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Metabolism of terpenes in the response of grape (*Vitis vinifera* L.) leaf tissues to UV-B radiation

Mariana Gil, Mariela Pontin, Federico Berli, Rubén Bottini, Patricia Piccoli *

Laboratorio de Bioquímica Vegetal, Instituto de Biología Agrícola de Mendoza, Consejo Nacional de Investigaciones Científicas y Tecnológicas-Universidad Nacional de Cuyo, Almirante Brown 500, M5528AHB Chacras de Coria, Argentina

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ABSTRACT

This study investigated the terpene profiles as determined by GC–EIMS analysis of *in vitro* cultured plants of *Vitis vinifera* exposed to a "field-like" dose of UV-B (4.75 kJ m^{-2} d^{-1}) administered at two different fluence rates (low, 16 h at 8.25 μW cm^{-2}, and high 4 h at 33 μW cm^{-2}). Low UV-B treatment increased levels of the membrane-related triterpenes sitosterol, stigmasterol and lupeol, more notable in young leaves, suggesting elicitation of a mechanism for grapevine acclimation. By contrast, accumulation of compounds with antioxidant properties, diterpenes α and γ tocopherol and phytol, the sesquiterpene E-nerolidol and the monoterpenes carene, α-pinene and terpinolene had maximum accumulation under high UV-B, which was accentuated in mature leaves. Also the levels of the sesquiterpenic stress-related hormone abscisic acid (ABA) increased under high UV-B, although 24 h post irradiation ABA concentrations decreased. Such increments of antioxidant terpenes along with ABA suggest elicitation of mechanism of defense. The adaptative responses induced by relatively low UV-B irradiations as suggested by synthesis of terpenes related with membrane stability correlated with augments in terpene synthase activity.
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1. Introduction

Ultraviolet solar radiation (UV) is mostly absorbed by the stratospheric ozone layer and other atmospheric gases, being UV-B (wavelength 280–315 nm) only 0.5% of the total solar radiation energy reaching the earth's ground. Environmental UV-B levels are regulated by altitude, latitude, season, day time and cloud cover (McKenzie et al., 2003). Despite the relatively low levels of UV-B irradiance that reach the vegetation canopy, the biological impact on plant tissues may be important and depend on a number of factors, including UV-B irradiation level, duration, and wavelength of the UV-B treatments (Blanding et al., 2007; Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003; Jenkins and Brown, 2007). It has been reported that elevated UV-B irradiation levels induce expression of many genes usually involved in defense, wounding, or general stress responses (A-H-Mackerness, 2000; Brosché and Strid, 2003; Kilian et al., 2007; Ulm and Nagy, 2005). High fluence rates of UV-B can damage macromolecules like DNA and proteins, as well as membrane lipids, the photosynthetic apparatus and even generate tissue necrosis (Casati and Walbot, 2004; Jansen et al., 1998; Julkunen-Titto et al., 2005; Rozema et al., 1997). It has also been demonstrated that UV-B generates reactive oxygen species (ROS) (Allan and Fluhr, 1997; Barta et al., 2004; Hideg et al., 2002), eventually producing oxidative damage (Brosché and Strid, 2003). Plants respond to this damage through induction of a complex antioxidant defense system involving different enzymes and secondary metabolites (Jansen et al., 1998, 2001; Brosché and Strid, 2003; Berli et al., 2010).

UV-B radiation is not always a damage-inducing source of stress but it also can act as a key environmental signal regulating diverse metabolic responses of defense and development in plants (Brosché and Strid, 2003; Caldwell et al., 2003; Frohnmeyer and Staiger, 2003; Jenkins, 2009). Transcriptome analyses show that UV-B increases expression of several genes related with reducing oxidative stress (Brown et al., 2005; Casati and Walbot, 2003, 2004; Kilian et al., 2007; Pontin et al., 2010; Ulm et al., 2004). However, although changes in the transcriptome are involved in modulation of secondary metabolites by UV-B, variations in enzyme activity and accumulation of defense metabolites do not necessarily correlate with changes in gene expression (Dolzhenko et al., 2010). Also, UV-B stimulates protective responses that affect the plant's resistance to other biotic and abiotic stresses (Ballaré, 2003; Frohnmeyer et al., 1999; Kim et al., 1998). Additionally, UV-B-generated ROS may act as signaling molecules mediating the acquisition of tolerance to both biotic and abiotic stresses (Leshem et al., 2007; Torres and Dangl, 2005).

