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Fruit-localized photoreceptors increase phenolic compounds in berry skins of field-grown *Vitis vinifera* L. cv. Malbec

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ABSTRACT

Sunlight exposure has multiple effect on fruits, as it affects the light climate perceived by fruit photoreceptors and fruit tissue temperature. In grapes (*Vitis vinifera* L.), light exposure can have a strong effect on fruit quality and commercial value; however, the mechanisms of light action are not well understood. The role of fruit-localized photoreceptors in the control of berry quality traits was evaluated under field conditions in a commercial vineyard in Mendoza (Argentina). Characterization of the diurnal dynamics of the fruit light environment in a vertical trellis system indicated that clusters were shaded by leaves during most of the photoperiod. Supplementation of the fruit light environment from 20 days before veraison until technological harvest showed that red (R, 660 nm) and blue (B, 470 nm) light strongly increased total phenolic compound levels at harvest in the berry skins without affecting sugar content, acidity or berry size. Far-red (FR, 730 nm) and green (G, 560 nm) light supplementation had relatively small effects. The stimulation of berry phytochromes and cryptochromes favored accumulation of flavonoid and non-flavonoid compounds, including anthocyanins, flavonols, flavanols, phenolic acids and stilbenes. These results demonstrate that the chemical composition of grape berries is modulated by the light quality received by the clusters under field conditions, and that fruit photoreceptors are not saturated even in areas of high insolation and under management systems that are considered to result in a relatively high exposure of fruits to solar radiation. Therefore, manipulation of the light environment or the light sensitivity of fruits could have significant effects on critical grape quality traits.

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1. Introduction

Fruits are important for seed dispersal and represent a key component of the diet of many animals, including humans. Fleshy fruits are particularly rich in sugars, acids, pigments, minerals and vitamins. A series of coordinated changes in color, texture, flavor, aroma, and chemical characteristics takes place during ripening, rendering the fruits more attractive and nutritionally valuable (Giovannoni, 2004).

Grape berries are fleshy fruits and represent an important source of phytonutrients. Grape berries are particularly rich in antioxidants like phenolic compounds, including flavonoids such as anthocyanins, flavonols (quercetin, kaempferol, etc.), and flavanols (catechins, epicatechins and tannins), as well as non-flavo-

Abbreviations: d, day/s; DAF, days after flowering; h, hour; DW, dry weight; FW, fresh weight; LEDs, light emitting diodes; LMWPC, low molecular weight phenolic compounds; PAR, photosynthetic active radiation; UV-A, UV-A radiation; UV-B, UV-B radiation.

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noids, such as stilbenes, hydroxycinnamic and hydroxybenzoic acids and their derivatives. These compounds are of particular interest since they define organoleptic, nutritional and nutraceutical characteristics of grape berries. Adequate concentrations of phenols, sugars, acids and volatile compounds are desirable attributes in berries, both for fresh consumption and for winemaking (Downey et al., 2006). Anthocyanins have a high antioxidant capacity and, as components of the human diet, they have strong health-promoting effects offering protection against cancer and various age-related degenerative diseases (Martin et al., 2013). Therefore, a better understanding of the mechanisms controlling the accumulation of phenolic compounds could be useful to optimize phytonutrient content of fruits, with potential benefits to reduce the incidence of chronic diseases, and improve the organoleptic characteristics of wines.

Grape berry ripening and, consequently, fruit growth and composition are affected by many environmental factors. The best studied of these factors are light, water status, temperature and pathogens. In general, moderate water deficits, ultraviolet-B (UV-B, 280–315 nm) radiation, and low temperatures positively affect ripening by increasing the content of soluble solids and anthocyanins; while high temperature, pathogens and shade have negative effects on berry quality (Kuhn et al., 2014).

The light environment of grape berries can be influenced by site characteristics, season and the cultural practices that directly affect light penetration through the leaf canopy (i.e. training and trellis system, row orientation, plant density, pruning, shoot thinning and positioning, leaf removal, etc.) (Matus et al., 2009; Smart, 1985, 1988). There are many reports on the effect of light on berry development and metabolite composition. Most of the experimental approaches involved the application of shade treatments, either to the whole plant (Kliewer, 1977; Kliewer and Antcliff, 1970; Smart et al., 1988) or only to the clusters (Cortell and Kennedy, 2006; Dokoozlian and Kliewer, 1996; Downey et al., 2004; Jeong et al., 2004; Koyama and Goto-Yamamoto, 2008; Morrison and Noble, 1990; Niu et al., 2013; Ristic et al., 2007). In other studies, the effect of light on berry development and composition was evaluated by sampling berries from different canopy positions – i.e. shade or sun-exposed berries – (Bergqvist et al., 2001; Crippen and Morrison, 1986a,b; Haselgrove et al., 2000; Kliewer and Lider, 1968; Price et al., 1995; Spayd et al., 2002; Tarara et al., 2008) and also by using different levels of defoliation in the fruit zone (Hunter et al., 1991, 1995; Kliewer and Antcliff, 1970; Matus et al., 2009). All of these studies concluded that sunlight-exposed fruits have (in general) higher levels of total soluble solids, anthocyanins and phenolics, and lower values of titrable acidity, malate, juice pH and berry weight, as compared to shaded fruits. Excessively shaded fruits may even show a delayed ripening and herbaceous aroma, and they may also be affected by fungal diseases (Smart et al., 1988). Additionally, it has been reported that the expression of genes of the flavonoid pathway and various transcription factors involved in its regulation (i.e. *MYBA1*) were up-regulated by visible light and UV radiation (Azuma et al., 2012; Downey et al., 2004; Jeong et al., 2004; Koyama and Goto-Yamamoto, 2008; Koyama et al., 2012; Matus et al., 2009; Zhang et al., 2012). Summarizing, although many studies have characterized the effect of total solar radiation on berry quality traits, the effect

