilates transport to accumulate sugars, the aim of this work was to study the combined effects of climate change conditions (elevated CO₂ and temperature) and UV-B radiation throughout grape development on leaf physiology and berry ripening rate of *Vitis vinifera* cv. Tempranillo. Three doses of UV-B: 0, 5.98, 9.66 kJ m⁻² d⁻¹, and two temperature-CO₂ regimes: 24/14°C (day/night) - ambient CO₂ (current situation) and 28/18°C - 700 ppm CO₂ (climate change) were imposed to grapevine fruit-bearing cuttings from fruit set to ripeness under glasshouse-controlled conditions. Gas exchange and chlorophyll a fluorescence under climate change conditions were always higher. High levels of UV-B radiation inhibited carbon fixation during the first days of treatments; however this was not observed at the onset of ripening, when acclimation took place, through the accumulation of flavonoids and increased antioxidant enzyme activities. Close to maturity, photosynthetic performance showed evidences of leaf senescence, especially under climate change conditions (e.g. Fv/Fm decrease). Interacting effects between UV-B and climate change conditions were observed for the lipid peroxidation, which was lower in the plants exposed to UV-B under climate change conditions. This suggests that UV-B treated plants were less susceptible to senescence-related oxidative damage induced under elevated CO₂-temperature. Average photosynthetic rates were correlated to overall ripening rates. Thereby, the hastening effect of climate change conditions on grape ripening, associated to higher rates of carbon fixation, was attenuated by UV-B radiation.

Keywords: Climate change, ripening, phenology, UV-B radiation, carbon assimilation, grapevine, cross tolerance

1. INTRODUCCION

Historical records of harvest dates in viticulture have been used as an indicator in climate change research (Chuine et al., 2004). Compared to pre-industrialization records, the current date of grape harvest for wine production is approximately 2-3 weeks earlier. Since the pre-industrial era, carbon dioxide (CO₂) levels have raised from 280 ppm to 400 ppm concomitant to an average temperature rise of 1-2°C (Mira de Orduña, 2010). By the end of this century CO₂ levels are expected to rise up to 700 ppm, with a subsequent increase in temperature up to 4°C (IPCC, 2013). The way environmental factors affect the rate of grape ripening (i.e. phenology) is largely unknown. Although, it has been reported that changes in the source to sink ratio affect ripening rates in grapevine (Palliotti et al., 2013), changes in fruit physiology have been usually associated to the direct effect of environmental conditions on the fruit itself and not to the indirect impact, through carbon assimilation (Spayd, 2002).

Carbon fixation process is highly conditioned by environmental factors. For C₃ plants such as grapevine, carbon fixation is limited by CO₂ levels at least up to 800 ppm (Long et al., 2006). Whereas short-term exposition to elevated CO₂ is followed by a sudden increase in photosynthetic rates, long-term exposures often lead to biochemical and molecular changes that result in acclimation, marked by a photosynthetic activity decrease, lower stomatal conductance and/or lower leaf protein levels (Erice et al., 2006; Long et al., 2004; Morales et al., 2014; Salazar-Parra et al., 2012b). Under CO₂ acclimation, an over-accumulation of carbohydrates in the leaves results in photosynthesis inhibition (Paul and Pellny, 2003). Additional carbon sinks, such as leguminous nodules may prevent acclimation to elevated CO₂ (Bertrand et al., 2007; Erice et al., 2006). However, the acclimation process has been rarely tested on fleshy-fruited crops, whose response may be different from that of herbaceous species (Ainsworth and Rogers, 2007). In addition, photosynthetic acclimation is not a generalised process. Responses in long-term studies performed in free air systems do not show signs of acclimation after several years (Springer and Thomas, 2007; Zotz et al., 2005).

Temperature has been considered as the major factor affecting grape ripening, based on the thermal optimum for the different grapevine varieties proposed by Jones (2006) (Hannah et al., 2013) and given the dependence of grape phenology on daily temperatures (Godwin et al., 2002; Webb et al., 2007). However, studies focused on the effects of temperature on grapevine show contradictory results. Photosynthesis rates may increase up to 25-30°C, but higher temperatures may reduce the efficiency of the carbon assimilation process (Greer and Weedon, 2013; Huglin and Schneider, 1998). In addition, such increases in photosynthesis reported under elevated temperatures may not result in higher sugar accumulation rates (Greer and Weedon, 2013).

Solar UV-B radiation levels may oscillate together with PAR and infrared radiation when factors such as time of the day, season, latitude and cloud coverage are considered. In addition, UV-B levels may also be susceptible to changes according to the thickness of the ozone layer. For instance, due to their lower ozone levels, southern hemisphere UV-B radiation levels are generally higher. Given the success of the Montreal Protocol phasing out ozone-depleting substances, ozone levels may return to pre-1980s levels before the first half of the century (UNEP, 2012), which in turn may reduce present UV-B levels. UV-B is regarded as a growth modulator, able to turn metabolic switches between primary and secondary metabolism (Jansen and Bornman, 2012). Under the current situation, UV-B radiation levels may constitute a strong limitation for biomass production (Ballaré et al., 2011). Acclimation to UV-B radiation undertakes the biosynthesis of phenolic compounds that protect plant tissues from UV-B harming effects (Jansen et al., 2012). In addition, UV-B radiation triggers antioxidant enzymes, and even photosynthetic activity may be transiently reduced during the UV-B acclimation process (Martínez-Lüscher et al., 2013). Thereby, the UV-B acclimation process may represent a significant carbon sink (Snell et al., 2009), which may compete with berry sugar storage, delaying ripening.

Therefore, the objective of the current study was to investigate the combined effects of climate change conditions (elevated CO₂ and temperature) and UV-B radiation throughout

grape development on leaf physiology and berry ripening rate of *Vitis vinifera* cv. Tempranillo. Given the difficulties of studying the combined effects of different environmental factors under natural conditions, the present study was carried out under controlled conditions using the fruit-bearing cuttings of grapevine as a model system (Mullins, 1966). The fruit-bearing cuttings model allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines, under controlled environmental conditions. In addition, the chronology of flowering and fructification of the fruit-bearing cuttings is similar to that of the vineyard-grown grapevines (Geny et al., 1998; Lebon et al., 2008). Indeed, this model has been previously useful in the evaluation of the physiological response of grapevine, as well as in the study of the response of berry ripening to environmental or developmental factors (Antolín et al., 2010; Carbonell-Bejerano et al., 2013; Dai et al., 2013; Martínez-Lüscher et al., 2013; Martínez-Lüscher et al., 2014; Salazar-Parra et al., 2012a, b).

2. MATERIAL AND METHODS

Growing conditions and experimental design

Fruit-bearing cuttings were produced as in Martínez-Lüscher et al. (2013). Briefly, three-node segments of grapevine cv. Tempranillo were collected from Institute of Sciences of Vine and Wine (Logroño, Spain). Rooting was induced using indol butiric acid in a heat-bed (27°C) kept in a cool room (5°C). When cuttings developed enough roots, they were transplanted to 6.5 L pots containing 2:1 peat: perlite (v/v). Only one flowering stem was allowed to develop on each plant. Shoots were fixed to horizontal rods compelling leaves to face upwards. Vegetative growth was controlled by pruning, thus maintaining a leaf area to grape mass ratio optimal for berry ripening in all the treatments (12 leaves per plant) (Jackson and Lombard, 1993). The experiment was carried out in glasshouses at the University of Navarra (Pamplona, Spain, 42°48'14" N, 1°39'54" W) from April to September 2012. Until fruit set, growth conditions in the glasshouses were 25/15°C and 50/90% relative humidity (day/night).

Glasshouse walls and structure filtered up to 99.9% of UV-B, 35% of UV-A and 15% of the photosynthetically active radiation (PAR) coming from the sun. A supplemental system of high-pressure sodium lamps (HQI-TS 400W/D Osram, Augsburg, Germany) was triggered when PAR dropped below a photon flux density of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (14 h photoperiod). When fruit set was complete for all plants, three levels of biologically effective UV-B radiation (0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$), calculated using the generalized plant action spectrum of Caldwell (1971) as formulated by Green et al. (1974); and two ambient conditions combining two CO₂ levels (ambient CO₂ or 700 ppm) and two temperature regimes (24/14°C or 28/18°C) were applied. This resulted in two conditions imposed in two different glasshouses: current situation (CS; Ambient CO₂- 24/14°C) and climate change (CC; 700 ppm CO₂- 28/18°C). This made a total of 6 treatments with 12 plants each. Relative humidity was programmed in each glasshouse to maintain the same vapour pressure deficit among treatments. Irrigation was performed with the nutritive solution described by Ollat et al. (1998).

UV-B was applied by means of narrow band lamps TL100W/01 (311-313 spectrum peaking; Philips, Netherlands) hanging above the canopy. Spectral scans were performed with a double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd., Livingstone, Scotland). UV-A radiation emitted by the lamps accounted for less than 1 % of the solar UV-A irradiance (W m^{-2}) reaching the plants during the irradiation period. In the 0 $\text{kJ m}^{-2} \text{d}^{-1}$ treatment, lamps were unlit. The UV-B doses of 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ were reached after 3h and 4h 51m of irradiation (0.55 W m^{-2}), respectively. The applied biologically effective dose of 5.98 $\text{kJ m}^{-2} \text{d}^{-1}$ corresponded to 86% of the maximum daily dose at the study site, whereas 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ is only reached in viticulture suitable sites of the southern hemisphere, such as Argentina, South Africa or Australia (Quick TUV Calculator; <http://cprm.acd.ucar.edu/Models/TUV/>).

Gas exchange, chlorophyll *a* fluorescence and UV-B absorbing compounds were measured: i) one day after fruit set (fruit set+1d), ii) 10 days after fruit set (fruit set+10d), iii) at the onset

of veraison (veraison), when berries of each plant started to turn colour; and iv) at maturity, when the grapes in the cluster of each plant reached a total soluble solids (TSS) content of ca. 20°Brix. Leaf photosynthetic pigments, antioxidant enzyme activities, lipid peroxidation and multiparametric fluorescence measurements (Multiplex®) were determined at maturity. Given the importance of comparing leaves of the same physiological age in UV studies (Majer and Hideg, 2012), young fully expanded leaves of the same age (around four week old) were chosen. Leaves measured one and ten days after fruit set, had been fully developed before the beginning of the treatments under the glasshouse conditions described previously, whereas leaves measured at veraison and maturity were completely developed during the treatments.

Phenological development and plant growth

Three events, fruit set, onset of veraison and maturity, were selected for the study of phenological development, determined as the elapsed time (days) between fruit set and the onset of veraison, between the onset of veraison and maturity and between fruit set and maturity. Berry total soluble solids were recorded at the beginning of the treatments (around fruit set), and maturity. At this point, clusters and leaves were detached, to determine fruit mass and leaf area per plant.

Gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence measurements were conducted using a portable photosynthesis system (GFS-3000, Walz, Germany) with a 3 cm² cuvette. Dark respiration (R_D) measurements were performed 2 h before the beginning of the light period, with the CO₂ concentration, temperature and RH set to maintain growing conditions for each case. Gas exchange characteristics in illuminated leaves were measured 3 h after the beginning of the light period, under a photon flux density of 1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, with the CO₂ concentration, temperature and RH set to maintain growing conditions for each case. Dark respiration (R_D), net photosynthesis (A_{net}), stomatal conductance (g_s) and sub-stomatal CO₂

concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). Chl fluorescence was measured immediately after gas exchange measurements (in the dark and in the light) with a fluorescence module (PAM-fluorometer 3055-FL, Walz, Germany) attached to the photosynthesis equipment. The minimal and maximal fluorescence (F_o and F_m, respectively) were measured in dark-adapted leaves, whereas F_o' and F_m' were measured in the same leaves, with a photon flux density of 1200 μmol m⁻² s⁻¹, also measuring steady-state fluorescence signal (F_s). The maximum potential PSII efficiency was calculated as F_v/F_m, where F_v is F_m-F_o (Morales et al., 1991). Actual PSII efficiency (Φ_{PSII}) was calculated as (F_m'-F_s)/F_m'. Non-photochemical quenching (NPQ) as (F_m/F_m')-1 (Larbi et al., 2004). Electron transport rate (ETR) was calculated according to Krall and Edwards (1992) as Φ_{PSII} x PPFD x 0.5 x 0.84, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII (Ogren and Evans, 1993) and 0.84 is the fractional leaf absorptance, considered the most common absorptance coefficient for *V. vinifera* leaves under a wide range of environmental conditions and ages (Schultz, 1996). Multiplying 0.84 x 0.5 gives a value of 0.42, a value very similar to the α term used by other researchers to calculate ETR, which includes the product of leaf absorptance and the partitioning of absorbed quanta between PSI and PSII, and determined as the slope of the relationship between Φ_{PSII} and Φ_{CO₂} (i.e. the quantum efficiency of gross CO₂ fixation), obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing 1% O₂ (Valentini et al., 1995). For grapevine cv. Tempranillo, α was reported to be 0.425 (Pérez-Martín et al., 2009). Photorespiration (R_L) was estimated as 1/12(ETR - 4 x (A_{net} + R_D)), according to Valentini et al. (1995). Mesophyll conductance (g_m) was estimated from combined gas exchange and Chl fluorescence measurements (Harley et al., 1992) as A_{net} / (C_i-(Γ * (ETR + 8 (A_{net} + R_D))/(ETR - 4 (A_{net} + R_D)))) by the variable J method. Γ was taken from Bernacchi et al. (2002)

Sampling for biochemical assays

The same leaves used for photosynthesis and Chl fluorescence measurements were used for the biochemical analysis. Two leaf disks of 1 cm² were obtained immediately after gas exchange and Chl fluorescence measurements, cut with a calibrated cork borer, wrapped in aluminum foil, immediately plunged into liquid N₂ and stored at -80°C until photosynthetic pigments and methanol extractable UV-B absorbing compounds (MEUVAC) analyses. Afterwards, the leaf was detached and frozen in liquid N₂ for lipid peroxidation (thiobarbituric acid reacting substances, TBARS), antioxidant enzyme activities and protein determinations.