Despite the many reports on damage of UV-B to different plant tissues, there is a rather limited understanding regarding the role of secondary metabolites in protective mechanisms against potentially harmful UV-B irradiation. Most information available deals with biosynthesis and accumulation of phenolics in epidermal leaf

* Corresponding author. Tel.: +54 261 4135010x1228; fax: +54 261 4960469.
E-mail addresses: ppiccoli@fca.uncu.edu.ar, ppicco013@umn.edu (P. Piccoli).

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tissues that attenuate penetration of UV-B into deep cell layers, including grapevines (Berli et al., 2008, 2010; Rozema et al., 1997). However, little is known about the UV-B radiation protective role played by other compounds, such as isoprenoid-derived terpenes (Chapman, 2009; Zavala and Raveta, 2002). In autotrophic metabolism, isoprenoids act as photosynthetic pigments (chlorophylls and carotenoids), electron carriers (quinones), radical scavengers (tocopherols), membrane components (sterols), as well as the stress-related phytohormone abscisic acid (ABA; Zhu, 2002). Notwithstanding, the role of ABA in UV-B-induced responses has just begun to be studied (Berli et al., 2010, 2011).

In a previous paper (Pontin et al., 2010) *in vitro* grape plants were treated with a single UV-B dose equivalent to that grape plants receive in a vineyard but in two different intensities: low UV-B (16 h at 8.25 μW cm^{-2}) or high UV-B (4 h at 33 μW cm^{-2} UVB). Although a group of *ca.* 650 probe sets were up- or down-regulated by both UV-B intensities, high UV-B specifically regulated a group of more than 2000 probe sets while low UV-B induced differential expression of other group of approximately 650 probe sets. These results suggested that different "environmental" intensities regulate gene expression in diverse ways; purportedly, relatively low intensities may induce responses of adaptation while high intensities could induce defense-related genes.

The hypothesis of this study was that grapevine tissues have two kind of metabolic responses, one in which the signal induces adaptation of the leaf tissues to UV-B, other in which the tissue responds to the UV-B injury. The aim was to investigate the effects of a "field-like" dose of UV-B administered at two different fluence rates (low and high), as compared with a control without UV-B, on terpene profiles in young and mature leaves of *in vitro* cultured grapevines. Changes in terpene levels and terpene synthase (TPS) activity were monitored in order to discriminate between two purported responses, acclimation and response to UV-B-induced damage.

2. Results

Only data for those compounds which levels varied in more than double as compared with the values found in control (without UV-B) tissues are presented.

2.1. UV-B induces synthesis of membrane-related sterols

As shown in Fig. 1, the amount of sterols (1–3) measured in leaves of grape plants significantly increased after both UV-B treatments as compared with the control in which UV-B was filtered, and these amounts were also affected by leaf ontogeny. It has been reported that the most abundant plant sterols in nature are sitosterol (1) and stigmasterol (2) (Croteau et al., 2000), which regulate membrane fluidity and play a role in the adaptation of membranes to different stresses (Piironen et al., 2000; Schaller, 2003). Consistent with this, sitosterol (1) was the most abundant sterol and increased 16.4-fold in young leaves and 8.0-fold in mature leaves under low UV-B irradiance, and 4.8-fold in young leaves and 1.8-fold in mature leaves by high UV-B irradiance, as compared with the control (Fig. 1a). Thus, sitosterol (1) levels were higher in younger leaves than in mature leaves under both, low and high UV-B irradiance treatments. The concentration of stigmasterol (2) was augmented 3.2-fold under low UV-B in young leaves as compared to control, and 2.3-fold with respect to high UV-B, without differences in the rest of the treatments (Fig. 1b). Levels of another triterpenoid related with antioxidant defense system, lupeol (3) (Bracco et al., 1981), increased by low UV-B, 4.2-fold in young leaves and 3.2-fold in mature leaves as compared with control plants, without any effect of high UV-B (Fig. 1c). Under the three