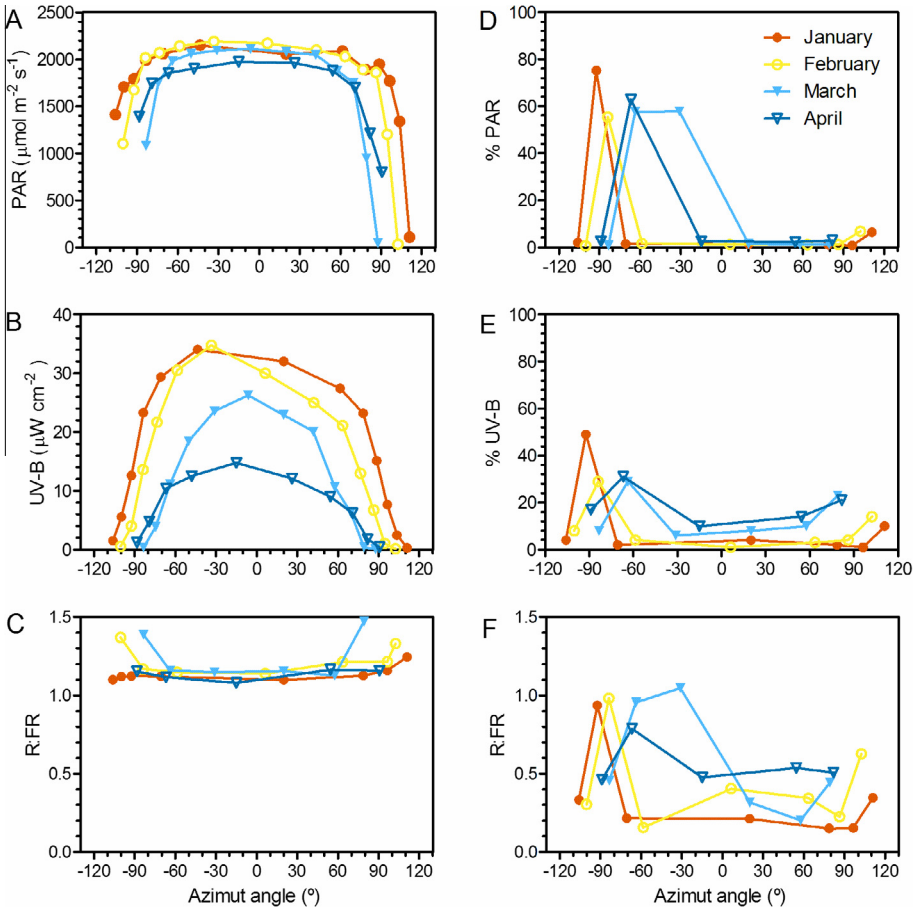

Fig. 1. Cluster zone light environment. Daily course of PAR (A and D), UV-B radiation (B and E) and R:FR ratio (C and F) recorded over the grapevine canopy (left panels) and in the cluster zone (right panels). PAR and UV-B in the cluster zone are expressed as a % of the incident PAR and UV-B, respectively. Solar noon is at 0° azimuth. Date are means ± SE of five measurements.

of specific wavelengths and photoreceptors on berry composition has not been fully elucidated.

Plants have evolved a sophisticated photosensory system which allows them to monitor the irradiance (light quantity), spectral composition (light quality), direction and timing of the incoming solar radiation. At least four groups of photoreceptors are involved in the perception of these light signals. The R-(660 nm) and FR-(730 nm) absorbing phytochromes, the ultraviolet-A (UV-A)/blue (B)-(315–500 nm) absorbing cryptochromes and phototropins, and the UV-B absorbing UVR8 photoreceptor (Ballaré et al., 2012; Casal, 2013). Leaf-shading involves the wavelength-dependent attenuation of the solar spectrum. When sunlight reaches the leaves, leaf pigments strongly absorb UV radiation and photosynthetically-active radiation (PAR, 400–700 nm), particularly the R and B (450 nm) wavelengths, whereas FR and green (G; 550 nm) photons are either transmitted or reflected. The R:FR ratio (660 ± 10 nm:730 ± 10 nm) can be used by the plant to detect, via phytochrome, direct sunlight exposure (R:FR = 1.1) or leaf-shading (R:FR < 1.1) (Ballaré, 2014; Casal, 2013). Attenuation of B light, perceived by cryptochromes, is also an indication of leaf shading and modulates important adaptive responses in plants, such as stem elongation and leaf orientation (Keller et al., 2011; Keuskamp et al., 2011; Sellaro et al., 2010).

Extensive literature exists on how plant photoreceptors are involved in many biological processes throughout the plant's life cycle, from germination to flowering (Chen et al., 2004). However, the role of photoreceptors in fruit physiology has received comparatively little attention, although phytochromes and cryptochromes have been reported to be involved in fruit pigmentation in tomato and apple fruits (Azari et al., 2009; Li et al., 2013a; Toledo-Ortiz et al., 2010). In grapes, previous studies have demonstrated the effects of UV radiation on fruit characteristics (Berli et al., 2008, 2011; Carbonell-Bejerano et al., 2014; Gil et al., 2013), but the effect of longer wavelengths is not well documented. The aim of this study was to investigate the effects of specific wavelengths, within the visible and FR spectrum, perceived by fruit-localized photoreceptors, on the phenolic composition of field-grown grape berries.

2. Results

2.1. Clusters develop in a shaded environment

The diurnal patterns of the light environment within the cluster zone of a commercial vineyard (vertical trellis system in north–south orientated rows) located in the Mendoza region (Western