Leaf pigments and methanol extractable UV-B absorbing compounds (MEUVAC)

Leaf photosynthetic pigments were extracted with 100% acetone in the presence of Na ascorbate, filtered through a 0.45 µm filter and quantified by HPLC (Larbi et al., 2004). Two steps were used: mobile phase A (acetonitrile:methanol, 7:1, v/v) was pumped for 3.5 min, and then mobile phase B (acetonitrile:methanol:water:ethyl acetate, 7:0.96:0.04:8 by volume) was pumped for 4.5 min. To both solvents, 0.7% (volume) of the modified triethylamine (TEA) was added to improve pigment stability during separation (Hill and Kind, 1993). Depoxidation state of the xanthophyll cycle (DES) was calculated as the sum of antheraxanthin and zeaxanthin concentrations divided by the sum of violaxanthin, antheraxanthin and zeaxanthin concentrations. For extraction of MEUVAC, frozen leaf disks were extracted with 10 mL of methanol:water:7M HCl, equivalent to 70:29:1 (v/v/v). After 15 min at 85°C in the dark, the extract was centrifuged for 15 min at 2,000 g. The absorbance spectrum was measured in a spectrophotometer UV/Vis (UVMini 1240, Shimadzu, Kyoto, Japan). The amount of MEUVAC was expressed in arbitrary units as the area under the absorbance curve in the UV-B interval (AUC₂₈₀₋₃₁₅) calculated per unit of leaf area (Dai et al., 2004).

Determination of total soluble proteins and antioxidant enzymes

Frozen leaf tissue (1 g) was homogenized with 10 mL of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA-Na₂, 0.5 mM ascorbate and 1% PVPP (polyvinyl polypyrrolidone) in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min. The supernatant was used for determinations of protein content and antioxidant enzyme activity. Total soluble protein concentration was determined as described by Bradford (1976) using bovine serum albumin as standard. Superoxide dismutase (SOD, EC 1.15.1.1) was determined by the nitroblue tetrazolium (NBT) method (Dhindsa et al., 1981) with some modifications. Guaiacol peroxidase (GPX, EC 1.11.1.7) assay was performed using the method described by Pütter (1974). Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of H₂O₂ disappearance at 260 nm as described by Aebi (1984) with some modifications. Ascorbate peroxidase (APX, CE 1.11.1.11) activity was determined as described by Nakano and Asada (1981) with some modifications. For more details on enzyme activity determination and methods modifications see Martínez-Lüscher et al. (2013).

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reacting substances (TBARS), as described by Dhindsa et al. (1981). Frozen leaf tissue (0.5 g) was homogenized with 10 mL trichloroacetic acid (TCA) 0.1% (w/v) in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min. Two mL of extract were mixed with 2 mL of either (i) 20% (w/v) TCA solution or (ii) 20% TCA solution containing 0.5% (w/v) TBA. The mixtures were heated at 95°C for 30 min, quickly cooled and centrifuged at 10,000 g and 4°C for 10 min. The absorbance of the supernatant was read at 532 nm with the values for non-specific absorption at 600 nm subtracted. TBARS concentration was calculated using the following formula: $[(A_{532} \times 1000) - (A_{600} \times 1000)] / 155$, being 155 the extinction coefficient in mM⁻¹ cm⁻¹ (Heath and Packer, 1965).

Multiparametric fluorescence measurements

The levels of chlorophyll, flavonols and anthocyanins were assessed by non-destructive fluorescence measurements using a multiparametric portable optical sensor (Multiplex_Research, FORCE-A, Orsay, France) (Ghozlen et al., 2010). Chlorophyll concentration is correlated to a parameter resulting from the ratio of far-red to red fluorescence (SFR_R index) (Gitelson et al., 1999). Flavonol concentrations were determined considering the logarithm of the red to UV excitation ratio of far-red chlorophyll fluorescence (FLAV index), which is correlated to the flavonol content of the leaves (Agati et al., 2007). Anthocyanin concentrations were determined by the ratio between the logarithm of the red to green excitation ratio of far-red chlorophyll fluorescence (ANTH_RG index), which is correlated to the anthocyanin content in the tissue (Agati et al., 2007).

Statistical analyses

Statistical analysis was performed using XLstat-Pro (Addissoft). The data were subjected to a three-factor analysis (ANOVA 3x2) for each sampling point to partition the variance into the main effects (UV-B and water deficit) and the interaction among them. In case of significant interaction among factors, treatments were compared using the least significant difference (LSD) post-hoc test ($p < 0.05$).

3. RESULTS

Phenological development and plant growth

Plant leaf area, fruit mass per plant and leaf to fruit ratio were not significantly modified by UV-B exposure (Table 1). The elapsed time from the beginning of the treatments (around fruit set) to the onset of veraison was greatly reduced by CC conditions and increased by UV-B only under CS (Table 1). The elapsed time from fruit set to maturity was shortened by CC and significantly increased by UV-B.

Gas exchange

Leaf net photosynthesis was significantly higher under CC conditions compared with CS throughout the development (Fig.1 A). Plants exposed to UV-B had significantly lower A_{net} 1 d and 10 d after fruit set and at maturity compared with the non-exposed plants, regardless of CO₂ and temperature conditions. Stomatal conductance was significantly lower in plants under CC conditions 1 d after fruit set, at the onset of veraison and at maturity (Fig 1 B). CC significantly reduced mesophyll conductance only at veraison, whereas UV-B decreased g_m 1 d and 10 d after fruit set and at maturity (Fig. 1D). In general, CC conditions significantly increased both R_L and R_D (Fig 1 E and F).

Chlorophyll a fluorescence

Photosystem II yield significantly increased with CC, except at maturity (Fig 2 A). UV-B significantly reduced Φ_{PSII} only on day 10 after fruit set. CC conditions increased Fv/Fm 10 d after fruit set and at veraison, and strongly decreased this ratio at maturity compared with CS (Fig 2 B). UV-B reduced Fv/Fm only 1 d after fruit set. A significant interaction was observed between UV-B and CC 1 d after fruit set for Fv/Fm. NPQ decreased on day 1 after fruit set and at veraison under CC (Fig. 2 C).

Protein concentration, lipid peroxidation and enzyme activity

Neither UV-B nor CC conditions had an effect on TSP, however soluble protein levels under 0 kJ m⁻² d⁻¹ of UV-B and CC conditions were the highest (Table 2). Lipid peroxidation (TBARS) was significantly lower in the dose of 9.66 kJ m⁻² d⁻¹ of UV-B, concomitant to higher SOD, APX, GPX and CAT activities under CS conditions. Significant interactions were observed between UV-B radiation and CC in TPS, TBARS, SOD, APX and CAT (Table 2).

Leaf pigments and MEUVAC

Chlorophylls tended to decrease with UV-B radiation and to increase under CC conditions (not significant differences), the highest values were observed in those plants non-exposed to UV-B and under CC conditions (Table 3). Chl a/ Chl b significantly increased under CC

conditions. Neoxanthin and lutein were the only two carotenoid pigments affected by CC (reductions of less than 10% on average) (Table 3). Violaxanthin, antheraxanthin and zeaxanthin concentrations and the de-epoxidation state (DES) of xanthophyll cycle were not affected by CC and UV-B. MEUVAC values were higher in UV-B treated plants from 10 d after fruit set to maturity (Fig. 3). CC conditions significantly reduced the concentration of MEUVAC at maturity.

Multiparametric fluorescence measurements

FLAV index was significantly higher in plants treated with UV-B radiation and lower under CC conditions (Table 4). ANTH_RG index had a tendency to increase with UV-B, and in fact the 9.66 kJ m⁻² d⁻¹ CS treatment had significantly higher values than the 0 kJ m⁻² d⁻¹ CS treatment. SFR_R index significantly decreased with UV-B and increased under CC conditions, the highest values were observed in those plants non-exposed to UV-B and under CC conditions.

Ripening rates

Ripening rates were significantly higher under CC conditions ($p < 0.001$), and slightly lower under UV-B ($p = 0.001$). These rates were strongly correlated to the average photosynthetic rate ($r^2 = 0.97$; Fig. 4).

4. DISCUSSION

Effect of elevated CO₂-temperature (CC) on grapevine photosynthetic performance

Results show that leaf photosynthetic performance is likely to change greatly within the conditions predicted by IPCC (2013). Carbon fixation rates (A_{net}) significantly increased under CC conditions, and most importantly, no signs of acclimation were observed. Contrasting results were found by Salazar-Parra et al. (2012b), who reported an acclimation (i.e. decrease in A_{net}) to the same CC conditions after 21 days of treatments. One of the

differences between the studies, vegetation control down to 12 leaves carried out in the present work, may have contributed to prevent CO₂ acclimation (Erice et al., 2006). The increase in net photosynthesis was accompanied by higher sub-stomatal CO₂ levels and dark respiration; and lower stomatal conductance and photorespiration. Decrease in stomatal conductance has been reported widely in response to elevated CO₂ (Ainsworth and Rogers, 2007). One of the processes regulating stomatal conductance is occlusive cells intracellular CO₂ concentration (Mott, 1988), which is most certainly affected by sub-stomatal CO₂ levels. Elevated CO₂ is also known to competitively inhibit the oxygenation reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase, resulting in lower photorespiration rates (Long et al., 2004). Increases in dark respiration rates have been reported in response to CO₂, mediated by higher carbohydrate levels and the increased energy status of the plant (Li et al., 2013). In addition, temperature is more widely regarded to affect respiratory rates of plants (Azcón-Bieto, 1992). In summary, both elevated CO₂ and temperature may enhance the same processes. However, the impact of 4 degrees increase in temperature has showed to be negligible compared to the effect of an increase of CO₂ levels up to 700 ppm (Sanz-Saez et al., 2010).

Effect of UV-B and its interaction with elevated CO₂-temperature on grapevine photosynthetic performance

As it was demonstrated in previous research, grapevine leaves may experience, an initial decrease in photosynthetic rate after UV-B exposure (Martínez-Lüscher et al., 2013). However, acclimation takes place in a few weeks period. UV-B acclimation process is characterized by the accumulation of phenolic compounds, most likely flavonols (Martínez-Lüscher et al., 2014), which protect plant cells from the most harming UV wavelengths. In the present work, photosynthesis was transiently reduced 1 and 10 days after fruit set, and recovery took place concomitant to an increase in UV-shielding compounds, as indicated by MEUVAC and FLAV index. In addition, flavonols may have another role in UV acclimation, due to their antioxidant activity but also due to their relatively free distribution in the sub-

cellular domains (Agati and Tattini, 2010; Jansen et al., 2012). The synthesis of flavonols by flavonol synthase is mediated by UV-B photoreception (Cloix and Jenkins, 2008), being this just one of the multiple responses downwards in the UV-B specific signalling (Stracke et al., 2010). For instance, UV-B signaling cascade may up-regulate genes encoding for antioxidant enzymes (Brosche et al., 2002; Brown et al., 2005; Ulm et al., 2004), and contrarily to other stress factors, this may be triggered without the mediation of reactive oxygen species, which may lead to cross tolerance to other stresses (Hideg et al., 2013 and references therein). In the present study, despite the acclimation to UV-B, plants showed elevated enzyme activity in response to UV-B. This fact, combined with higher non-enzymatic antioxidant levels, indicated by the increased MEUVAC and FLAV index, conferred to UV-B treated plants a strong antioxidant machinery ready to scavenge reactive oxygen species produced in the cells (Agati et al., 2013). Moreover, under CC conditions leaf senescence was more evident at maturity (e.g. lower Fv/Fm and higher lipid peroxidation). When combined both factors, UV-B and elevated CO₂-temperature, the antioxidant response triggered by UV-B may have contributed to reduce the oxidative damage under CC conditions, as indicated the absence of changes in Fv/Fm and TBARS at maturity.

Effect of UV-B and elevated CO₂-temperature on berry ripening

Grapevine phenology and berry ripening is traditionally assessed as a temperature-dependent process (e.g. growing degree days or biologically effective degree days) (Gladstones, 1992; Winkler et al., 1974). Several models have been performed in order to assess the impact of climate change on viticulture, exclusively based on the effect of increasing air temperatures on grapevine (Hannah et al., 2013; Webb et al., 2007). Also rising global temperatures are considered to be responsible for the widely reported phenomenon of early grape ripening nowadays (Ganichot, 2002; Stock et al., 2005). However, sugar accumulation during ripening relies on carbon fixation and translocation from mature leaves. Carbon assimilation may be affected by temperature, although there are other climate change related factors that may affect grapevine photosynthesis to a greater

extent (Greer and Weedon, 2012; Martínez-Lüscher et al., 2013; Moutinho-Pereira et al., 2009).

In previous research, Salazar-Parra (2011) showed light effect of temperature on berry ripening, and this was only significant when combined with elevated CO₂. Similarly, in the present study plants under the same conditions of elevated CO₂-temperature, as in the study of Salazar-Parra (2011) reached maturity earlier than those under CS and this was mediated by higher photosynthetic rates. By contrast, UV-B delayed grape berry ripening, associated with a reduction in net photosynthesis, which counteracted the effect of CC conditions. As the treatments did not affect leaf to fruit ratio (Table 1) and vegetation was controlled to maintain a maximum of 12 leaves, we consider fair to correlate average photosynthetic capacity to overall ripening rates in order to establish the relation between these two processes (carbon fixation and carbon storage). Our results show a great dependence of the ripening rates on the carbon fixation process ($R^2=0.97$), regardless of the environmental factor assessed. Leaf removal has been proposed as an strategy to regulate sugar accumulation in the berries and/or to decelerate an overly quick and unbalanced ripening (Martinez de Toda et al., 2013; Palliotti et al., 2013) . Thus, while source to sink ratio is often considered to affect grape berry ripening (Diago et al., 2012; Kliewer and Dokoozlian, 2005; Pastore et al., 2013; Pastore et al., 2011), photosynthetic performance is rarely assessed. Growers have already implemented adaptive strategies, in response to changes in environmental conditions (van Leeuwen et al., 2013). Therefore, a proper knowledge of grapevine ecophysiology may be crucial to determine the procedures committed to maintain wine grape quality standards taking into account the combined effects of environmental factors.