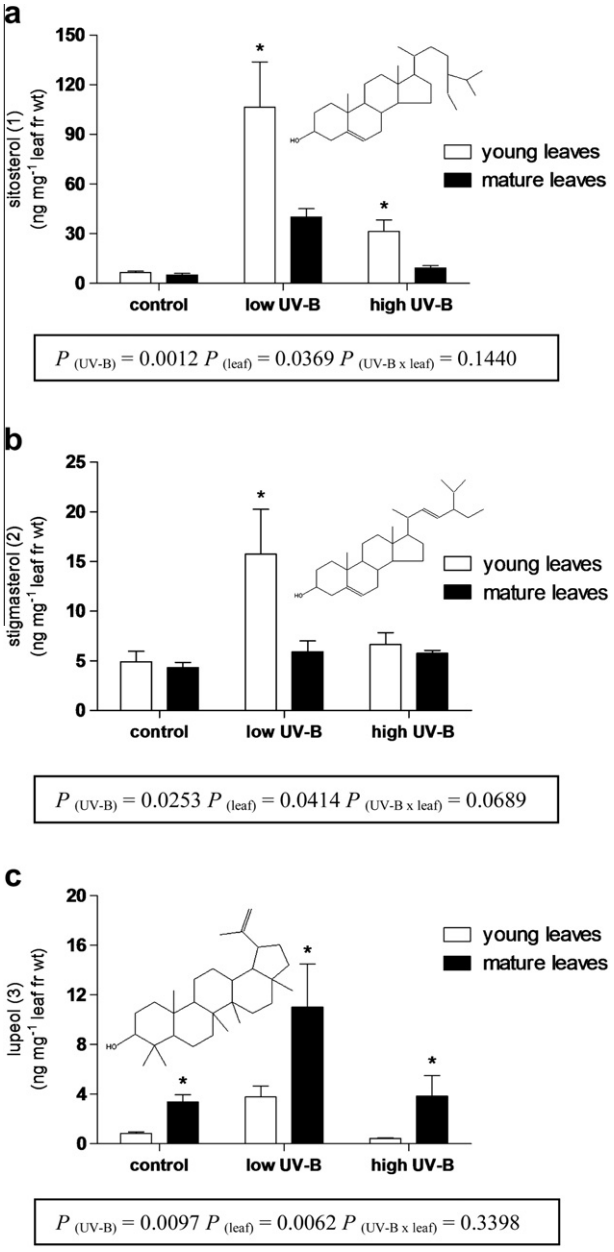

Fig. 1. Triterpene (1–3) levels as assessed by GC–EIMS in ng mg^{-1} leaf FW. Sitosterol (1) (a), stigmasterol (2) (b) and lupeol (3) (c) in young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered. $P_{(UV-B)}$: UV-B effect; $P_{(leaf)}$: leaf ontogeny effect; $P_{(UV-B\ \times\ leaf)}$: UV-B × leaf ontogeny interaction effect. Asterisk means significantly differences comparing upper with lower leaves for the same treatment. Values are means ± SE, n = 3.

light treatments, lupeol (3) levels were always higher in mature leaves than in young leaves.

2.2. High UV-B irradiance increases antioxidant diterpenes in mature leaf tissues

Tocopherols (4–6) are antioxidant diterpenes with a well-recognized ability to protect cells from oxidative stress (Fryer, 1992). Levels of γ-tocopherol (4), α-tocopherol (5) and phytol (6) increased in grape plants exposed to high UV-B irradiation (Fig. 2). γ-Tocopherol (4) was augmented 1.9-fold in young leaves and

3.3-fold in mature leaves under high UV-B respect to low UV-B irradiation, and 3.6- and 6.1-fold as compared to their respective controls (Fig. 2a). α-Tocopherol (5) levels were 2-fold increased in mature leaves under high fluence UV-B conditions with regard to the rest of the treatment. The highest concentration was measured in mature leaves exposed to high UV-B (Fig. 2b). Among diterpenes measured in grapevine leaf tissue, phytol (6) was the most abundant and its level was augmented 2.8-fold under high UV-B as compared with controls and low UV-B irradiation, irrespective of leaf ontogeny (young or mature, Fig. 2c).