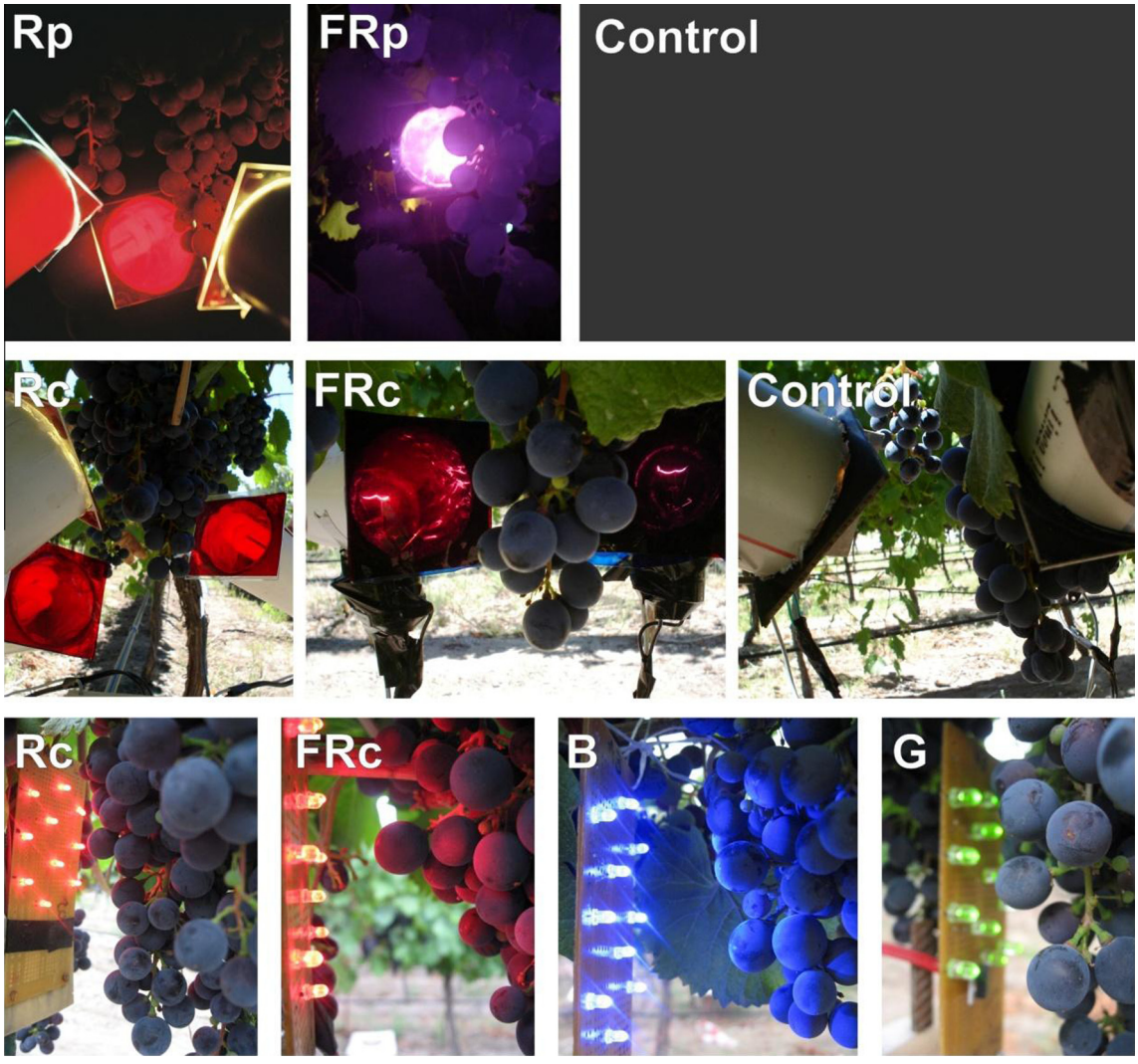

Fig. 2. Three types of light supplementation treatments carried out in the field since 20 days before veraison (52 ± 5 DAF) until harvest (133 DAF). I – End of day (EOD) Red and Far Red light pulses: Rp and FRp (upper panels), II – Continuous Red and Far-Red light (Rc and FRc) during the natural photoperiod which were provided by lamps and filters or by LEDs (middle and left bottom panels), III – Continuous Blue (B) and Green (G) light during the natural photoperiod (right bottom panels). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Argentina), was characterized during the berry ripening period. As expected, the fruit light environment was dramatically altered in quantity and also in quality as compared to full sunlight (Fig. 1, Fig. S1). Clusters were exposed to virtually unfiltered solar radiation during the morning hours (60% of PAR, 40% UV-B and R:FR = 1), but they were heavily shaded during the rest of the photoperiod (Fig. 1D–F). PAR and UV-B radiation within the cluster zone were reduced by 90% and 80%, respectively, compared to ambient sunlight levels during most of the photoperiod (Fig. 1A–E). The R:FR ratio was very low early in the season (January), but values tended to increase toward the end of the summer (March), following the onset of leaf senescence (Fig. 1E). These data are generally consistent with previous reports showing that clusters receive low PAR and R:FR levels at solar noon in a vertical trellis system with east–west orientated rows in the northern hemisphere (Dokoozlian and Kliewer, 1995a,b).

2.2. R light increased the content of anthocyanin and total phenolic compounds in berry skins

We used three local light supplementation treatments (Fig. 2) to stimulate fruit photoreceptors under field conditions throughout the berry ripening period (from 20 days before veraison until harvest, 50 DAF to 133 DAF). As a first approach to study the effects of phytochrome manipulation on berry quality, end-of-day (EOD) pulses of R and FR radiation (Rp and FRp) were used (Fig. 2, upper panels). It was found that the Rp treatment increased the contents of anthocyanin and total phenolic compounds in berry skins without affecting sugar content, acidity, or berry size at harvest in the three growing seasons: 2008, 2009 and 2010 (Fig. 3). In contrast, the FRp treatment showed no significant effects on the content of phenolic compounds, and a decrease in anthocyanin content in one season (2008) (Fig. 3A). Complementary evaluation of anthocyanin profiles of skin grapes at harvest confirmed that the Rp treatment increased anthocyanin accumulation but without affecting the relative abundance of each group of anthocyanins (malvidins, delphinidins, cyanidins, petunidins and peonidins) (Fig. 4; Supplementary Table S1).

Previous studies using seedlings of other plant species demonstrated that daytime manipulations of the light environment (i.e. supplementation treatments that are maintained during the whole photoperiod) often have more pronounced effects on plant photomorphogenesis than EOD pulses alone (Morgan and Smith, 1978). Because it was found that the fruit light environment is shaded by leaves during most of the photoperiod (Fig. 1), the effects of daytime manipulation of local light conditions on fruit traits was studied. In three different growing seasons, it was found that continuous supplementation with R light during the photoperiod (Rc) increased the contents of anthocyanins and total phenolic compounds in berry skins without affecting the sugar content of the fruits, titrable acidity or berry size at harvest (Fig. 5). In contrast, continuous supplementation with FR (FRc) failed to affect berry skin phenolics, with the exception of a small decline in anthocyanin content in one of the seasons (Fig. 5). HPLC-DAD analysis demonstrated that Rc had a strong effect in increasing anthocyanin content, with FRc having a weaker but significant effect (Fig. 6A).

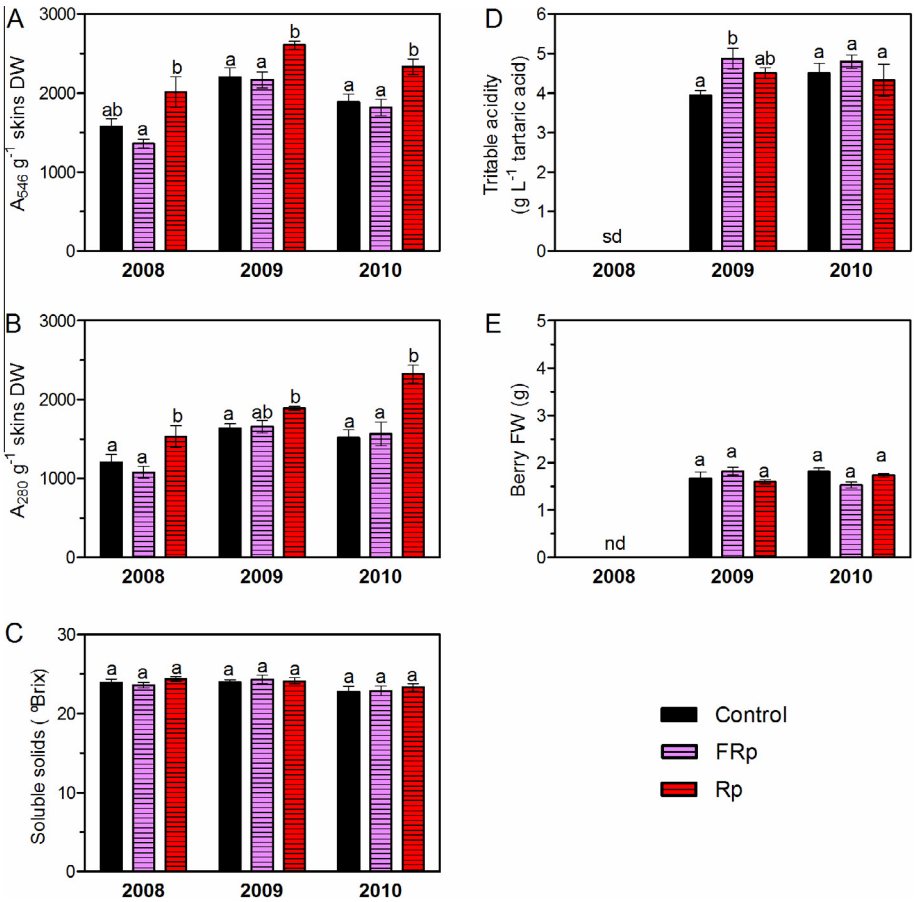

Fig. 3. EOD Rp and FRp experiment. Concentration of (A) anthocyanins ($A_{546\ nm}$ g^{-1} skin DW), (B) total phenolic compounds ($A_{280\ nm}$ g^{-1} skin DW), (C) total soluble solids (°Brix), (D) titrable acidity (g L^{-1} tartaric acid) and (E) berry FW (g) of field-grown grapes at harvest (133 DAF) supplemented with EOD Rp and FRp treatments. Data are means ± SE of eight biological replicates (2008, 2009 and 2010 growing seasons). Different letters indicate significant differences between treatments as calculated by Tukey HSD statistical analysis ($P < 0.05$). *Note:* nd = not available data.