5. CONCLUSIONS

UV-B treated plants suffered a transient down-regulation of photosynthesis. Even though carbon fixation rates were always higher under elevated CO₂-temperature, these plants

showed signs of early leaf senescence at maturity. UV-B radiation elicited both enzymatic and non-enzymatic antioxidant responses, leading to a eustress state, which may have contributed to reduce senescence-related damage induced by elevated CO₂-temperature. The photosynthetic performance of fruit-bearing cuttings was strongly correlated to changes in berry ripening rates. The hastening effect of climate change conditions on grape ripening, associated to higher rates of carbon fixation, was attenuated by UV-B radiation.

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Table 1. Total leaf area per plant, fruit mass per plant, leaf to fruit area ratio and elapsed days from the start of the treatments (fruit set) to veraison and to maturity of plants grown under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Values are means±SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$. In case of significant interaction between factors, different letters within the same parameter indicate significant differences (p<0.05) according to LSD test.

	Current situation (CO ₂ Amb- 24/14°C)			Climate change (700ppm CO ₂ - 28/18°C)			$P_{(UV-B)}$	$P_{(CC)}$	$P_{(UV-B \times CC)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
Leaf área (m ² plant ⁻¹)	0.33±0.01	0.33±0.02	0.31±0.02	0.34±0.02	0.33±0.03	0.32±0.03	0.643	0.815	0.932
Fruit mass (g plant ⁻¹)	305±13	269±15	290±5	292±18	260±16	268±19	0.115	0.289	0.936
Leaf to fruit ratio (cm ² g ⁻¹)	11.0±0.6	12.6±0.9	10.6±0.6	12.3±1.2	13.3±2.1	12.5±1.4	0.524	0.261	0.926
Days to veraison (d)	48.1±0.6b	47.8±0.6b	52.1±1.0a	39.6±0.9c	40.9±0.8c	40.0±0.7c	0.018	< 0.001	0.005
Days to maturity (d)	101.0±6.9	103.2±6.8	108.8±2.5	78.0±2.7	83.0±5.5	82.6±5.2	0.017	< 0.001	0.361

Table 2. Total soluble proteins (TSP), lipid peroxidation (thiobarbituric acid reactive substances; TBARS), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPX) activities measured in leaves from plants grown under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Values are means±SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$. In case of significant interaction between factors, different letters within the same parameter indicate significant differences (p<0.05) according to LSD test.

	Current situation (CO ₂ Amb- 24/14°C)			Climate change (700ppm CO ₂ - 28/18°C)			$P_{(UV-B)}$	$P_{(CC)}$	$P_{(UV-B \times CC)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
TSP (mg g ⁻¹ dry wt)	2.35±0.21b	2.70±0.40ab	2.91±0.50 ab	3.53±0.68 a	1.87±0.13 b	2.23±0.12 b	0.274	0.742	0.033
TBARS (nmol MDA g ⁻¹ dry wt)	152±29 bc	210±38 ab	84±16 c	261±60 a	130±40 bc	111±19 c	0.012	0.518	0.034
SOD (uSOD mg ⁻¹ TSP min ⁻¹)	280±32 d	424±65 bc	577±64 ab	368±48 cd	613±26 a	444±45 bc	< 0.001	0.220	0.005
APX (μmol ascorbate mg ⁻¹ TSP min ⁻¹)	0.87±0.19 bc	0.87±0.21 bc	1.51±0.33 ab	0.73±0.15 c	2.28±0.52 a	1.18±0.26 bc	0.021	0.174	0.006
CAT (μmol H ₂ O ₂ mg ⁻¹ TSP min ⁻¹)	596±11 b	664±30 b	804±81 a	786±45 a	660±19 b	643±23 b	0.349	0.810	0.000
GPX (nmol guaiacol mg ⁻¹ TSP min ⁻¹)	61.7±13.4	86.7±16.8	65.1±16.0	31.5±4.8	78.5±18.2	81.7±8.7	0.033	0.529	0.282

Table 3. Chlorophylls and carotenoids concentration and de-epoxidation state (DES) of xanthophyll cycle measured in leaves from plants grown under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻²d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Values are means±SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$. In case of significant interaction between factors, different letters within the same parameter indicate significant differences (p<0.05) according to LSD test.

	Current situation (CO ₂ Amb- 24/14°C)			Climate change (700 ppm CO ₂ - 28/18°C)			$P_{(UV-B)}$	$P_{(CC)}$	$P_{(UV-B \times CC)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
Chla+b (μmol m ⁻²)	702.0±56.5	553.1±63.2	617.1±53.3	752.2±61.6	660.6±79.2	642.6±59.2	0.148	0.245	0.800
Chla/Chlb	2.97±0.04	2.95±0.04	2.96±0.04	3.11±0.04	3.25±0.06	3.11±0.05	0.295	<0.001	0.163
a-Caroten (mmol mol ⁻¹ Chl)	4.61±1.64	4.40±0.46	3.68±0.71	4.30±0.86	2.14±0.55	3.60±1.33	0.277	0.461	0.715
b-Caroten (mmol mol ⁻¹ Chl)	89.0±2.8	87.8±2.7	90.6±1.5	89.8±2.8	89.0±1.7	90.7±3.0	0.752	0.980	0.823
Neoxanthin (mmol mol ⁻¹ Chl)	40.1±1.2	39.9±2.3	41.9±1.2	36.8±1.2	37.1±1.1	37.5±0.6	0.586	0.004	0.841
Lutein (mmol mol ⁻¹ Chl)	153.5±4.8	159.4±4.5	160.5±1.7	144.1±2.3	146.5±3.5	147.7±2.8	0.294	0.000	0.848
Violaxanthin(V) (mmol mol ⁻¹ Chl)	44.0±1.5	41.6±2.7	41.3±3.2	50.3±5.6	51.3±5.1	38.5±4.0	0.149	0.188	0.281
Antheraxantin(A) (mmol mol ⁻¹ Chl)	9.54±1.38	9.65±1.98	8.19±0.96	7.33±1.31	8.13±1.70	9.26±0.55	0.948	0.444	0.475
Zeaxanthin(Z) (mmol mol ⁻¹ Chl)	17.5±2.0	12.5±3.4	19.4±2.6	18.6±3.6	20.7±3.4	14.5±5.0	0.523	0.171	0.719
V+A+Z (mmol mol ⁻¹ Chl)	71.1±3.9	72.0±6.0	68.9±3.4	70.2±2.6	73.9±4.1	66.3±3.0	0.419	0.875	0.852
DES(A+Z)/(V+A+Z)	0.38±0.03	0.41±0.05	0.40±0.04	0.29±0.07	0.30±0.08	0.42±0.05	0.354	0.181	0.434

Table 4. Multiparametric fluorescence measurements of flavonols (FLAV), anthocyanins (ANTH RG) and Chlorophylls (SFR R) in leaves from plants at maturity (ca.20 °Brix) grown under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Values are means±SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$. In case of significant interaction between factors, different letters within the same parameter indicate significant differences (p<0.05) according to LSD test.

Multiplex™ Parameters (Arbitrary units)	Current situation (CO ₂ Amb-24/14°C)			Climate change (700 ppm CO ₂ -28/18°C)			$P_{(UV-B)}$	$P_{(CC)}$	$P_{(UV-B \times CC)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
	FLAV	0.31±0.15	1.05±0.27	1.27±0.21	0.07±0.05	0.946±0.30			
ANTH_RG	0.31±0.04	0.41±0.12	0.34±0.07	0.29±0.02	0.32±0.04	0.37±0.10	0.087	0.263	0.110
SFR_R	5.94±0.15	5.18±0.24	5.58±0.18	6.09±0.14	5.80±0.17	5.87±0.16	0.016	0.017	0.404

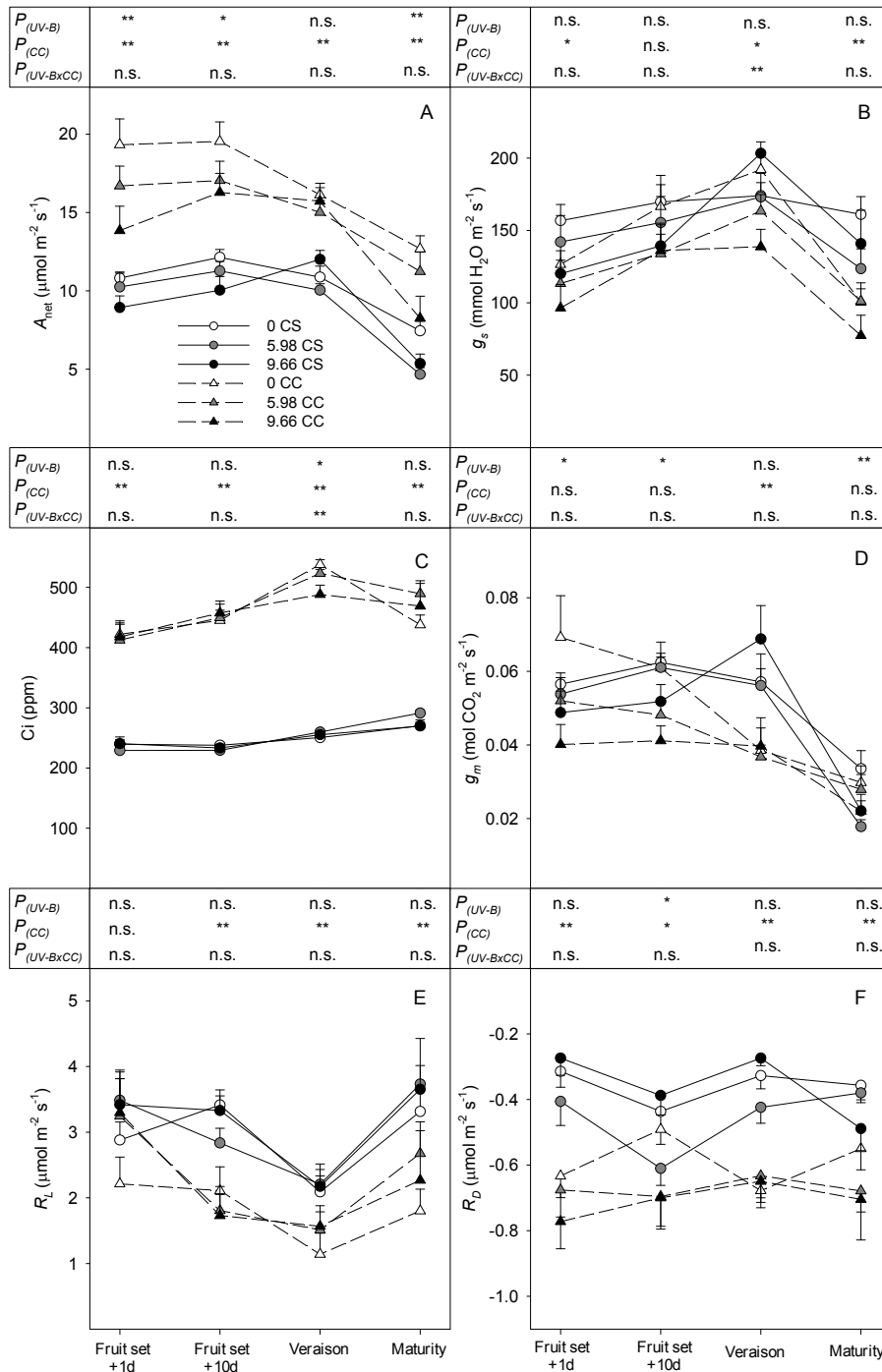


Figure 1. Photosynthetic rate (A_{net} ; A), stomatal conductance (g_s ; B), sub-estomatal CO₂ concentration (C_i ; C), mesophyll conductance (g_m ; D), photorespiration (R_L ; E) and dark respiration (R_D ; F) throughout the development of plants under three levels of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Symbols are means ± SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$, and their interaction, $P_{(UV-B \times CC)}$. **, $P < 0.01$; *, $P < 0.05$ and n.s. not significant.