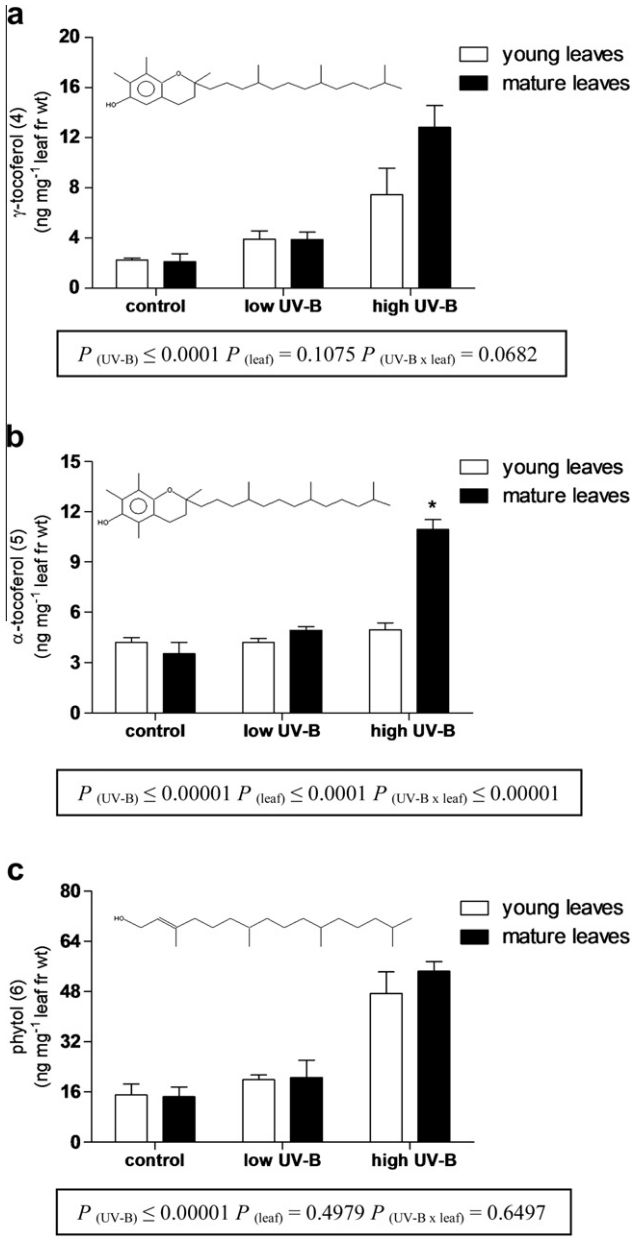

Fig. 2. Diterpene levels assessed by GC–EIMS in ng mg^{-1} leaf FW. γ-Tocopherols (4) (a), α-tocopherol (5) (b) and phytol (6) (c) in young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered. $P_{(UV\text{-}B)}$: UV-B effect; $P_{(leaf)}$: leaf ontogeny effect; $P_{(UV\text{-}B \times leaf)}$: UV-B × leaf ontogeny interaction effect. Asterisk means significantly differences comparing upper with lower leaves for the same treatment. Values are means ± SE, $n = 3$.

2.3. UV-B increases level of defense-related terpenes

Levels of the sesquiterpene E-nerolidol (7), which is primarily related with mechanisms of plant defense against pathogens (Park et al., 2009), was increased according to the fluence rate of UV-B with no differences between young and mature leaves (Fig. 3). Although other sesquiterpenes such us α-farnesene and *trans* α-bergamotene were identified in grapevine leaf tissue, they did not show any difference among the treatments (data not shown).

The volatile monoterpenes α-pinene (8), terpinolene (9) and carene (10) were also identified by GC–EIMS in leaf tissue of plants submitted to low and high UV-B irradiance, but not in the control plants. As shown in Fig. 4a–c their levels were 2-fold higher under high UV-B as compared with low UV-B irradiance treated-plants.

2.4. UV-B induces fluence rate-dependent ABA accumulation

ABA (11) is a sesquiterpene hormone which mediates adaptive responses to different abiotic and biotic stresses in vegetative tissues (Crozier et al., 2000). The highest ABA (11) levels, measured immediately after the end of the UV-B treatments, were obtained in young leaves exposed to high UV-B (Fig. 5a). ABA (11) levels measured in young leaves increased 2.4-fold under low UV-B and 7.9 times with high UV-B irradiation, as compared to controls. In mature leaves, ABA concentration was 3.7-fold and 3.4-fold higher under high and low UV-B irradiance, respectively, as compared to controls. However, 24 h after the UV-B treatments, the ABA (11) concentration decreased to levels lower than the controls, been highest in the control mature leaves (Fig. 5b).

2.5. UV-B increases TPS activity

The committed step in the biosynthesis of the different skeletal types of terpenes is catalyzed by terpene synthases (TPS), a class of enzymes that forms a large variety of terpenoids products from prenyl diphosphate precursors (Croteau et al., 2000). To determine if UV-B-induced terpene accumulation was due to *de novo* biosynthesis, the activity of TPS on farnesyl diphosphate (FPP) (12) as substrate was measured. The potential fates of FPP (12) are shown in Fig. 8. TPS activity, assessed in terms a radioactivity in the hexane-soluble fraction from tritiated FPP (12) increased 8-fold in