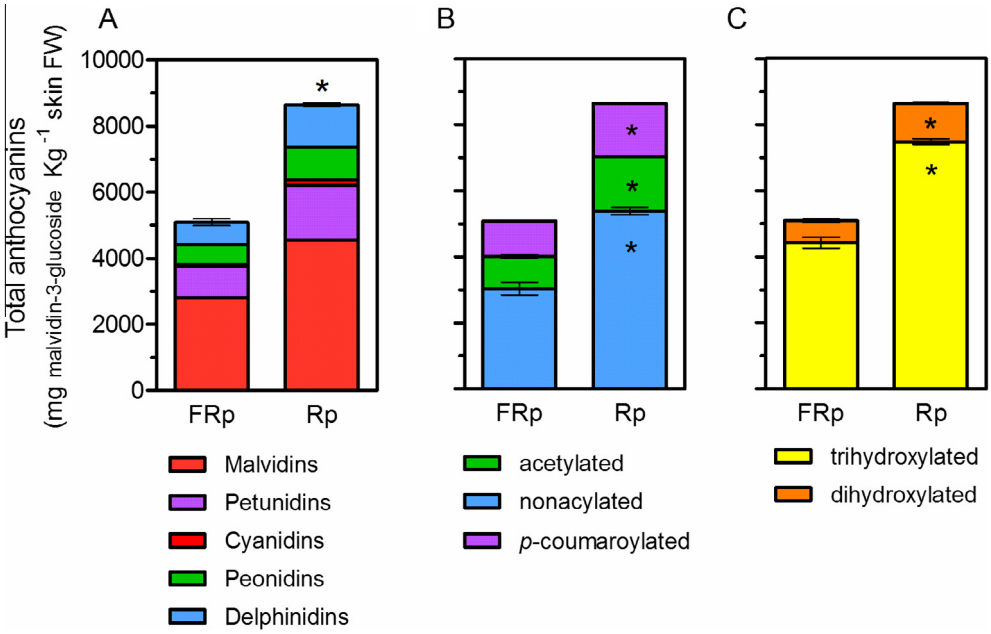

Fig. 4. Concentration of total anthocyanin glucosides (mg malvidin-3-glucoside kg^{-1} skin FW) of field-grown grapes at harvest (133 DAF, 2010 growing season) supplemented with Rp and FRp. Stacked bars show (A) malvidins, peonidins, petunidins, cyanidins and delphinidins, (B) non-acylated, acetylated and *p*-coumaroylated, and (C) tri and dihydroxylated anthocyanin glucosides concentration. Data are means ± SE of three biological replicates. Asterisks indicate significant differences between treatments as calculated by *t*-test analysis ($P < 0.05$).

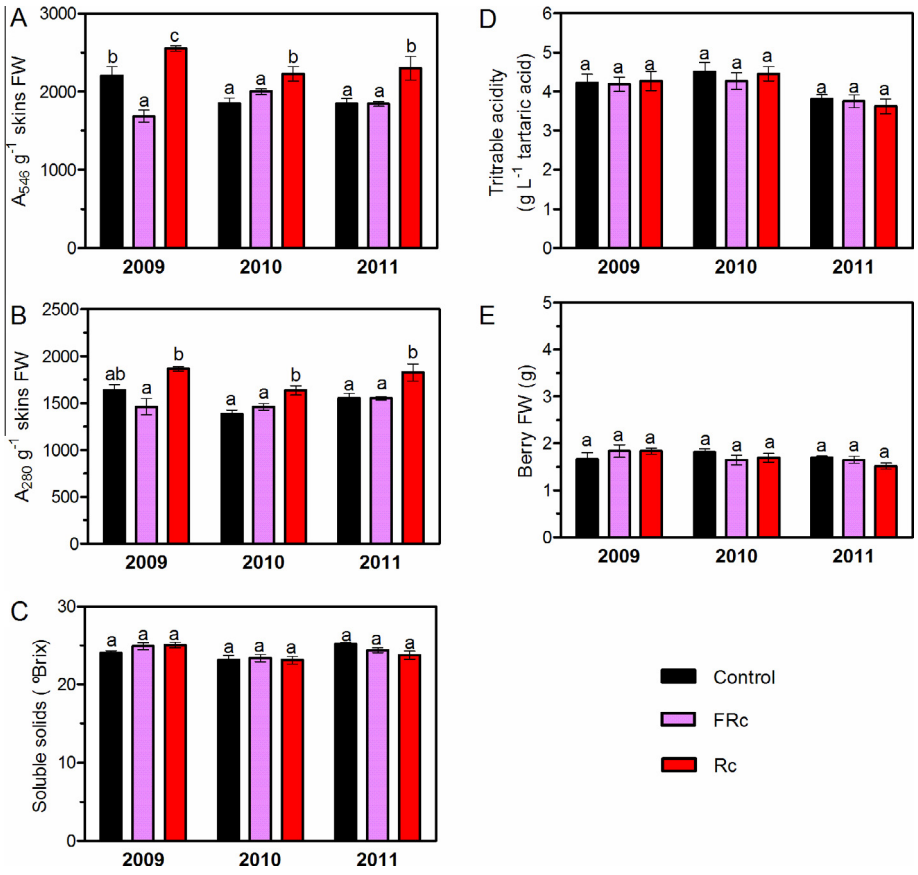

Fig. 5. Rc and FRc experiment. Concentration of (A) anthocyanins ($A_{546\ nm}\ g^{-1}$ skin DW), (B) total phenolic compounds ($A_{280\ nm}\ g^{-1}$ skin DW), (C) total soluble solids (°Brix), (D) titrable acidity (g L^{-1} tartaric acid) and (E) berry FW (g) of field-grown grapes at harvest (133 DAF) supplemented with Rc and FRc treatment. Data are means ± SE of eight (2009 and 2010) or five (2011) biological replicates. Different letters indicate significant differences between treatments as calculated by Tukey HSD statistical analysis ($P < 0.05$).