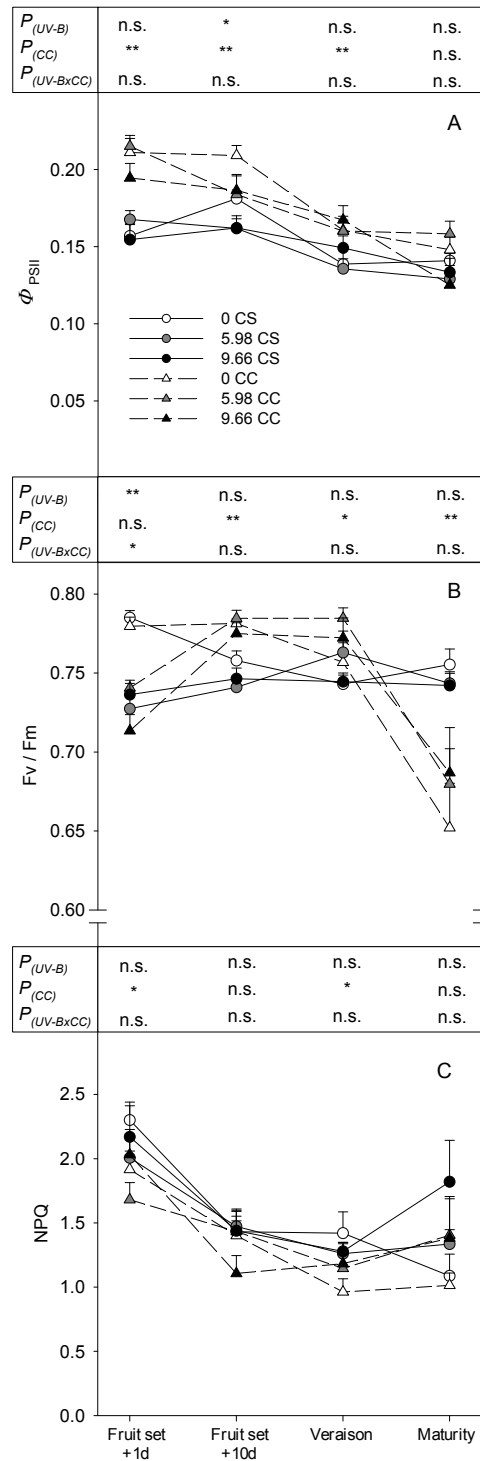


Figure 2. Actual photosystem II efficiency (ϕ_{PSII} ; A), Maximum potential photosystem II efficiency (Fv/Fm; B) and non-photochemical quenching (NPQ; C) throughout the development of plants under three levels of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Symbols are means \pm SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$, and their interaction, $P_{(UV-B \times CC)}$. **, $P < 0.01$; *, $P < 0.05$ and n.s. not significant.

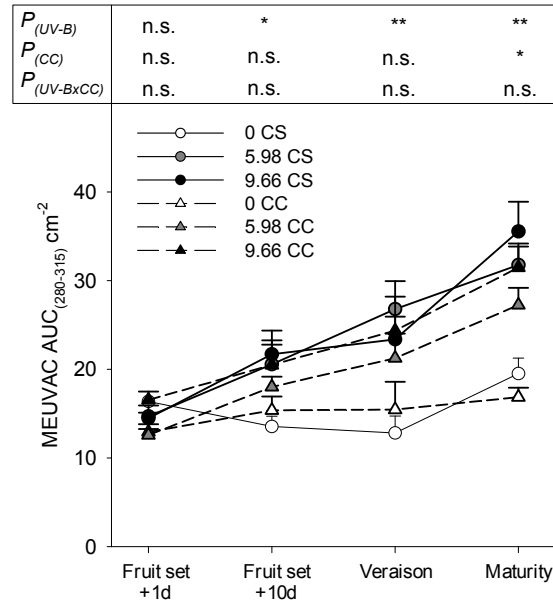


Figure 3. Methanol extractable UV-B absorbing compounds (area under the curve between 280 and 315 nm) of leaves throughout the development of plants under three levels of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CO₂ Amb- 24/14°C day/night) and climate change (700 ppm CO₂ and 28/18°C). Symbols are means ± SE, n=9-10. Main effects of UV-B, $P_{(UV-B)}$, climate change, $P_{(CC)}$, and their interaction, $P_{(UV-B \times CC)}$. **, $P < 0.01$; *, $P < 0.05$ and n.s. not significant.

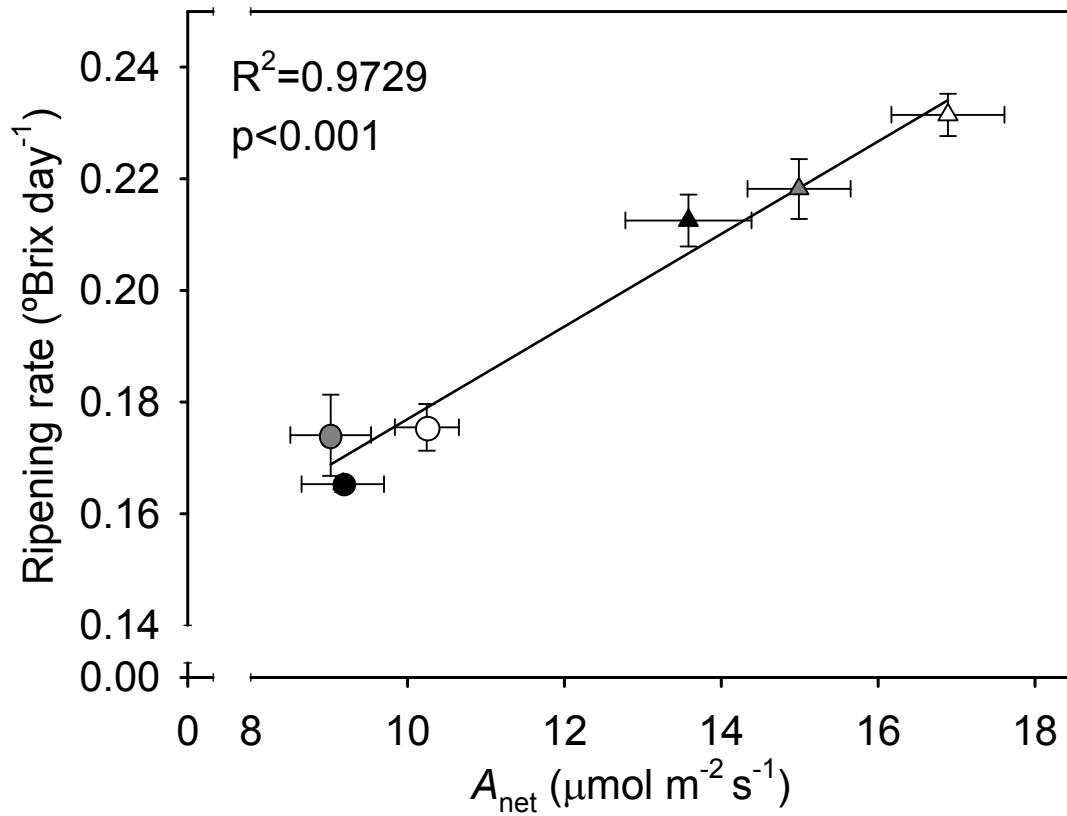


Figure 4. Correlation between berry ripening rates from fruit set (3.8°Brix) to maturity (ca. 20°Brix) and the mean net photosynthesis at light saturation. Circles represent plants grown under ambient CO₂ – 24°/14°C day/night and triangles plants under 700 ppm CO₂-28/18°C; open, grey and black symbols represent 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B radiation treatments, respectively. Symbols are means ± SE, n=9-10.

CHAPTER 6

UV-B alleviates the uncoupling effect of climate change conditions (elevated CO₂-temperature) on grape berry (*Vitis vinifera* cv. Tempranillo) anthocyanin-sugar accumulation

Article in preparation

UV-B alleviates the uncoupling effect of climate change conditions (elevated CO₂-temperature) on grape berry (*Vitis vinifera* cv. Tempranillo) anthocyanin-sugar accumulation

ABSTRACT

Modeling of future environmental conditions predicts an increase in temperature for most viticultural regions, mainly mediated by the anthropic release of CO₂. Therefore, some wine grape quality traits may be altered. UV-B has been previously proposed as a growth modulator with a positive effect on grape quality. The aim of the present study was to explore the potential interactions between climate change conditions (elevated CO₂-temperature) and UV-B radiation on anthocyanin and flavonol biosynthesis to understand the mechanisms leading to changes in grape quality under the forecasted future climatic conditions. Three doses of UV-B: 0, 5.98, 9.66 kJ m⁻² d⁻¹, and two CO₂-temperature regimes: Ambient CO₂ - 24/14°C (day/night) (current situation) and 700 ppm CO₂ - 28/18°C (climate change) were imposed to grapevine (*Vitis vinifera* cv. Tempranillo) fruit-bearing cuttings from fruit set to maturity. Grape skin flavonol and anthocyanin contents were determined throughout development and transcript levels of genes involved in flavonoid biosynthesis, one week after veraison. Quality related traits were determined at maturity. UV-B was the main factor driving flavonol biosynthesis, as their concentration was several times higher in UV-B treated plants. Anthocyanin biosynthesis was up-regulated by elevated CO₂-temperature. However, total soluble sugar accumulation in the berries (TSS) was further enhanced than anthocyanin accumulation, which constitutes a decoupling between anthocyanin and sugar accumulation. UV-B always resulted in higher anthocyanin concentration, especially under climate change conditions, which alleviated the anthocyanin-sugar decoupling. Transcript levels analyzed one week after veraison revealed the up-regulation of structural (*FLS1*, *CHS*, *OMT2* and *GST*) and regulatory (*MYBF1* and *MYBA1*) genes of flavonol and anthocyanin biosynthesis under UV-B radiation. *MYBA1* was further up-regulated under climate change conditions. The effect of UV-B modulating grape sugar accumulation and anthocyanin-related gene up-

regulation, clearly contributed to alleviate the deleterious effects of elevated CO₂-temperature on anthocyanin content at maturity.

Keywords: Climate change, UV-B, flavonoids, organic acids, nitrogen compounds, anthocyanin-sugar decoupling

1. INTRODUCTION

CO₂ levels have risen over the last decades at an increasing rate (IPCC, 2013), concomitant to the temperature increase. Predictions estimate that at the current rate, CO₂ levels will reach 700 ppm by the end of this century, which may correspond to a rise in temperatures by 4°C (IPCC, 2007). Studies concerning crop development records, have not dissociated these two factors that coexist in current conditions and may increase together within the future climate change scenario. Moreover, cloud patterns are also expected to change, increasing the irradiation periods in places such as the Mediterranean region (Alcamo et al., 2007). UV-B levels may vary to a great extent, according to stratospheric ozone levels, latitude, altitude, time of the year and cloudiness (McKenzie et al., 2007), which may have an impact on crop production (Ballaré et al., 2011).

Changes in CO₂ and temperature may significantly impact photosynthetic rates, carbon assimilation, sugar accumulation, and, therefore, grape ripening (reviewed by Mira de Orduña, 2010). This may result in altered primary and secondary metabolite concentrations at harvest (Kuhn et al., 2013), their balance being crucial for wine quality (Conde et al., 2007). Sugar levels have been increasing over the last decades (Bock et al., 2013), and currently developmental events such as bud break, flowering and veraison occur earlier in the season than ever (Bock et al., 2011). Sugar accumulation hastening plays a key role in the mediation in berry quality changes and may results in flavonoid biosynthesis up-regulation (Castellarin et al., 2007a). Further shortening in ripening period may cause anthocyanin content reduction (Salazar et al., 2010). Thereby, recovering or maintaining the balance between sugar accumulation and other quality traits, such as colour, flavour and aromas in future climate conditions constitutes a major challenge to viticulture over the next half century (Teixeira et al., 2013).

Climate change impact on viticulture has been recently assessed worldwide by Hannah et al. (2013) as an increase in temperature above the current thermal distribution of cultivars,

which may force growers to implement new strategies in order to maintain current standards of quality (van Leeuwen et al., 2013). Conversely to temperature, the effect of elevated CO₂ has not been considered in viticulture climate change impact assessments (Hannah et al., 2013; Webb et al., 2007; White et al., 2006).

In previous research it has been shown that UV-B induces an acclimation response, with an initial decrease in photosynthesis and a later recovery, due to an increase in UV-B absorbing compounds and antioxidant enzyme activity (Martínez-Lüscher et al., 2013). UV-B was also found to affect berry composition, increasing grape flavonol and anthocyanin content (Martínez-Lüscher et al., 2014). In fact, UV-B is claimed to be responsible for a great part of the quality improvement attributed to a good sun exposure of the clusters (Teixeira et al., 2013).

The aim of this work was to study the influence of CO₂ and temperature on the dynamic of anthocyanin-sugar accumulation, as well as to assess whether UV-B radiation can modulate the effects of climate change conditions on grape quality traits.

2. MATERIAL AND METHODS

Growing conditions and experimental design

Vitis vinifera cv. Tempranillo fruit bearing cuttings were produced as in Martínez-Lüscher et al. (2013). Briefly, three-node segments were collected from Institute of Sciences of Vine and Wine (Logroño, Spain). Rooting was induced using indol butiric acid in a heat-bed (27°C) kept in a cool room (5°C). When cuttings developed enough roots, they were transplanted to 6.5 L pots containing 2:1 peat: perlite (v/v). Only one flowering stem was allowed to develop on each plant. Vegetative growth was controlled by pruning, thus maintaining a leaf area to grape mass ratio optimal for berry ripening in all the treatments (12 leaves per plant) (Jackson and Lombard, 1993). Shoots were fixed to horizontal rods compelling leaves to face upwards. The experiment was carried out in glasshouses at the University of Navarra (Pamplona, Spain, 42°48'14" N, 1°39'54" W) from April to September 2012. Growth

conditions in the glasshouses up to fruit set were 25/15°C and 50/90% relative humidity (day/night). Glasshouse walls and structure filtered up to 99.9% of UV-B, 35% of UV-A and 15% of the photosynthetically active radiation (PAR) coming from the sun. A supplemental system of high-pressure sodium lamps (HQI-TS 400W/D Osram, Augsburg, Germany) was triggered when PAR dropped below a photon flux density of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (14 h photoperiod). When fruit set was complete for all plants, three levels of biologically effective UV-B (0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$), calculated using the generalized plant action spectrum of Caldwell (1971) as formulated by Green et al. (1974); and two ambient conditions combining two CO_2 levels (ambient CO_2 or 700 ppm) and two temperature regimes (24/14°C day/night or 28/18°C) were imposed. This resulted in two conditions in two different glasshouses: Current situation (CS; Ambient CO_2 - 24/14°C) and climate change (CC; 700 ppm CO_2 - 28/18°C). This made a total of 6 treatments with 12 plants in each. Relative humidity was programmed in each glasshouse to maintain the same vapour pressure deficit among treatments. Irrigation was performed with the nutritive solution described by Ollat et al. (1998).