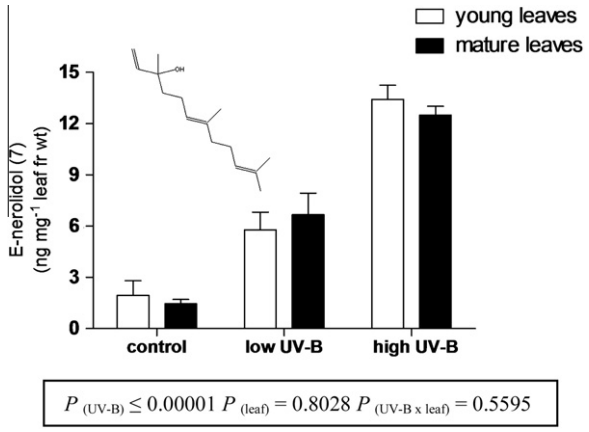

Fig. 3. Sesquiterpene E-nerolidol (7) content assessed by GC–EIMS in ng mg^{-1} leaf FW in young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered. $P_{(UV\text{-}B)}$: UV-B effect; $P_{(leaf)}$: leaf ontogeny effect; $P_{(UV\text{-}B \times leaf)}$: UV-B × leaf ontogeny interaction effect. Asterisk means significantly differences comparing upper with lower leaves for the same treatment. Values are means ± SE, $n = 3$.

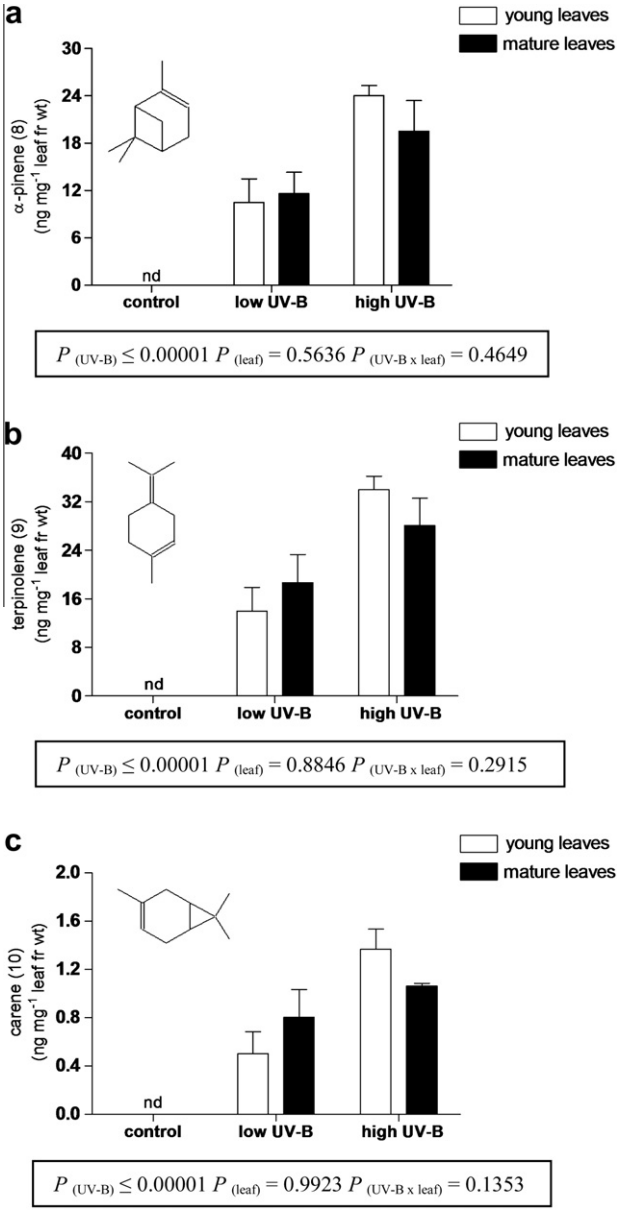

Fig. 4. Monoterpene levels assessed by GC–EIMS in ng mg^{-1} leaf FW. α-Pinene (8) (a), terpinolene (9) (b) and carene (10) (c) in young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered. $P_{(UV\text{-}B)}$: UV-B effect; $P_{(leaf)}$: leaf ontogeny effect; $P_{(UV\text{-}B \times leaf)}$: UV-B × leaf ontogeny interaction effect. Asterisk means significantly differences comparing upper with lower leaves for the same treatment. Values are means ± SE, $n = 3$.