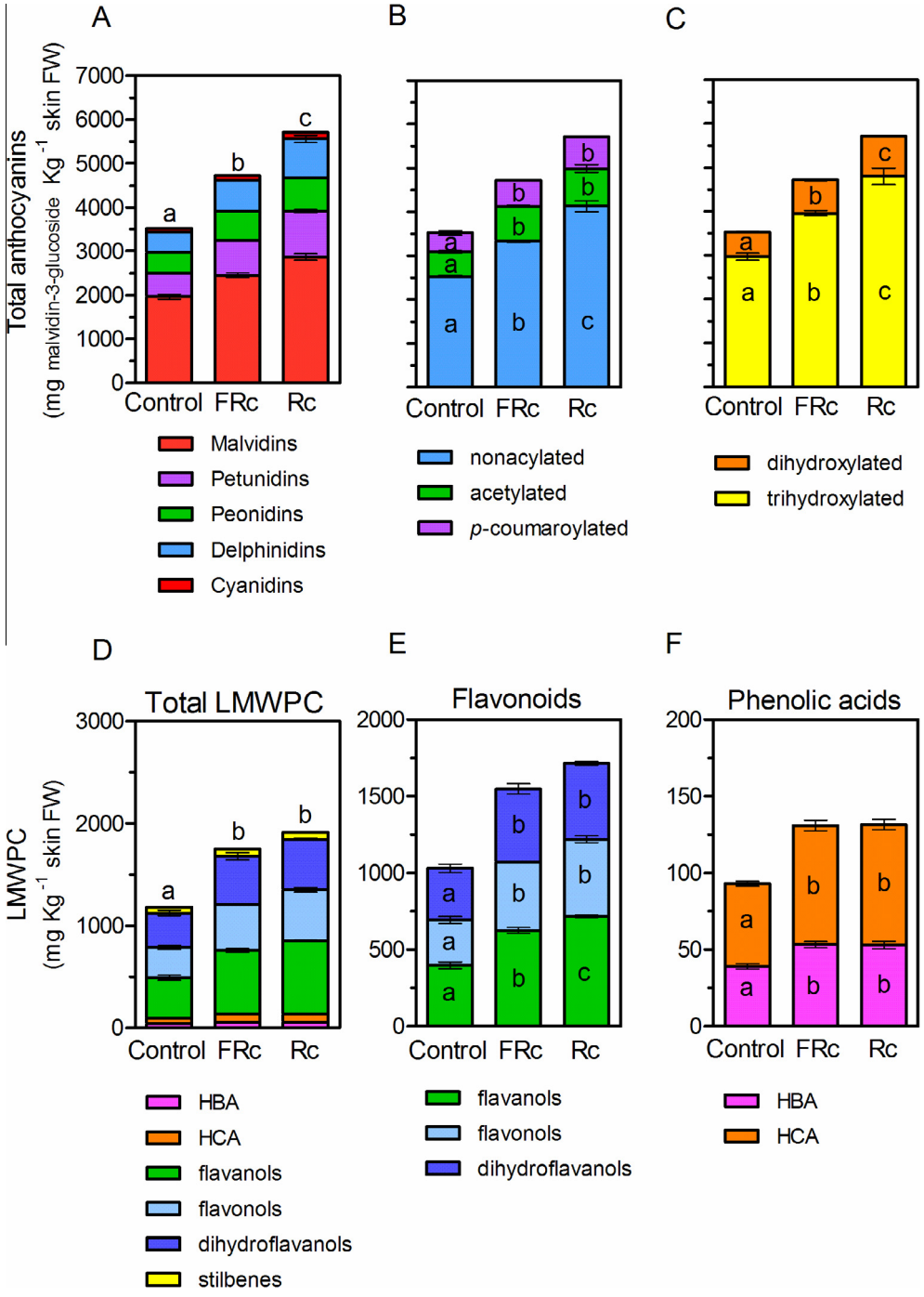

Fig. 6. Concentration of total anthocyanin (A–C) and non-anthocyanin phenols (D–E) of field-grown grapes at harvest (133 DAF, 2011 growing season) supplemented with Rc and FRc treatments. Stacked bars show concentration of (A) malvidins, peonidins, petunidins, cyanidins and delphinidins; (B) nonacylated, acetylated and *p*-coumaroylated; and (C) tri and dihydroxylated anthocyanin glucosides (mg malvidin-3-glucoside kg^{-1} skin FW). (D) Total LMWPC, (E) Flavonoids, (F) Phenolic acids (mg kg^{-1} skin FW). Data are means ± SE of three biological replicates. Different letters indicate significant differences between treatments as calculated by Tukey HSD analysis ($P < 0.05$).

In general, the relative abundances of the various anthocyanins were not affected by the light treatments (Fig. 6A–C and Table S2). Both Rc and FRc enhanced LMWPC (Fig. 6D), including flavonoids, phenolic acids and stilbenes (Fig. 6E–F and Supplementary Table S3). Of note, the effect of FRc on the stilbene content (*trans*-resveratrol glucoside) was higher than that of Rc (Supplementary Table S3). The results of the EOD and daytime manipulations of R and FR levels are consistent with the involvement of phyB-like phytochromes in controlling berry skin phenolics under natural conditions, and suggest that unstable (i.e. phyA-like) phytochromes could also play a role in the response to continuous FR.

2.3. B light increased anthocyanin and total phenolic compounds in berry skins

Leaf-shading causes a decrease in B light irradiance received by the fruits (Fig. S3). To study the effects of changes in blue light on berry quality traits, the berry light environment was supplemented with B and G light (Fig. 2). In two growing seasons (2010 and 2011), it was found that B light increased levels of anthocyanin and total phenolic compounds in berry skins without affecting sugar content, acidity or berry size at harvest (Fig. 7). No effects of the G light treatment were observed in these experiments

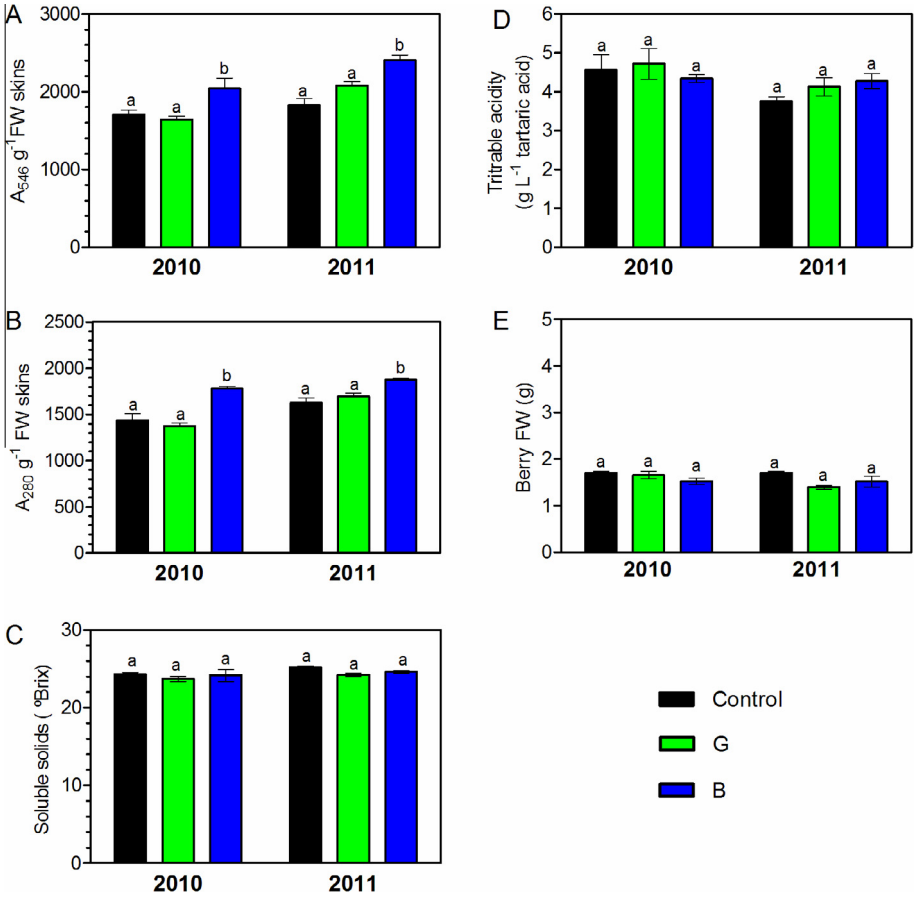

Fig. 7. B and G experiments. Concentration of (A) anthocyanins ($A_{546\ nm}\ g^{-1}$ skin DW), (B) total phenolic compounds ($A_{280\ nm}\ g^{-1}$ skin DW), (C) total soluble solids (°Brix), (D) titrable acidity (g L^{-1} tartaric acid) and (E) berry FW (g) of field-grown grapes at harvest (133 DAF) supplemented with B and G light treatments. Data are means ± SE of five biological replicates. Different letters indicate significant differences between treatments as calculated by Tukey HSD statistical analysis ($P < 0.05$).

(Fig. 7). HPLC-DAD analyses confirmed that B light increased total anthocyanin content without modifying the relative abundance of the various compounds (Fig. 8A–C and Table S4). The G treatment showed a weaker enhancement of total anthocyanins (Fig. 8A–C). B light also increased non-anthocyanin phenolic compounds, including flavonoids, phenolics acids and *trans*-resveratrol glucoside (Fig. 8D–F and Supplementary Table S5), whereas G treatment stimulated flavanoids and phenolic acids but not stilbenes (Fig. 8 and Supplementary Table S5). These results suggest that cryptochromes are also involved in regulating the accumulation of phenolic compounds in berry skins under field conditions.

The HPLC-DAD analyses generally confirmed the results obtained by UV–Vis spectrophotometry, and also indicated an increase in total anthocyanin and LMWPC concentration under the FR and G treatments (Figs. 6 and 8, respectively). These small responses to FR and G supplementation were probably too weak to be detected by the less sensitive UV–Vis approach (Figs. 5 and 7).