UV-B was applied by means of narrow band lamps TL100W/01 (311-313 spectrum peaking; Philips, Netherlands) hanging above the canopy. Spectral scans were performed with a double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd., Livingstone, Scotland). UV-A radiation emitted by the lamps accounted for less than 1 % of the solar UV-A irradiance (W m^{-2}) reaching the plants during the irradiation period. In the treatment of 0 $\text{kJ m}^{-2} \text{d}^{-1}$, lamps underneath were unlit. The UV-B doses of 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ were reached after 3h and 4h 51m of irradiation (0.55 W m^{-2}), respectively. The applied biologically effective dose of 5.98 $\text{kJ m}^{-2} \text{d}^{-1}$ corresponded to 86% of the maximum daily dose at the study site, whereas 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ is only reached in viticulture suitable sites of the southern hemisphere, such as Argentina, South Africa or Australia (Quick TUV Calculator; <http://cprm.acd.ucar.edu/Models/TUV/>).

Grape berry sampling

Grape berry samplings were performed at 4 developmental stages: i) pea size; ii) onset of veraison, when berries started to turn colour; iii) two weeks after the onset of veraison, before anthocyanin accumulation reach a saturation kinetic (Coombe and McCarthy, 2000); and iv) maturity, when the grapes in the cluster of each plant reached a total soluble solids (TSS) content of ca. 22°Brix. Berry samples containing 15 berries from 3 different plants were collected one week after the onset of veraison for quantitative real-time RT-PCR (n=4), when transcript levels of the target genes are around their maximum (Castellarin et al., 2007b). All samples were collected right after the daily UV-B irradiation period, frozen immediately in liquid nitrogen and stored at -80°C until analysis.

Grape must composition analysis

At maturity, 25 berries per cluster were crushed and centrifuged to obtain the must. Must samples were then run through a must FTIR analyzer (Fourier Transformed Infrared Spectrometer, Winescan 120™, FOSS, Hillerød, Denmark) to determine pH, titratable acidity, malic acid, tartaric acid, assimilable nitrogen, α -amino nitrogen, ammonia nitrogen and potassium.

Flavonol and anthocyanin determination

Grape skins from samples collected at pea size, at the onset of veraison and at maturity were freeze dried, ground and extracted in methanol containing 0.1% HCl (v/v). Extracts were filtered through a 0.45 μ m polypropylene syringe filter (Pall Gelman Corp., Ann Arbor, USA) for HPLC routine analysis of individual anthocyanins and flavonols by means of a summit HPLC System consisting of P680 pump, ASI-100T™ autosampler and UVD 340U UV-Vis diode array detector operating at 520 nm and at 360 nm (Dionex Corporation, Sunnyvale, CA, USA) as described in Acevedo De la Cruz et al. (2012). Malvidin-3-O-glucoside was used as common external standard for all the quantified anthocyanins (at 520 nm), and quercetin-3-O-glucoside was used for all the quantified flavonols (at 360 nm)

(Extrasynthese, Genay, France). Formal identification of flavonoids was carried out using the peak identification provided in (Martínez-Lüscher et al., 2014).

Quantitative real-time RT-PCR

Berry samples collected one week after the onset of veraison were powdered in a ball grinder MM200 (Retsch, Haan, Germany). Total RNA was extracted according to the method described in Lecourieux et al. (2010). RNA isolation was followed by DNase I treatment. Reverse transcription was performed from 2 µg of purified RNA using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. Resulting cDNA was diluted 1:10 in ultrapure water. After a literature search, primers of candidate genes *chalcone synthase 2 (CHS 2)*, *flavonoid 3' hydroxylase (F3'H)*, *flavonoid 3' 5' hydroxylase (F3'5'H)*, *flavonol synthase 1 (FLS1)*, *dihydroflavonol reductase (DFR)*, *leucoanthocyanidin dioxygenase (LDOX)*, *UDP-glucose: flavonoid glucosyltransferase (UGT)*, *flavonoid O-methyl transferase 2 (OMT2)*, *MYBF1* and *MYBA1* were obtained (Castellarin et al., 2006; Czemplin et al., 2009; Fournier-Level et al., 2011; Fujita et al., 2006; Jeong et al., 2006; Jeong et al., 2004). Amplification efficiencies were tested for the cv. Tempranillo samples (Table S1). Quantitative real-time RT-PCR (qRT-PCR) expression analysis was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad). Reaction mixes (10 µL) were prepared in triplicate, including 5 µL of iQ™SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer, and 2 µL of diluted (1:10) cDNA. Four candidate reference genes proposed by Reid et al. (2006) were evaluated for expression stability by means of GeNorm M value within the qBase PLUS software (Biogazelle), and as a result glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen for normalization. Thereby, gene transcripts were quantified upon normalization to GAPDH by comparing the cycle threshold (C_T) of the target gene with that of GAPDH.

Statistical treatment

Statistical analysis was performed with XLstat-Pro (Addinsoft). TSS, berry weight, berry diameter, flavonol and anthocyanin skin concentrations were subjected to a two-way anova (ANOVA 3x2) in order to partition the variance into the main effects (UV-B radiation dose, UV-B; CO₂-temperature, CC and the interaction between them, UV-BxCC) independently at every sampling point. Gene expression levels were subjected to a two-way ANOVA (3X2) in order to partition the variance into the main effects (UV-B radiation dose, UV-B; CO₂-temperature, CC and the interaction between them, UV-BxCC). A linear regression was performed to analyze the relationship between anthocyanin skin concentration and TSS.

3. RESULTS

Phenology

Berry development was hastened by CC conditions, shortening the time to reach veraison and maturity by 9 and 23 days, respectively (Table 1). Contrarily UV-B radiation induced a delay in veraison and maturity, compared to non-exposed plants (0 kJ m⁻² d⁻¹).

Grape must Fournier Transformed Infrared (FTIR) spectroscopy analysis

Grape must FTIR spectroscopy analysis revealed a significant reduction of organic acid must concentration under CC conditions (total acidity and malic acid) (Table 2). However, this effect was not concomitant with an increase in pH. UV-B had significant effect in grape must, increasing pH, and reducing total acidity. Potassium levels were higher under UV-B radiation. Yeast assimilable nitrogen and α -amino nitrogen were significantly lower under CC conditions.

Berry total soluble solids, weight and diameter

Berry traits show differences in TSS only for CC conditions, the main effect being noticed at veraison and 2 weeks after veraison (Fig. 1). TSS of berries under CS conditions were higher

than those of CC at veraison, but 2 weeks after veraison berries under CC conditions presented significantly higher levels of TSS than berries under CS conditions. Berry weight remained unchanged 20 days after the beginning of the treatments (Pea size stage), and two weeks after veraison berry weight and diameter were not significantly different among treatments. However, berries under CS conditions reached veraison with a higher berry weight and larger diameter than those under CC conditions. When maturity was reached for each individual cluster, berry size and diameter were lower in UV-B treated plants compared to non-exposed ones.

Flavonols and anthocyanins

Flavonol content of grape berry skins was always several times higher in berries under 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B (Fig. 2 B). On average, under CC conditions flavonol content was significantly lower compared to CS conditions. Total grape skin anthocyanins started to accumulate at the onset of ripening (Fig. 2 A). Two weeks after veraison anthocyanin content was higher in berries under CC conditions and UV-B radiation compared to berries under CS conditions. There was a significant positive interaction between UV-B and CO_2 -temperature regime, further increasing anthocyanin levels in 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ under CC conditions. Conversely, total skin anthocyanin levels were higher under CS conditions at maturity. UV-B also increased significantly total anthocyanin skin concentration at maturity.

Skin anthocyanins and TSS always presented strong linear correlations (Fig. 3). The correlation between skin anthocyanins and TSS presented a higher slope value for CS samples than for CC samples ($p < 0.001$; Fig. 3 A). When comparing the different UV-B exposures (0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$) under CC conditions, 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ had the highest slope ($p = 0.005$; Fig. 3 B), and in fact, the slopes of the treatment with 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B and CC conditions were not statistically different from those of the treatment with 9.66 kJ m^{-2} of UV-B and CS conditions ($p = 0.126$) (data not shown).

Transcript profiling

Transcript profiling one week after veraison showed significantly higher expression levels of the structural genes *CHS*, *FLS1*, *OMT2* and *GST* with UV-B (Fig 4). Regulatory genes *MYBF1* and *MYBA1* were significantly higher under UV-B compared to control UV-B ($0 \text{ kJ m}^{-2} \text{ d}^{-1}$) and *MYBA1* transcript levels were also significantly higher under CC conditions, than CS conditions.

4. DISCUSSION

Effect of UV-B and elevated CO₂-temperature on must quality parameters

Organic acid levels in the must were affected by the treatments (Table 2). However this effect was induced in different ways by UV-B and CC conditions. Whereas, UV-B radiation increased potassium levels, which most likely affected grape must pH; malic acid and total acidity concentration were lowered by CC conditions. UV-B may be involved somehow in potassium accumulation in the berries. However, it seems more likely that the lower berry weights and higher relative skin mass under UV-B may have increased potassium levels in the must, as potassium concentration in the skins is much higher than in the flesh (Conde et al., 2007). The effect of CC conditions could be explained by the temperature-dependency of organic acid metabolism. The balance of malic acid synthesis and catabolism is directly affected by temperature. Indeed, cytosolic malate dehydrogenase, which is responsible for malate synthesis, operates more efficiently at low temperatures; while the mitochondrial isoform, which contributes to malic acid degradation works better under high temperature conditions (Etienne et al., 2013; Lakso and Kliewer, 1975).

All the parameters derived from nitrogen compounds but ammonia nitrogen (i.e. yeast assimilable and alpha amino nitrogen) were lower in the must of berries grown under elevated CO₂-temperature. This may be a sign of decrease in nitrogen concentration in the tissues, which constitutes a widely reported response to elevated CO₂ (reviewed by Leakey et al., 2009). Hence, elevated CO₂ may induce a priority in the storage of nitrogen - free

compounds (i.e. carbohydrates), to nitrogen - containing compounds (i.e. amino acids and proteins) (Aranjuelo et al., 2008; Dijkstra et al., 2010; Zavala and Ravetta, 2002).

Effect of UV-B and elevated CO₂-temperature on grape berry skin flavonol content

Flavonol biosynthesis is not as strongly linked to phenological events in grape development, as anthocyanins or proanthocyanidins are (Czemmel et al., 2012). High *FLS* expression levels have been reported mostly around fruit set and late ripening (Downey et al., 2003; Fujita et al., 2006), but also around veraison (Castellarin et al., 2007a; Deluc et al., 2007). Developmental increase in sugar levels (Fig. 1A) in the berry may have also induced flavonol biosynthesis up-regulation, as it occurs in a greater manner for anthocyanins (Dai et al., 2014). In absence of UV-B radiation (0 kJ m⁻² d⁻¹ treatments) flavonol biosynthesis was negligible up to the onset of veraison, followed by an increase in skin flavonol concentration during berry ripening, which suggest certain developmental regulation of flavonol biosynthesis (Czemmel et al., 2012; Downey et al., 2003; Fujita et al., 2006). On the other hand, flavonol accumulation is known to be highly responsive to light, and more precisely to the UV portion of the solar spectrum (Koyama et al., 2012; Martínez-Lüscher et al., 2014). In the present study, grape skin flavonol content increased at any stage with UV-B radiation (Fig. 2B). Increase in berry size did not induce a dilution effect on the concentration of flavonols as reported in previous research (Downey et al., 2003). In addition, Under CC conditions flavonol levels were significantly lower. However, this was most likely due to the lower exposure times to UV-B, rather than biosynthesis down-regulation (Fig. 4), as berries under CC conditions ripened faster than under CS conditions (Table 1).

Sugars accumulation and anthocyanin biosynthesis under elevated CO₂-temperature and UV-B

The onset of ripening (veraison) marks the start of the second growth phase of berries and is characterized by a strong increase in sugar levels in the mesocarp (pulp) and the start of anthocyanin accumulation in the exocarp (skin) (Conde et al., 2007). Grape berry is a non-

climacteric fruit and molecular signals leading to the onset of ripening are still not clear (Kuhn et al., 2013). Nevertheless, it is known that, among others, hormone levels activate relevant genes encoding proteins for sugar import into the berry (Lecourieux et al., 2014). At this point, anthocyanin content starts to increase concomitantly to a faster sugar accumulation (Coombe and McCarthy, 2000). Hereby, anthocyanin biosynthesis appears to be highly sugar level dependent (Dai et al., 2014), as well as other phenolic compounds are (Ferri et al., 2011). In the present experiment, the onset of veraison was triggered earlier in time under CC conditions (Table 1), but also with lower sugar levels in pulp (Fig. 1). In the following two weeks after veraison, sugar accumulation was greatly enhanced under CC conditions; and in fact, due to their higher sugar accumulation rates, CC grapes reached maturity on average 23 days earlier, compared to those under CS conditions. The fact is that elevated CO₂-temperature enhanced both anthocyanin biosynthesis and sugar accumulation in the berries, but, indeed, sugar levels were further increased, resulting in an anthocyanin-sugar accumulation decoupling (Fig. 3). Anthocyanin-sugar uncoupling phenomenon has been described under elevated temperature (Sadras and Moran, 2012) as an increase in TSS level at the onset of veraison, making the ripening phase shorter, and therefore, reducing the time window for anthocyanin biosynthesis, before reaching a certain level of TSS before harvest. Our results showed a different uncoupling behaviour, as veraison was triggered 9 days earlier on average (Table 1) and with lower TSS (Fig 1) under climate change conditions. Plants under CC conditions had a faster vegetative growth (time to reach 12 leaves per cluster; $p < 0.001$), and higher photosynthetic rates (Chapter 5), than those grown under CS conditions, which may have contributed to increase overall carbon assimilation. The moderate range of temperatures tested (24-28°C), rule out degradation as a main cause of anthocyanin decrease at harvest under CC conditions (Mori et al., 2007). In addition, for a fixed amount of time (2 weeks after veraison), anthocyanins concentration and *MYBA1* transcript levels were much higher under CC compared to CS conditions, which suggests an up-regulation of anthocyanin biosynthesis. Our results also indicate that the up-regulation of anthocyanin biosynthesis under elevated CO₂-temperature may not be

proportional to the increase in sugar inflow into the berries induced by elevated CO₂-temperature (Fig. 3).