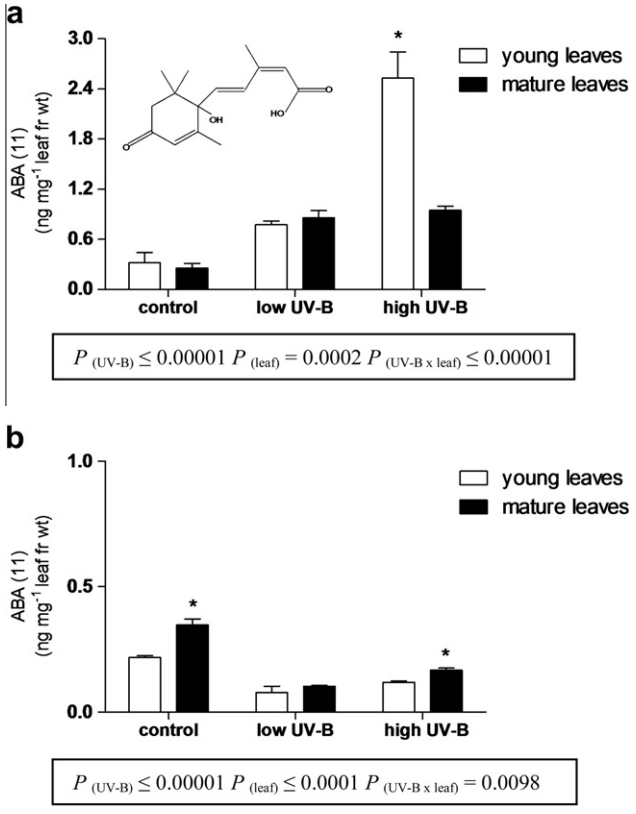

Fig. 5. Abscisic acid (ABA) (11) levels assessed by GC–EIMS in ng mg^{-1} leaf FW in young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered. $P_{(UV\text{-}B)}$: UV-B effect; $P_{(leaf)}$: leaf ontogeny effect; $P_{(UV\text{-}B \times leaf)}$: UV-B × leaf ontogeny interaction effect. Determinations were done directly after the treatments (a) and after 24 h (b). Asterisk means significantly differences comparing upper with lower leaves for the same treatment. Values are means ± SE, $n = 3$.

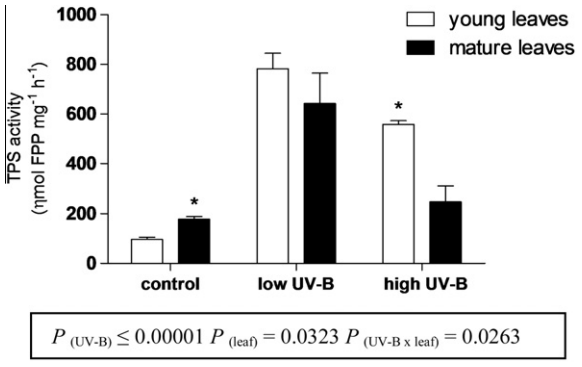

Fig. 6. Terpene synthase activity (TPS) expressed as nmol [^{3}H]-FPP (12) transformed mg of protein^{-1} h^{-1} (see Section 5 for details) of young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered. $P_{(UV\text{-}B)}$: UV-B effect; $P_{(leaf)}$: leaf ontogeny effect; $P_{(UV\text{-}B \times leaf)}$: UV-B × leaf ontogeny interaction effect. Asterisk means significantly differences comparing upper with lower leaves for the same treatment. Values are means ± SE, $n = 3$.

young leaves and 3-fold in mature leaves, under low UV-B as compared to their controls, whereas those exposed to high UV-B irradiance increased 5.8-fold in young leaves compared to control but in the mature ones did not differ (Fig. 6). In general, TPS activity was greater in the young leaves exposed to UV-B. TPS activity measured in root tissue presented the same behavior, but with 10-fold lower levels than leaf tissue (data not shown).

2.6. Principal component analysis (PCA) of metabolites

For an overall interpretation of the results obtained a PCA was used (Fig. 7). The matrix for the analysis consisted of 6 cases corresponding to the combination of the three UV-B regimes and the leaves ontogeny (young and mature leaves), and 12 variables (compounds which levels, assessed by GC–EIMS, varied in more than double as compared with control, and TPS activity). PC1 explained