3. Discussion

The light environment influences the growth and composition of a wide variety of fruits, including grapes. Many reports suggest that leaf-shading impairs ripening-associated processes, affecting fruit quality and commercial value. Under the experimental conditions herein, the characterization of the diurnal dynamics of the fruit light environment indicated that clusters (in a vertical trellis system) were shaded by leaves during most of the photoperiod (Fig. 1). Different local light supplementation experiments were performed in the field to stimulate fruit-localized phytochromes and B light photoreceptors (Fig. 2). The results demonstrate that: (a) changes in the fruit light environment perceived by berry-localized photoreceptors are directly involved in controlling the accumulation of phenolic compounds in berry skins, as local light quality treatments modified the levels of flavonoid and non-flavonoid compounds without affecting total soluble solids, acidity or berry size (Figs. 3, 5 and 7), (b) R light, perceived by stable phytochromes (i.e. phyB-like) increased the contents of anthocyanins and LMWPC (Figs. 4, 6 and 8), (c) continuous FR, presumably perceived by phyA-like phytochromes, caused a weaker but significant increase in anthocyanins and LMWPC (Fig. 6), (d) daily supplementation of B light, presumably perceived by cryptochromes, had effects on berry skin chemistry that were similar to those of R light supplementation (Figs. 7 and 8), and (e) activation of fruit-localized photoreceptors causes a generalized up-regulation of the phenylpropanoid pathway, without affecting the relative abundance of the various anthocyanins or LMWPC measured in this study (Figs. 4, 6 and 8). The effects of R light (Figs. 4 and 6) are likely to be mediated by a LFR (low fluence response) mechanism, whereas those of continuous FR may reflect the activation of a HIR (high irradiance response) mechanism (Fig. 6).

This study contributes to improve the understanding of the role of photoreceptors in controlling fruit physiology in a woody perennial species, which is a relatively poorly explored area of plant biology. In grapevine, although photoreceptor genes have not been fully described, two genes that are homologous to the Arabidopsis *PHYA* and *PHYB* have been identified. Both phytochromes were proposed to regulate entry and exit of bud from endodormancy

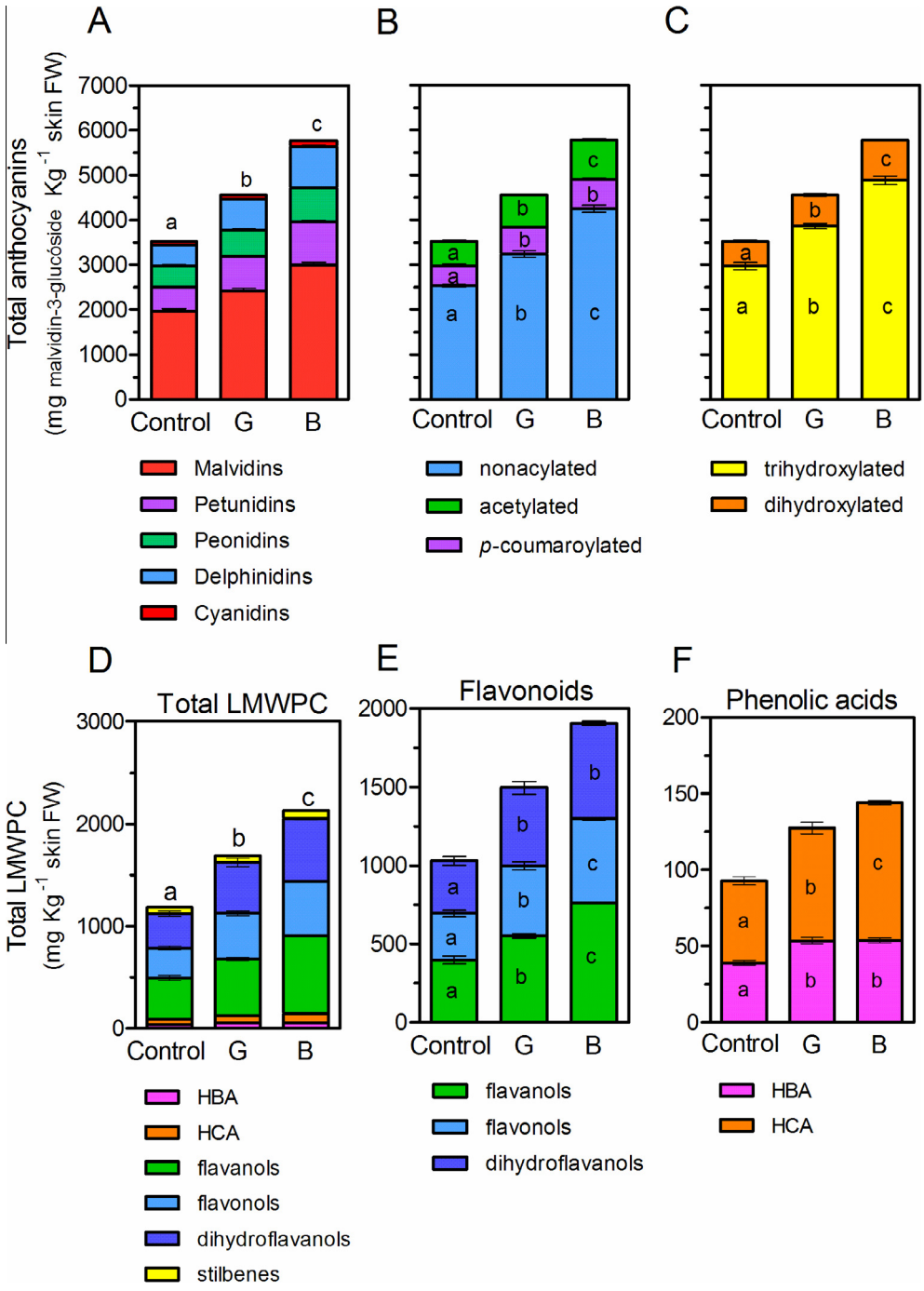

Fig. 8. Concentration of total anthocyanin (A–C) and non-anthocyanin phenols (E–F) of field-grown grapes at harvest (133 DAF, 2011 growing season) supplemented with B and G treatments. Stacked bars show concentration of (A) malvidins, peonidins, petunidins, cyanidins and delphinidins; (B) nonacylated, acetylated and *p*-coumaroylated; and (C) tri and dihydroxylated anthocyanin glucosides (mg malvidin-3-glucoside kg^{-1} skin FW). (D) Total LMWPC, (E) Flavonoids, (F) Phenolic acids (mg kg-1 skin FW). Data are means ± SE of three biological replicates. Different letters indicate significant differences between treatments as calculated by Tukey HSD analysis ($P < 0.05$).

(Kuhn et al., 2009; Pérez et al., 2009, 2011). Similarly, phytochromes have been implicated in the mechanisms that trigger growth cessation and development of dormancy in some tree species in response to photoperiod (Welling and Palva, 2006). In apple (*Malus domestica*), recent studies described *MdCRY1* and *MdCRY2* from fruit skins, and analyzed the functional roles of these photoreceptor genes in transgenic Arabidopsis (Li et al., 2013a,b). The observation here that fruit-localized photoreceptors are involved in grape berry pigmentation is consistent with previous findings in other types of fleshy fruits from unrelated taxa, such as, tomato (*Solanum lycopersicum*, Solanaceae) (Azari et al., 2009) and apple (*M. domestica*, Rosaceae) (Li et al., 2012). The ecological importance of this conserved response in fruits of different species may be linked to the accumulation of phenolic sunscreens (Flint et al., 1985) that may protect fruit tissues from the harmful effects of UV-B-radiation, or pigments that increase fruit visibility to potential dispersers (Cazetta et al., 2009).

It was found that the responses of grape fruit photoreceptors are not saturated under field conditions. This result provides theoretical support for cultural practices aimed to increase light penetration through the canopy, such as leaf removal in the fruit zone. From these experiments, it could be inferred that very small

Table 1
Flowering, veraison, experiment set and harvest dates during the growing seasons assessed.