Interestingly, when plants grown under CC conditions were exposed to UV-B radiation, the anthocyanin-sugar uncoupling was alleviated, and anthocyanin biosynthesis in response to sugar accumulation was partially restored as UV-B radiation exposure increased. This effect might be mediated by the MYBF1 transcription factor, with affinity for *CHS* promoter (Czemmel et al., 2009), which was strongly induced by UV-B (Fig. 4). Indeed, CHS is responsible for the synthesis of precursors of a broad number of flavonoid compounds, anthocyanins being among them. In Dai et al. (2014), anthocyanin precursors (i.e. phenylalanine) showed a clear tendency to depletion as anthocyanin biosynthesis was enhanced by increasing sugar concentration. Therefore an up-regulation of genes encoding for enzymes of the central flavonoid biosynthesis; such as CHS, responsible for the synthesis of precursors, may result in higher anthocyanin content (Li and Strid, 2005). Other important genes were up-regulated under UV-B radiation (Fig. 4). Notably, transcript levels of the gene encoding glutathione-S-transferase (GST), a protein involved in vacuolar accumulation of anthocyanins (Castellarin et al., 2007a), were higher in berries treated with UV-B, as well as the transcript levels of the anthocyanin accumulation master regulatory gene *MYBA1*, reported to trans-activate the *UFGT* promoter (Ageorges et al., 2006). The up-regulation of these two genes may have also contributed to higher anthocyanin content in UV-B treated plants at harvest.

Strategies involving viticulture practices to alter sugar inflow rate to the berries have been reported before (Martínez de Toda and Balda Manzanos, 2011). Reduced source-sink ratios may slow down considerably ripening rates (Martinez de Toda and Balda Manzanos, 2013; Martinez de Toda et al., 2013; Palliotti et al., 2013), and the timing of the onset of ripening has also been manipulated by means of water management (Sadras and Moran, 2012). Even so, producers concerns grow as grape sugar levels required to achieve a determined aroma or polyphenolic maturity continue to increase (Bock et al., 2013; Mira de Orduña,

2010). According to this work, moderate or high levels of UV-B radiation exposure may represent an exploitable advantage in vineyard management in response to a changing climate, through canopy manipulation, for instance.

5. CONCLUSION

Elevated CO₂-temperature hastened grape berry development, resulting in an earlier onset of veraison and higher sugar levels at maturity. Under such conditions anthocyanin–sugar accumulation was uncoupled, leading to a lower anthocyanin/sugar ratio at harvest. UV-B radiation contributed to enhance anthocyanin biosynthesis, and therefore alleviate anthocyanin/sugar accumulation uncoupling, that could be explained by the up-regulation of structural (*CHS*, *OMT2* and *GST*) and regulatory (*MYBA1* and *MYBF1*) genes. This study, which to our best knowledge is the first to address the combined effects of three factors linked to climate change on grapevine berry composition, reveals an anthocyanin biosynthesis limitation under elevated CO₂-temperature conditions, which can be reverted to a great extent by UV-B radiation exposure.

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Table 1. Elapsed days from the beginning of treatments (fruit set) to each sampling point (pea size, veraison, 2 weeks after veraison [+2W] and maturity) of berries under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: current situation (CS, CO₂ Amb - 24/14°C) and climate change (CC, 700 ppm CO₂ - 28/18°C). Data are means ± SE, n=10-12. Main effects of UV-B, $P_{(UV-B)}$; climate change, $P_{(CC)}$; and their interaction, $P_{(UV-B \times CC)}$.

	Current situation (CO ₂ Amb- 24/14°C)			Climate change (700ppm CO ₂ - 28/18°C)			Main factor		
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	$P_{(UV-B)}$	$P_{(CC)}$	$P_{(UV-B \times CC)}$
Pea size	20.0±0.0	20.0±0.0	20.0±0.0	20.0±0.0	20.0±0.0	20.0±0.0			
Veraison	48.1±1.7	47.8±1.8	52.1±3.1	39.6±2.5	40.9±2.4	40.0±1.4	0.007	< 0.001	0.005
Veraison+2W	62.1±1.7	61.8±1.8	66.1±3.1	53.6±2.5	54.9±2.4	54.0±1.4	0.007	< 0.001	0.005
Maturity	101.0±6.9	103.2±6.8	108.8±2.5	78.0±2.7	83.0±5.5	82.6±5.2	0.008	< 0.001	0.361

Table 2. FTIR spectroscopy must determinations (pH, total acidity, malic acid, assimilable nitrogen, α-amino nitrogen, ammonia nitrogen and potassium) of berries under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) two CO₂ and temperature conditions: current situation (CS, CO₂ Amb - 24/14°C) and climate change (CC, 700 ppm CO₂ - 28/18°C), at maturity. Data are means ± SE, n=4. Main effects of UV-B, $P_{(UV-B)}$; climate change, $P_{(CC)}$; and their interaction, $P_{(UV-B \times CC)}$.

	Current situation (CO ₂ Amb- 24/14°C)			Climate change (700 ppm CO ₂ - 28/18°C)			Main factor		
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	$P_{(UV-B)}$	$P_{(CC)}$	$P_{(UV-B \times CC)}$
pH	3.60±0.01	3.63±0.03	3.70±0.01	3.63±0.01	3.63±0.03	3.70±0.03	0.007	0.612	0.851
Total acidity (g L ⁻¹)	8.60±0.39	8.56±0.42	7.61±0.39	7.98±0.14	7.52±0.24	6.79±0.51	0.010	0.007	0.818
Malic acid (g L ⁻¹)	3.96±0.16	4.11±0.15	3.97±0.21	3.30±0.14	2.88±0.06	2.84±0.31	0.550	< 0.001	0.363
Potassium (mg L ⁻¹)	1985±25	2100±66	2239±63	2095±28	1977±50	2146±49	0.018	0.451	0.103
Assimilable nitrogen (mg L ⁻¹)	290.8±17.1	314.0±5.8	295.8±9.3	248.3±8.1	237.0±20.5	229.5±24.4	0.716	< 0.001	0.540
α-amino nitrogen (mg L ⁻¹)	335.8±12.7ab	356.8±7.4	335.5±8.2	298.0±7.4	290.5±21.2	283.0±22.7	0.654	0.001	0.659
Amonia nitrogen (mg L ⁻¹)	137.8±10.1	139.5±6.8	129.8±9.9	144.3±4.0	144.5±9.5	126.5±4.6	0.247	0.709	0.841

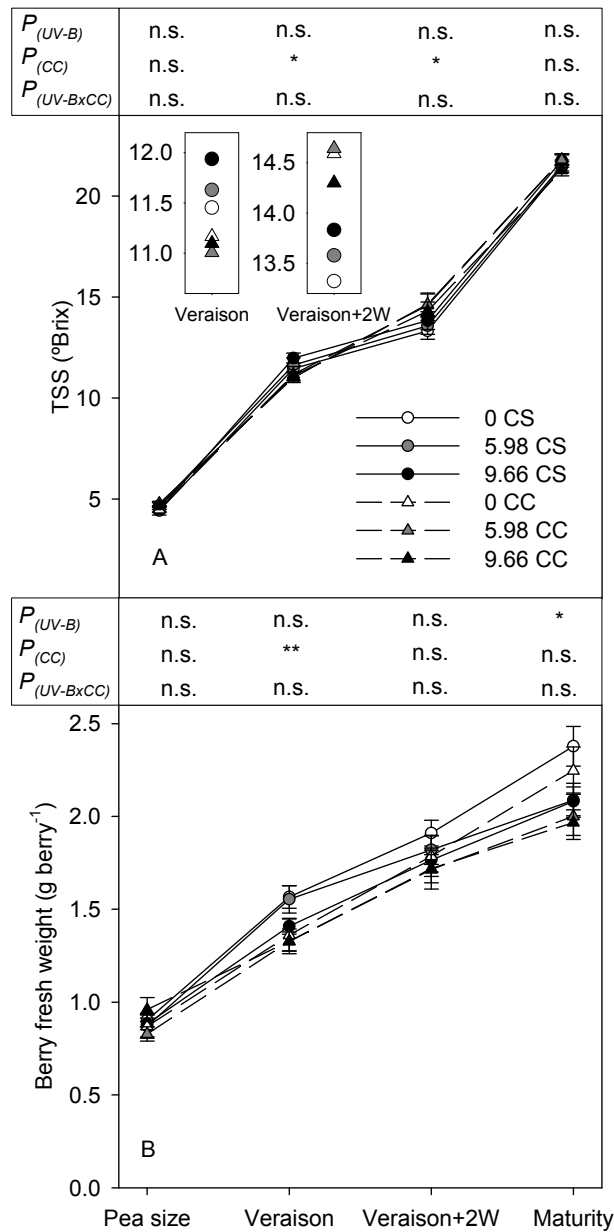


Figure 1. Total soluble solids (A) and berry fresh weight (B) evolution throughout the development of berries, under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻²d⁻¹) and two CO₂ and temperature conditions: current situation (CS, CO₂ Amb - 24/14°C) and climate change (CC, 700 ppm CO₂ - 28/18°C). Inset: Close up of TSS at veraison and the 2 weeks after the onset of veraison. Symbols are means ± SE, n=4-5. Main effects of UV-B, $P_{(UV-B)}$; climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$. **, $P < 0.01$; *, $P < 0.05$ and $P > 0.05$, n.s. not significant.

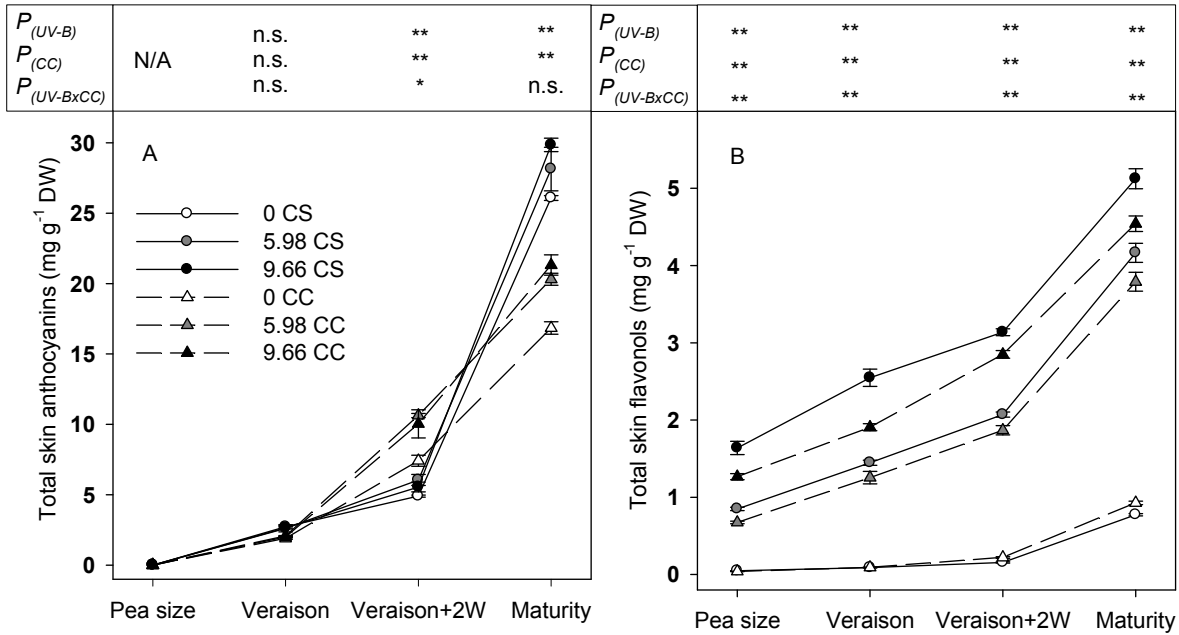


Figure 2. Total skin anthocyanins (A) and flavonols (B) evolution throughout the development of berries, under three doses of UV-B (0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$) and two CO_2 and temperature conditions: current situation (CS, CO_2 Amb - 24/14°C) and climate change (CC, 700 ppm CO_2 - 28/18°C). Symbols are means \pm SE, $n=4-5$. Main effects of UV-B, $P_{(UV-B)}$; climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$. **, $P < 0.01$; *, $P < 0.05$ and $P > 0.05$, n.s. not significant.