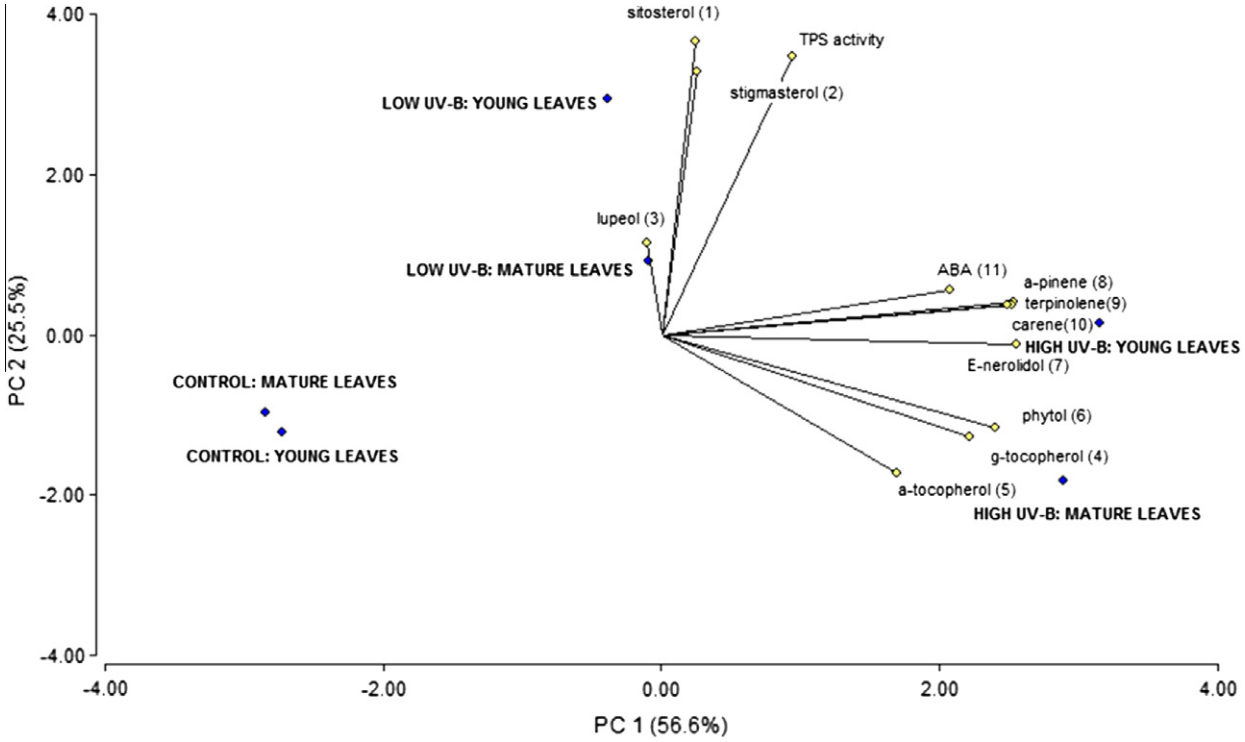

Fig. 7. Biplot display of the principal component analysis (PCA) of the metabolites analyzed in young and mature leaves of 45 d old *Vitis vinifera* cv. Malbec plants. Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low (8.25 μW cm^{-2} during a 16 h per day photoperiod) and high (33 μW cm^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a control in which UV-B was filtered.

56.6% of the variance and separated the treatments by UV-B regimes. PC2 explained 25.5% of the variance and separated the treatments by the leaves ontogeny in the low and high UV-B treatments, not in the control. Monoterpenes, diterpenes and ABA (measured immediately after the end of the UV-B treatments) levels were associated with the high UV-B treatment. E-nerolidol (7), α-pinene (8), terpinolene (9), carene (10) and ABA (11) were more associated with the young leaves, while phytol (6), γ-tocopherol (4) and α-tocopherol (5) were more associated with mature leaves. Finally, the variables of TPS activity (12), membrane-related sterols (sitosterol (1), stigmasterol (2)) and lupeol (3) were associated with low UV-B irradiance (TPS activity and sterols with young leaves and lupeol (3) with the mature leaves).

3. Discussion

The results can be compared with the gene expression profile reported in former work in which the grape whole transcriptome response to UV-B was analyzed in an analogous experiment (Pontin et al., 2010). The experimental design used allowed to compare two situations; one in which a field-like UV-B dose (4.75 kJ m^{-2} d^{-1}) was administrated at a low irradiance, other where the same dose was given in a rather high irradiance but in a narrower period, and a control where UV-B was excluded. This experimental approach allowed comparison and contrast of two purported responses, one in which the UV-B signal would induce adaptation of the plants to this radiation, and the other in which the plant may respond to UV-B-induced damage. In plant tissues, UV-B exposure causes an increase of the excitation energy, which may lead to reduction of oxygen and therefore to generation of ROS. These compounds are mainly responsible for UV-B-mediated damage, but can also act as second messengers in different signaling pathways (Hideg et al., 2002). In fact, ROS are considered signaling molecules that initiate defense responses and their levels increase after UV-B pulses (Allan and Fluhr, 1997). In this report, it is shown that UV-B induces in grape leaf tissues a modulating effect on isoprenoid metabolism and this was differentially affected by fluence rate of UV-B.