Growing season	2008		2009		2010		2011	
	Date	DAF	Date	DAF	Date	DAF	Date	DAF
50% flowering	19/11/07	0	13/11/08	0	10/11/09	0	19/11/10	0
Experiment set	09/01/08	50	08/01/09	55	07/01/10	57	06/01/11	47
50% veraison	30/01/08	71	24/01/09	71	18/01/10	68	30/01/11	71
Harvest	02/04/08	133	01/04/09	138	23/03/10	133	01/04/11	133

amounts of extra light (5 or 15 μmol m^{-2} s^{-1}) can be enough to stimulate photoreceptor responses. Severe defoliation, in contrast, could have the effect of increasing temperature especially in areas of high insolation, with potentially negative consequences on pigment accumulation (Mori et al., 2007). Since the present study was performed in a vineyard conducted in a vertical trellis system, it is predicted that in different systems (e.g., in an overhead trellis system or under a high density canopy), larger pigmentation responses to photomorphogenic light treatments are likely to occur. In addition to canopy manipulations, the fruit light environment can also be modified by plastic mulches or photo-selective nets. Kasperbauer (2000) and Kasperbauer et al. (2001) showed that light reflected from red mulches increased yield, aroma, sugar and organic acid concentrations in strawberry. Colored photo-selective hail nets are an emerging approach in protected agriculture, and these nets can be designed to selectively filter different spectral bands of solar radiation, and to transform direct light into scattered light. In fact, it has been reported that selective netting increases fruit quality and yield in many fruit species (kiwifruit, apples, sweet pepper) (Stamps, 2009). Taken together, these results add weight to recent suggestions that manipulation of photomorphogenic processes in plant canopies can have important beneficial effects for crop production and product quality (Ballaré et al., 2012; Wargent and Jordan, 2013).

The results obtained in this study could have implications for plant breeding by manipulation of photoreceptor genes or genes involved in light signal transduction. In tomato, *CRY2* overexpressing plants show a high-pigment phenotype, resulting in overproduction of anthocyanins and chlorophyll in leaves, and flavonoids and lycopene in fruits (Giliberto et al., 2005). Also, tomato mutants hypersensitive to light (*hp*, high pigment) present higher concentration of lycopene and other carotenoids in leaves and fruits. Apart from a darker pigmentation, *hp* mutants also overproduce several colorless flavonoid compounds, as well as vitamins C and E, thus enhancing their nutritional quality. Because of their enhanced fruit lycopene content, *hp* mutations have been introgressed into several processing tomato cultivars, which are cultivated as novel Lycopene Rich Tomatoes (LRT) (Azari et al., 2009). In the same vein, Li et al. (2012) showed that *MdCOP1* (ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1), a key regulator of light signaling, interacts with *MdMYB1* to regulate light-induced anthocyanin biosynthesis in red apples. These reports, along with our physiological results in grapevine, suggest that increasing fruit-sensitivity to light by targeted manipulation of fruit photomorphogenesis (e.g., through the utilization of fruit specific promoters) could represent a plausible way to improve fruit quality in many species.

4. Conclusion

To conclude, the results here demonstrate that the chemical composition of grape berries is modulated by the light quality perceived by phytochromes and blue light photoreceptors located in the berries, and that fruit photoreceptors in grapes are not saturated under field conditions. Therefore, manipulation of the light environment and light sensitivity of fruits are potential tools to increase the concentration of health-promoting compounds in fruits, such as resveratrol, anthocyanins, flavanols and flavonols.

5. Materials and methods

5.1. Plant material and experimental conditions

The study was performed in a commercial vineyard located in the Uco Valley (1450 m above sea level; 69° 15′ 37′ W and 33° 23′ 51″ S, Gualtallary, Tupungato, Mendoza, Argentina). The plant material was a selected clone of *Vitis vinifera* L. cv. Malbec, planted in 1997 without rootstock in sandy soil and drip irrigated plots, arranged in north–south oriented rows spaced 2 m apart, with a distance of 1.20 m between two consecutive plants on each row. The vines were trained on a vertical trellis system, pruned as Guyot and protected by antihail nets (black polyethylene) that reduced 17% of PAR without modification of the light quality. The vineyard was managed according to standard viticultural practices for the cultivar and region. Irrigation started before budbreak and ended about 1 week before harvest (September to April). Full bloom and veraison were on the dates indicated in Table 1, according to stages 23 and 35 described by Coombe (1995), respectively. During the experiments the plants were maintained without leaf removal or water restriction practices.

A randomized complete block design was used. Each row was considered as a block. Individual plants were used as experimental units, and only one cluster per plant was exposed to the light manipulation treatments. Clusters from the main cane with homogeneous size and placed in the middle of both sides of the canopy were selected. Experiments started approximately 20 days before veraison (52 ± 5 DAF), and lasted until harvest (133 DAF, 24–25°Brix). Complete information regarding sampling dates and berry stages is detailed in Table 1.

5.2. Light measurements

Light measurements were performed along the day (from −120° to +120° azimuth, approximately) during the berry ripening period on 2009 and 2010 growing seasons. Data recorded on four dates in 2010 are shown: 7 January (57 days after flowering; DAF), 12 February (92 DAF), 23 March (133 DAF) and 21 April (161 DAF). All measurements were taken under clear sky conditions, either above the grapevine canopy or in the cluster zone. To evaluate berry light environment, five wood-stakes were placed within the grapevine canopy mimicking the position of five imaginary clusters (Supplementary Fig. S1). Stakes were spaced 0.3 m apart in a single row. Light sensors were positioned vertically facing upward above the canopy or placed over the stakes in subsequent determinations.

A LI-250 light meter with a LI-190SA quantum sensor (Li-Cor Inc., Lincoln, NE, USA) and a PMA2200 radiometer with a PMA2102 UV-B detector (Solar Light Company Inc., Glenside, PA, USA) were used to measure PAR (400–700 nm) and UV-B radiation (280–315 nm), respectively. Light spectra was scanned between

300 and 1000 nm with an USB4000 spectroradiometer (Ocean Optics Inc., Dunedin, FL, USA) using an optical probe (0.4 mm diameter, 2 m length) with a CC-3-UV cosine corrector (Ocean Optics Inc.). Spectra were processed with SPECTRASUITE software (http://www.oceanoptics.com/Products/spectrasuite.asp). To calculate the R:FR (660 ± 10 nm:730 ± 10 nm) ratio and B (420–490 nm) irradiance, the spectral irradiance values within the appropriate wavelength range were integrated. PAR and UV-B within the cluster zone were expressed as the percentage of the incident radiation above the vineyard.

5.3. Light experiments

Three fruit-localized light supplementation treatments were performed in the field (Fig. 2):

(I) End of day (EOD) R and FR light pulses (Rp or FRp). Light pulses were given for 45 min at the end of the natural photoperiod (irradiance 5 μmol m^{-2} s^{-1}). The treatments were applied every day, starting 20 days before veraison and continuing until harvest. The experiment was carried out during the 2008, 2009 and 2010 growing seasons, and in each season there were 8 true replicates of each light treatment.