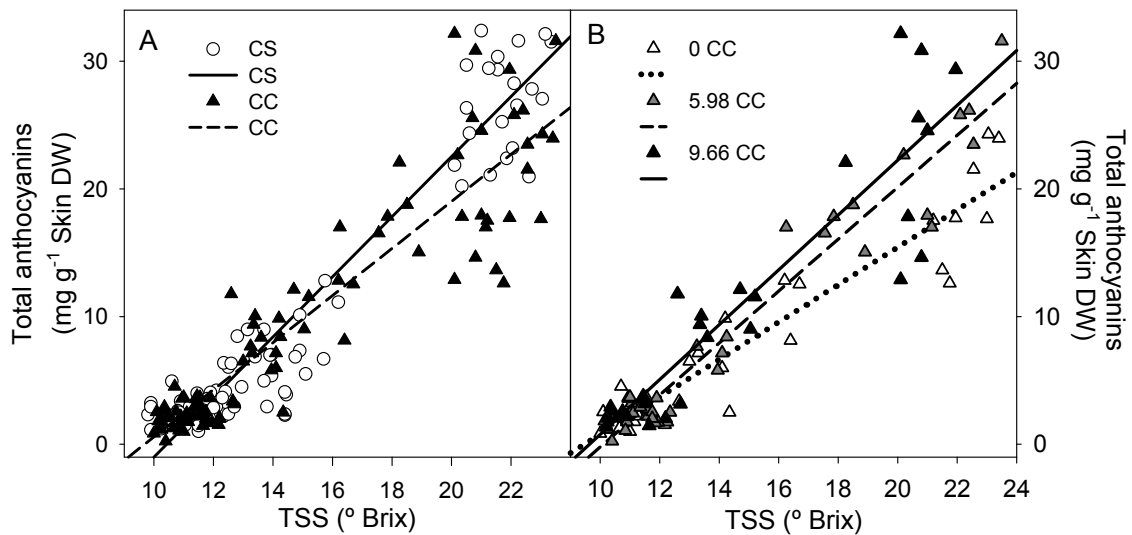


Figure 3. Effect of elevated CO_2 -temperature (CS, current situation, CO_2 Amb - 24/14°C and CC climate change, 700 ppm CO_2 - 28/18°C) on the relationship between the concentration of anthocyanins and total soluble solids (A); and effect of UV-B ($\text{kJ m}^{-2} \text{d}^{-1}$) on the relationship between the concentration of anthocyanins and total soluble solids under elevated CO_2 -temperature (B).

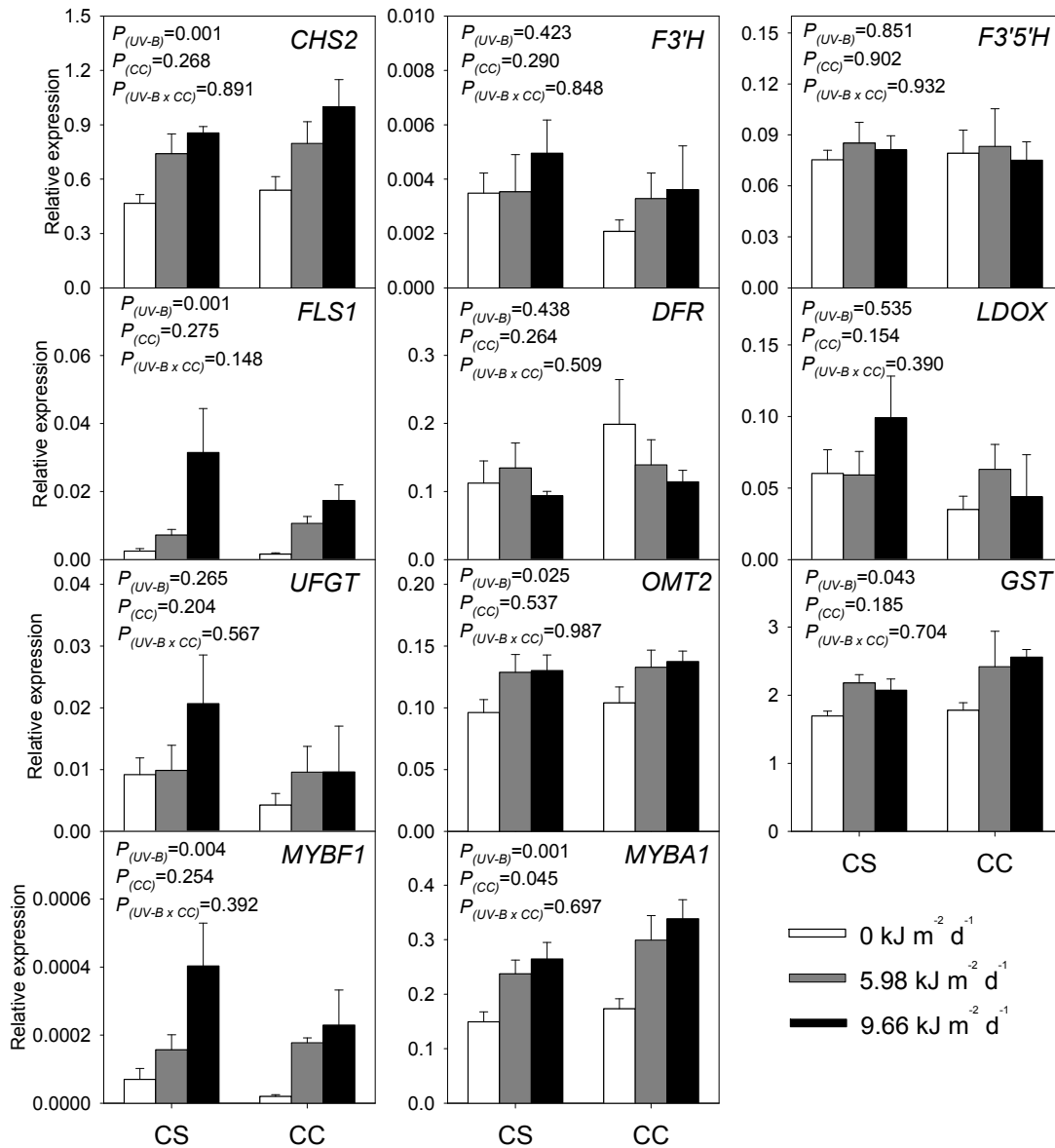


Figure 4. Transcript levels of structural and regulatory genes responsible for the synthesis and storage of phenolic compounds in berries one week after the onset of veraison, under three doses of UV-B (0, 5.98 and 9.66 kJ m⁻² d⁻¹) and two CO₂ and temperature conditions: Current situation (CS, CO₂ Amb - 24/14°C) and climate change (CC, 700 ppm CO₂ - 28/18°C). Symbols are means ± SE, n=4. Main effects of UV-B, $P_{(UV-B)}$; climate change, $P_{(CC)}$ and their interaction, $P_{(UV-B \times CC)}$.

Table S1. Accession ID, sequences and efficiency of primers used for real-time RT qPCR analyses

Gene name	GEN ID (GenBank Accession)	Source	Primers sequence	Primer efficiency
<i>CHS2</i>	AB066275	Jeong et al., 2004	F-.GAAGATGGGAATGGCTGCTG R-.AAGGCACAGGGACACAAAAG	90.9 %
<i>F3'H</i>	AB213603.1	Castellarin et al., 2006	F-.CAACAAGAGCTGGACGCAGT R-. AGCCGTTGATCTCACAGCTC	97.2 %
<i>F3'5'H</i>	AB213606	Jeong et al., 2006	F-. AAACCGCTCAGACCAAACC R-. ACTAAGCCACAGGAACTAA	88.4 %
<i>FLS1</i>	AB092591	Referred as FLS4; Fujita et al., 2006	F-. AAACCACCTACTTACAGAGC R-. ACC TAA CCC CAG TGA CAG AC	91.3 %
<i>DFR</i>	X75964	Jeong et al., 2004	F-.GGCCAAATCAAACCTACCAGA R-.GAAACCTGTAGATGGCAGGA	84.6 %
<i>LDOX</i>	X75966	Jeong et al., 2004	F-. TAGTCACCACTTCAAAAAGG R-.GAATGTGTTTGGGGTTTATC	95.6 %
<i>UFGT</i>	AF000372	Jeong et al., 2004	F-. GGGATGGTAATGGCTGTGG R-. ACATGGGTGGAGAGTGAGTT	83.0%
<i>OMT2</i>	HQ702997	Fournier-Level et al., 2011	F-. TGTCAGCTCAAGTCATAGGG R-. AGCTTCTAATAGAGGCGCCTGCAGAG	81.8 %
<i>MYBF1</i>	FJ948477	Czemmel et al., 2009	F-. GGAGGTTGAGGGTTGTG R-.AAGTTGGGGAAGAGCAGGAG	92.2 %
<i>MYBA1</i>	AB097923	Jeong et al., 2004	F-. TAGTCACCACTTCAAAAAGG R-.GAATGTGTTTGGGGTTTATC	95.8 %
<i>GAPDH</i>	CB973647	Reid et al., 2006	F-. TTCTCGTTGAGGGCTATTCCA R-. CCACAGACTTCATCGGTGACA	93.5 %

GENERAL DISCUSSION

In this work grapevine leaf physiology and berry ripening under a predicted scenario of climate change has been approached. The study integrates physiological aspects with metabolomic and transcriptional changes induced by the isolated or combined effects of the environmental conditions assayed. Results show that UV-B radiation triggered clear responses in grapevine leaf physiology and berry composition. These responses were modulated to a greater or lesser extent by other factors linked to climate change (water availability, atmospheric CO₂ levels and temperature).

Given the current distribution of the main viticultural regions, grapevines are exposed to high levels of solar radiation, including UV-B, combined with a seasonal dry and warm period, which coincides with the most actively growth period of the plants (Doupis et al., 2011). Growing concerns are building up around to the impact of changes in environmental conditions on grape berry quality (Hannah et al., 2013; IPCC, 2013; Webb et al., 2007; White et al., 2006).

Therefore, a better understanding of how climate change-related environmental factors target and alter grapevine physiological processes may constitute an advantage for the design of adaptive strategies to preserve high quality viticulture.

RESPONSE OF GRAPEVINE LEAF PHYSIOLOGY TO UV-B RADIATION IN COMBINATION WITH CLIMATE CHANGE FACTORS

UV-B acclimation process

The exposure of plants to moderate-high doses of UV-B has been reported to decrease chlorophyll levels, photosynthetic activity and plant biomass production, as well as increase phenolic compounds content and antioxidant enzyme activities (reviewed by Kakani et al., 2003). The results reviewed by Kakani et al. (2003) highlighted the hazard of the ozone layer depletion, as generally the doses of UV-B tested in those studies were above the realistic levels in the current environment. The doses tested in the present work (5.98 and 9.66 kJ m⁻²

d^{-1} of UV-B) are close to the highest doses that grapevine plants may face in the main viticultural regions under clear sky conditions. The dose of $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B induced a clear but transient photosynthesis down-regulation. This down-regulation was due to both stomatal/mesophyll conductance limitations and lower photosystem II activity. Results indicate that although these plants were not previously acclimated to UV-B, they were able to recover in a relatively short period of time (days to weeks), by means of the accumulation of UV-shielding compounds and the increased antioxidant enzyme activity. UV-B absorbing compounds, which are mainly flavonoids, are not only located in the vacuoles of epidermal cells, but also in more internal layers (mesophyll cells), and other subcellular domains (reviewed by Agati et al., 2013). B-ring di-substituted flavonoids, which were specifically exalted in berries, are found in the envelope membrane of chloroplasts (Agati et al., 2007), and together with antioxidant enzymes, constitute the major defence mechanism against the light-induced ROS accumulation in the chloroplasts (Triantaphylidès and Havaux, 2009). Once grapevine plants were acclimated to UV-B and carbon assimilation rates were re-established, the levels of lipid peroxidation were generally lower than those observed in plants never exposed to UV-B. The elicitation of the antioxidant response (antioxidant enzymes and UV-B absorbing compounds) observed in this study, may correspond to the “eustress” conception (a mild stress not causing permanent damages but rather promoting an adaptive response) reported by Hideg et al. (2013). Such response may result in a cross-tolerance to other stressors (Jansen et al., 2012). Our results suggest that, as long as UV-B doses are within the range tested ($0\text{-}9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$), UV-B radiation does not represent a real stressor for grapevine plants.

Modulation of UV-B response by other environmental factors

The combined action of UV-B radiation and other environmental factors can modify the plant response patterns to UV-B. Previous studies show that UV-B radiation can interact with water deficit to induce protective mechanisms, i.e. increased levels of UV-B absorbing compounds (mainly flavonoids), antioxidant enzymes (SOD, CAT and APX), osmolyte

accumulation (i.e. proline), increased leaf cuticle thickness or reduced stomatal conductance, which may substantially decrease UV-B and/or drought sensitivity when both factors are applied in combination (Alexieva et al., 2001; Drilias et al., 1997; Hofmann et al., 2003; Lu et al., 2007; Poulson et al., 2006). Both UV-B and water deficit cause the generation of ROS, nitrogen oxide, and induce the synthesis of abscisic acid, ethylene, jasmonic and salicylic acid. These molecules, controlling plant reactions to single water deficit and UV-B, may also be involved in cross-talk under their combined actions (Bandurska et al., 2013 and references therein; Kovács et al., 2014). Nevertheless, few interactions between UV-B and water deficit were observed in the photosynthetic performance of grapevine in the present work. In general, the adaptive response of grapevine leaves to UV-B, through the accumulation of UV-B absorbing compounds, was not altered by water deficit. Results agree with Nogués and Baker (2000), Sullivan and Teramura (1990), Duan et al. (2008) and Bernal et al. (2013), who found few significant interactions between UV-B and water stress on plant growth and photosynthetic activity.

UV-B and CO₂ interaction also shows some degree of mitigating effect, but there is no a general pattern (reviewed by Kakani et al., 2003). The suggested mechanisms underlying the interaction between UV-B and elevated temperature are discussed based on the great temperature dependence of enzymatic reactions, such as those involved in flavonoid biosynthesis (Tevini et al., 1991). Therefore, the interactions between UV-B and climate change factors (water deficit, elevated CO₂ and elevated temperature) may enhance UV-screening compounds accumulation, improving UV-B acclimation (Tevini et al., 1991). In our experiments, elevated CO₂-temperature conditions induced early leaf senescence. This was observed visually, but also suggested by the strongest decrease in CO₂ assimilation, chlorophyll fluorescence parameters (Fv/Fm) and increases in lipid peroxidation, a clear senescence indicator (Dhindsa et al., 1981). Interacting effects between UV-B and climate change conditions were observed for lipid peroxidation, which was lower in the plants exposed to UV-B under climate change conditions. Results suggest that UV-B contributed to

the alleviation of senescence induced by elevated CO₂-temperature. This may be explained by the higher flavonoid content (i.e. MEUVAC) and its higher antioxidant capacity (Agati et al., 2013).

EFFECTS OF ENVIRONMENTAL FACTORS ON PHENOLOGY AND RIPENING

Water status, UV-B radiation and temperature may hasten, delay or enhance ripening. Hormones seem to play a central role, as their concentrations change prior to and during ripening and in response to several environmental cues (Kuhn et al., 2013). Hormones, such as salicylic acid and auxin, inhibit ripening (Davies et al., 1997; Kraeva et al., 1998), while abscisic acid and ethylene may promote it (Coombe and Hale, 1973; Chervin et al., 2004). In the present study, plants grown under UV-B radiation, took up to 11 days longer to reach grape berry ripeness (ca. 22°Brix). A delay in plant developmental stages (flowering and ripening) induced by UV-B has been reported for other plant species such as *Arabidopsis thaliana* and wheat (Comont et al., 2012; Li et al., 1998). Schultz (2000) also reported that grapevine phenology may be affected by UV-B radiation.

Regarding water deficit, veraison was triggered earlier in berries under drought conditions, showing lower total soluble solids (TSS) content at veraison, compared with well irrigated plants. Under water deficit, abscisic acid biosynthesis is promoted (Stoll et al., 2000), which may have had mediated in the early veraison induction. Even though ripening process started sooner, sugar accumulation was clearly slower in plants under water deficit, thus delaying ripening up to 13 days. In chapter 3, it is discussed how lower photosynthetic rates under UV-B and water deficit may have contributed to expand the time required to reach maturity.

Carbon fixation and berry ripening were two processes clearly correlated (Chapter 5). When elevated CO₂-temperature was applied, ripening rates were significantly hastened. In that experiment leaf to fruit ratio, which is a determinant factor affecting ripening rate (Poni et al.,

2013), was maintained constant. Under such conditions, higher carbon fixation rates under elevated CO₂-temperature may result in a higher photoassimilate inflow to the berries (Greer and Weedon, 2013; Moutinho-Pereira et al., 2009), thus explaining to a greater extent the shortened ripening period. This phenomenon has been widely observed in vineyards, where grapes are harvested 2 to 3 weeks earlier, on average, than usual (Stock et al., 2005). Even though it is hard to uncouple the factors leading to these changes in phenology, this is widely attributed to rise in global air temperature between 1 and 2°C, compared with pre-industrialization references (Mira de Orduña, 2010). However, this increase in temperature has been concomitant to increases in atmospheric CO₂ levels (Graven et al., 2013). The extent to which each factor is controlling ripening was addressed in Salazar-Parra (2011). In that work, mild effects were attributed to elevated temperature or elevated CO₂ solely. However, in combination they reduced significantly the time to reach maturity (same TSS concentration). Interestingly, when UV-B radiation was applied simultaneously with elevated CO₂ and temperature, the hastening effect of climate change conditions on grape berry ripening was attenuated.

EFFECTS OF ENVIRONMENTAL FACTORS ON GRAPE COMPOSITION

Influence of environmental factors on grape acidity

Organic acids accumulate in the berry flesh during berry formation (before veraison) and they decline during berry ripening, due to both growth dilution and breakdown (Matthews and Anderson, 1989). Even though, final organic acid levels are mostly ruled by temperature during ripening, water deficit may also occasionally contribute to the breakdown of organic acids (malic acid mostly) (Lakso and Kliewer, 1975; Shellie, 2006). This was observed in the present work, where acidity was reduced by water deficit and by elevated CO₂-temperature. UV-B radiation also had a negative effect on the acidity of the must, but it did not affect malic acid concentration, which is regarded as the most fragile organic acid (Conde et al., 2007). Interestingly, potassium levels were higher in UV-B treated berries (Chapter 6). Potassium

level is a key factor to understand the actual acidity of musts, as must pH depends basically on 3 factors: the total amount of acids, the ratio of malic acid to tartaric acid, and the concentration of potassium (Conde et al., 2007). Potassium is the major cation in the grape must, and it binds organic acids, leading to the tartrate precipitation during the fermentation and ageing of wine (Conde et al., 2007). In addition, high potassium levels in the berry may decrease the rate of malate degradation by impairing malate transport from the storage pools in the vacuole to the mitochondria, where breakdown takes place (Etienne et al., 2013). As malate accumulates mainly in grape skins, the higher relative skin mass in UV-B treated berries, associated with a lower berry size, may have led to higher potassium levels, and, therefore, higher pH values in the must.

Anthocyanin and flavonol biosynthesis regulation by UV-B radiation

Flavonol elicitation by UV-B, through the up-regulation of FLS1 and its regulating transcription factor MYBF1, was one of the main responses observed in all the experiments performed in the present work. Berry skin flavonol concentration was linearly correlated to the accumulated dose of UV-B, and the strongest inductions were achieved after the onset of veraison. As reported by Liu et al. (2014) in *V. vinifera* cv. Sauvignon blanc berries, FLS1 is responsible for most of UV-B induced flavonol biosynthesis after veraison. The regulatory mechanism from UV-B interception by UVR8 to *MYBF1* (*AtMYB12* homologue) up-regulation have been largely described in *Arabidopsis*. *HY5* is up-regulated by the UVR8-COP1 complex (Tilbrook et al., 2013), and subsequently, *HY5* transcription factor targets specifically *AtMYB12* promoter region (Stracke et al., 2010). *V. vinifera* homologue (*VvMYBF1*) is most likely performing the same role in grape berries, but in addition, it shows a relatively high affinity for the promoter of some central flavonoid biosynthesis enzyme genes (*CHS*, *CHI* and *LDOX*) (Czemmel et al., 2009). Several authors have proposed a developmental regulation of grape berries flavonol biosynthesis, in which flavonols are mainly accumulated during the last part of ripening (Czemmel et al., 2012; Downey et al., 2003; Fujita et al., 2006). In the present work, berries not exposed to UV-B radiation (0 kJ m^{-2}

d⁻¹) showed that pattern, however, taking into account the great induction of flavonol biosynthesis by UV-B, results suggest that developmental regulation is clearly overridden by UV-B regulation.

Anthocyanin concentration was also higher in UV-B treated berries. Nevertheless, the effect of UV-B was not as marked as for flavonols. Anthocyanin biosynthesis regulation is similar to that described for flavonols, through the specific transcription factor MYBA1 (Kobayashi et al., 2002; Walker et al., 2006). However, while MYBF1 regulates both flavonol-specific (*FLS*) and flavonoid-generic (e.g. *chalcone synthase*, *CHS*) genes (Czemmel et al., 2009); MYBA1 putatively only regulates the anthocyanin-specific gene *UDP glucose flavonoid glucosyl transferase (UGFT)* (Walker et al., 2007). *MYBA1* expression levels were higher in UV-B treated plants (only significant differences in Chapter 6). In addition, *UGFT* and *glutathione S-transferase (GST) genes*, encoding for enzymes in charge of the last stage of anthocyanin biosynthesis and their storage respectively (Marrs et al., 1995), also displayed higher expression levels under UV-B. These changes in transcript levels induced by UV-B may contribute to explain the observed increase in anthocyanins. Furthermore, the up-regulation of genes encoding for enzymes in the central flavonoid pathway, such as *CHS*, may have also enhanced anthocyanin biosynthesis (Li and Strid, 2005), as a side effect of the higher flavonoid precursor availability (i.e. chalcone).

Effect of UV-B and climate change conditions on anthocyanin-sugar accumulation

Phenolic compounds, in general, and anthocyanins, in particular, are elicited by an increase in the tissue sugar levels (Dai et al., 2014; Ferri et al., 2011). Elevated CO₂-temperature conditions increased skin anthocyanin concentration two weeks after the onset of veraison, concomitantly to an increase in TSS levels. Contrarily, when comparing berries at the same sugar level (maturity, ca. 22°Brix), berries grown under elevated CO₂-temperature had lower skin anthocyanin concentration. Due to the great preponderance of sugar levels on anthocyanin biosynthesis regulation, sugar accumulation rates were responsible for the differences in anthocyanin concentration two weeks after the onset of veraison; whereas at

maturity, where all the berries were sampled at 22°Brix, the real effect of elevated CO₂-temperature on anthocyanin accumulation was revealed. Extreme temperatures (35°C) have been pointed out to enhance anthocyanin degradation and inhibit anthocyanin transcriptional regulation (Mori et al., 2007). However, this seems unlikely under a 4 degree increase (24 to 28°C), as in the present study. In our experiment, although elevated CO₂-temperature enhanced both, sugar and anthocyanin levels, sugar accumulation in the berries was further enhanced than anthocyanins accumulation, thus, leading to a decoupling between anthocyanin-sugar accumulation. Moreover, UV-B always resulted in higher anthocyanins concentration, especially under climate change conditions, which alleviated the observed anthocyanin and sugar decoupling induced by elevated CO₂-temperature.

Influence of UV-B and water deficit on flavonol and anthocyanin profiles

Changes in flavonol concentrations were always associated to changes in their spectrum. In fact, changes in flavonol profile were better correlated to UV-B exposures than the skin total flavonol concentration itself (Chapter 2). UV-B increased the relative abundance of mono- and disubstituted flavonols, (4' and 3'4' hydroxylated, respectively) and decreased the relative abundance of trisubstituted flavonols (3'4'5' hydroxylated). The changes in the expression levels of *F3'5'H* and/or the imbalance between *F3'H* and *F3'5'H* enzymes have been proposed to explain flavonoid profiles in previous researches (Bogs et al., 2006; Castellarin et al., 2007a; Castellarin et al., 2007b; Falginella et al., 2010). Nevertheless, given the exclusiveness of this effect for flavonols but not for anthocyanins, it was hypothesised that the up-regulation of the gene encoding for *FLS* rather than the balance between *F3'H* and *F3'5'H*, was the factor leading to qualitative changes in flavonol profile in response to UV-B. In Chapter 4, it was demonstrated that the effect of *F3'5'H/F3'H* balance was overridden by *FLS* up-regulation by UV-B. Water deficit increased the proportion of 3'4'5' hydroxylated flavonols and 3'5' hydroxylated anthocyanins with respect to well watered conditions, which was explained by the up-regulation of *F3'5'H* under water deficit, as reported by Castellarin et al. (2007a). Significant interactions between UV-B radiation and

water deficit were observed in all the flavonol diversification parameters (e.g. 3'4'5'substituted/3'4'substituted flavonols). This phenomenon was explained by independent up-regulation of flavonol synthase (*FLS1*) and flavonoid 3'5' hydroxylase (*F3'5'H*) by UV-B radiation and water deficit, respectively. F3'5'H enzyme hydroxylates flavonol precursors, diversifying their profile; while FLS enzyme takes those precursors to synthesize flavonols, which are no longer susceptible to be further hydroxylated. In other words, FLS up-regulation reduces the chances of flavonol precursors of being hydroxylated in 5' position, before being converted into flavonols. Thus, while competing for dihydroflavonol substrates, FLS and F3'5'H enzymes drove the metabolic flux to different directions. This interaction resulted in very specific flavonol profile signature for each condition simulated.

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CONCLUSIONS

1. The high, but realistic, doses of UV-B radiation applied in this study did not induce any permanent deleterious effect on the photosynthetic performance of grapevines. Only an early transient down-regulation of photosynthesis was observed, followed by an acclimation mediated by flavonoids and antioxidant enzymes accumulation.
2. In general, the acclimation process of grapevine leaves to a long-term exposure to UV-B was not altered by water deficit. No remarkable interacting mechanisms between these two factors were observed in the leaf physiology.
3. UV-B acclimated grapevines gained certain cross-tolerance to elevated CO₂ and temperature, through antioxidant response activation and flavonoid accumulation in leaves, thus avoiding senescence-related lipid peroxidation.
4. Grapevine phenology events and grape ripening were greatly influenced by environmental conditions, not only due to their direct effects on the grape berries, but also due to the impact on leaf carbon balance.
5. The combined exposure to UV-B and water deficit led to additive effects slowing-down grape ripening. By contrast, the hastening effect of elevated CO₂ and temperature on grape berry ripening was attenuated by UV-B radiation.
6. UV-B radiation and water deficit increased total anthocyanin concentration and decreased acidity, which may be partially attributed to a higher grape berry skin mass.
7. UV-B radiation, alone or combined with the other environmental factors, was able to up-regulate the expression of both regulatory and structural genes involved in the anthocyanin and flavonol biosynthesis, resulting in a higher concentration of these compounds in the skins.
8. The imbalance in the up-regulation of *FLS1*, *F3'H* and *F3'5'H* genes induced by UV-B changed the flavonol hydroxylation profile, linearly correlated to UV-B exposure.
9. Significant interactions between UV-B and water deficit were observed in the flavonol hydroxylation profile, since these factors up-regulated genes encoding different

enzymes that compete for the same substrates. This mechanism of flavonol profile diversification has not been reported before.

10. Elevated CO₂ and temperature enhanced anthocyanin biosynthesis and sugar accumulation. However the rate of sugar accumulation was greater than that of anthocyanins, which derived into a lower grape skin anthocyanin concentration at the same total soluble solids.
11. The decoupling in anthocyanin and sugar accumulation rates was alleviated to some extent by UV-B radiation, by means of the up-regulation of structural and regulatory genes of anthocyanin biosynthesis.
12. The results show a great influence of UV-B radiation on grapevine leaf physiology and berry composition. These responses were modulated, to a greater or lesser extent, by other factors linked to climate change (water availability, atmospheric CO₂ levels and temperature).
13. This study has contributed to a better understanding of how climate change-related environmental factors target and alter grapevine physiological processes and may help to optimize adaptive strategies to preserve high quality viticulture.