(II) Continuous R and FR (Rc or FRc) supplementation during the natural photoperiod. The experiment was carried out during the 2009 and 2010 growing seasons, with 8 true replicates in each season and an irradiance of 5 μmol m^{-2} s^{-1}. During the 2011 growing season the experiment was performed with 5 replicates and 15 μmol m^{-2} s^{-1}. In all cases the treatments were applied every day, during the course of the natural photoperiod starting 20 days before veraison and continuing until harvest.
In 2008, 2009 and 2010 experiments, R light sources of 5 μmol m^{-2} s^{-1} (R:FR = 1.597) were made by filtering the light provided by energy-saving light bulbs (11 W E27 230 V warm white, Phillips; China) through the combination of a R filter (Code 027; Lee Filters: http://leefilters.com/, CA, USA) and 2-mm thick clear acrylic sheets (Alos Acrilicos: http://www.alosacrilicos.com.ar; Mendoza, Argentina). FR light sources of 5 μmol m^{-2} s^{-1} (R:FR = 0.053) were made by filtering the light provided by incandescent bulbs (25 W Classic P CL 230 V clear bulb, Osram; Buenos Aires, Argentina) through the combination of a R filter (Code 027; Lee Filters) and a 2-mm thick dark blue acrylic sheet (Code 2031; Paolini http://paolini-sa.com, Buenos Aires, Argentina). The control (0 μmol m^{-2} s^{-1}) was made by blocking the light emitted from incandescent bulbs (25 W Classic P CL 230 V clear bulb, Osram; Buenos Aires, Argentina) with a sheet of black acrylic (Alos Acrílicos). R light sources were placed 10 cm away from the cluster and FR light and control sources were placed 6 cm away from the cluster. In the 2011 experiments, R and FR light sources of 15 μmol m^{-2} s^{-1} were obtained using light emitting diodes (LEDs). LEDs are well suited for plant photobiology studies because they emit narrow-bandwidth light, have a long life, require little maintenance, and do not radiate heat toward the clusters. Light sources were made of ten R (peak emission: 660 nm, B3b-446-30; Roithner Laser Technik GmbH, Vienna, Austria; http://www.roithner-laser.com/led.html) or ten FR (ELD 740-524, peak emission: 730 nm; Roithner Laser Technik GmbH) LEDs. LEDs were welded to a platelet (12 × 5 cm). LED platelets were placed 5 cm apart from the clusters' south side. The control consisted of a randomly selected cluster of similar characteristics as those used for light supplementation.

(III) Continuous B and G light supplementation during the natural photoperiod. The treatments were applied every day, starting 20 days before veraison and continuing until harvest. The experiment was performed with five true replicates during 2010 and 2011 growing seasons. B and G light sources (irradiance 15 μmol m^{-2} s^{-1}) were obtained by welding ten B (B3B-447-IX, peak emission: 460 nm; Roithner Laser Technik GmbH) and thirteen G (B5-433-20, peak emission: 550 nm; Roithner Laser Technik GmbH) LEDs to a platelet (12 × 5 cm). Platelets were placed 5 cm apart from the clusters' south side. Clusters not supplemented with light were used a controls, as explained above.

Representative spectral scans of all light sources are given in Supplementary Fig. S2. The duration of light treatments was controlled daily with programmable digital timers. Fruit temperature was also monitored daily throughout the course of the experiments by placing iButton sensors (Thermochron iButton DS1921; http://www.maxim-ic.com/products/ibutton/datalogging) within the clusters. No significant differences among light treatments were found in any of the experiments ($P > 0.05$; data not shown).

5.4. Berry sampling and determination of fresh weight

Five berries per experimental unit were randomly collected in nylon bags after sunset. Berry fresh weight (FW) was immediately determined in the field. Samples were collected on ice and finally stored at −18 °C.

5.5. Total content of anthocyanin and polyphenols

UV–Vis determinations of total phenolic compounds were performed according to Mazza et al. (2000) and total anthocyanins according to Lariguet et al. (2003) with slight modifications. Briefly, strips of five frozen grapes of each sample were peeled using a scalpel and subsequently placed in 10 mL of 1% (w/v) HCl/MeOH for 48 h at −20 °C. The extract solution was diluted 1:20 with 1% (w/v) HCl/MeOH and absorbance was measured at 280 and 546 nm for determinations of total phenolics and anthocyanins, respectively using a Cary-50 UV–Vis spectrophotometer (Varian Inc., CA, USA) and 10-mm optical path cells. Next, berry skin strips were dried at 65 °C during 48 h and weighted using an analytical balance. After peeling, berries were thawed at room temperature for determinations of sugars and titratable acidity (TA).

5.6. Sugar accumulation and acidity

The pulps of defrosted berries were hand-crushed and sieved through a mesh. The concentration of sugars (°Brix) was measured in the juice with a Pocket PAL-1 digital hand-held refractometer (Atago Co., Ltd., Tokyo, Japan). TA, expressed as g L^{-1} of tartaric acid, was measured by titrating samples of juice (5-mL, diluted with 10 mL of distilled H_2O) with 0.1 N NaOH to a final pH value of 8.2.

5.7. Anthocyanins and low molecular weight phenolic compounds in berry skins

The skins of 10 frozen berries from three biological replicates were peeled, weighted and extracted with aqueous solution (15 mL, EtOH:H_2O, 88:12, v/v, 6 g L^{-1} tartaric acid, pH 3.2) at 70 °C in darkness during 3 h according to Riou and Asselin (1996). The liquid fraction was separated by decanting, maintained 24 h at 4 °C, and centrifuged 10 min at 10,000*g* in order to eliminate sediments.

For anthocyanins, aliquots (150-μL) of berry skin extract solution were filtered through a 0.45-μm pore size nylon membrane, and 100 μL w injected in a high-performance liquid chromatography-photodiode array detector (HPLC-DAD; Series 200, PerkinElmer, Shelton, CT, USA) according to the conditions described in Fanzone et al. (2011). Diode array detection was performed from 210 to 600 nm, and the quantification was carried out by peak area measurements at 520 nm. Anthocyanin amount was expressed by using malvidin-3-glucoside chloride as standard for a calibration curve (R^2 = 0.99). Identification and confirmation of anthocyanin pigments were performed by HPLC-DAD/ESI-MS as described by Monagas et al. (2003).

For low molecular weight phenolic compounds (LMWPC), skin extract solutions (10 mL) were added with NaCl (1 g) and subjected to three successive extractions with Et_2O (5 mL × 3) and EtOAc (5 mL × 3). The organic fractions were combined, dehydrated with Na_2SO_4 anhydrous (2.5 g), filtered through a 3 μm pore size cellulose filter, and evaporated to dryness under a gentle N_2 gas stream at 30 °C. The solid residue was dissolved in MeOH:H_2O (0.4 mL, 1:1, v/v), filtered through a 0.45-μm pore size nylon membrane, and then 30 μL were injected in the HPLC-DAD system commensurate to the conditions described in Fanzone et al. (2011). Diode array detection was performed by scanning from 210 to 360 nm with an acquisition speed of 1 s. The identification of specific compounds was carried out by comparison of their spectra and retention time with those of standards. All the individual phenolic compounds were confirmed by HPLC-DAD/ESI-MS as described by Monagas et al. (2005). Quantitative determinations were made by using the external standard method with commercial standards. The calibration curves were obtained by injection of standard solutions, under the same conditions as for the samples analyzed, over the range of concentrations observed ($R^2 \geqslant 0.94$). The compounds for which no standards were available were quantified with the curves of quercetin (dihydroflavonols), quercetin-3-glucoside (quercetin and flavonol glycosides), myricetin (myricetin glycosides), resveratrol (trans and cis-resveratrol glucoside), caffeic acid (fertaric, caftaric and coutaric acids), gallic acid (gentisic acid), ethyl gallate (methyl gallate), and (+)-catechin (procyanidins).

5.8. Statistical analysis

Student's *t*-tests, one or two-way ANOVA followed by the Tukey honest significant difference (HSD) post hoc test were performed when appropriate in order to assess differences between means. Analyses were carried out with the InfoStat 2011 version software (Di Rienzo et al., 2011). A $p < 0.05$ was considered to be statistically significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.11.018.

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