The synthesis of the terpenes is initiated from isopentenyl and dimethylallyl diphosphate precursors. Two independent pathways contribute in higher plants to the formation of building block (C5) of isoprenoids. In general, the cytosolic mevalonate pathway (MVA) provides the precursors for sesquiterpenes, and sterols, whereas the plastidial methylerythritol pathway (MEP) furnishes the monoterpenes, diterpenes, tetraterpenes, prenyl moieties of chlorophyll, plastoquinone, and tocopherol (Hampel et al., 2005; McGarvey and Croteau, 1995; Tholl, 2006). The C5 units are condensed by prenyltransferases to synthesize geranyl diphosphate (C10), farnesyl diphosphate (C15) and geranylgeranyl diphosphate (C20). These prenyl diphosphates in turn undergo a wide range of cyclizations and other transformations to produce the various structural types of mono-, sesqui- and diterpenes. These steps are catalyzed by terpene synthases (TPS), a very large family of enzymes with multiple representatives in all plant species studied so far (Degenhardt et al., 2009; Facchini and Chappell, 1992; Steele et al., 1998; Wise et al., 1998), including grapevine (Lücker et al., 2004; Martin et al., 2010). Grapevine leaf tissues exposed to UV-B radiation showed increased TPS activity, and the response was different depending on the irradiance level and the leaves ontogeny. In this sense, the amount of substrate ^{3}H-FPP (12) transformed via the mevalonate pathway (MVA; Lichtenthaler et al., 1997) was higher under low UV-B, and mainly in young leaves exposed to both UV-B treatments. Such increase in TPS activity was associated with augments in the membrane content of sitosterol (1), stigmasterol (2) and lupeol (3), that is, enhancement of the sterol-structural defense. This is in agreement with Sabater-Jara et al. (2010), who observed that accumulation of sterols was induced as defense response in elicited cell cultures of *Capsicum annuum*.

Sitosterol (1) is usually the major sterol found in plant tissues, and an increased sitosterol (1) to stigmasterol (2) ratio has been suggested as a mechanism of plant adaptation to stress (Douglas, 1985). This, was confirmed in our study where the proportion of sitosterol (1) to stigmasterol (2) was about 7-fold higher in the young leaves of plants exposed to low UV-B irradiance. When their levels were compared in control plants no differences were observed between young and mature leaf tissues, and only under UV-B treatment an increase was in these compounds detected. In previous work, with grapevine mature leaves, the sitosterol (1) content was 5-fold higher than that of stigmasterol (2) (Berli et al., 2010). However, the ratio of sitosterol (1) to stigmasterol (2) could be influenced by the plant species, stress intensity (Douglas, 1985) or tissue ontogeny (Geuns, 1973). Additionally, levels of the triterpene lupeol (3) increased under low UV-B. However in this case, such an increase was more notable in mature leaves, suggesting metabolism from an immediate precursor and not *de novo* synthesis via isoprenoid metabolism. Squalene synthase (SS) catalyses the first step of the isoprenoid pathway towards sterol and triterpene biosynthesis (Abe et al., 1993). In plants, both sterols and triterpenes are synthesized as products of cyclization of 2,3-oxidosqualene (14), a reaction catalyzed by oxidosqualene cyclases (OSCs; see Fig. 8). It has been suggested that biosynthesis of triterpenes occurs when sterol formation has been sacrificed (Flores-Sánchez et al., 2002; Kamisako et al., 1984). However, simultaneous enhancement of sterols and triterpene biosynthesis has been reported (Han et al., 2010; Lee et al., 2004). Probably, overproduction of squalene (13) in UV-B irradiated plants stimulates both, phytosterol and triterpene biosynthesis. In any case, lupeol (3) and sterols, in addition to regulate fluidity and permeability of the membranes have demonstrated capacity to intercept free radicals (Bracco et al., 1981; Hartmann, 1998; Saleem et al., 2001). Although the biological function of lupeol (3) in plants is less clear, it may have protective functions. It has been shown in mammal tissues that lupane derivatives have antioxidant, anti-inflammatory and antiviral properties (Lee et al., 2007; Saleem et al., 2004, 2005a,b, 2008). Other studies indicate that lupeol (3) also plays a role in stabilization of lipids (Saratha et al., 2011) and changes the tissue redox balance by scavenging free radicals (Nagaraj et al., 2000; Sunitha et al., 2001). Thus, these triterpenes may protect leaf membranes against potential photo-oxidative damage generated by low UV-B as part of a mechanism for grape acclimation to UV-B radiation. When the UV-B dose was administrated at low fluence rate, the results suggested *de novo* synthesis of membrane-related sterols. In fact, the highest levels of sitosterol (1) and stigmasterol (2) were found in young leaves in correlation with high TPS activity. This type of reaction is consistent with an adaptative response that prepares tissues to cope with adverse environmental conditions. Notwithstanding, in previous experiments (Berli et al., 2010) it was found that such structural resistance throughout an enhancement of membrane sterols is more associated with ABA (11) effects than a direct consequence of UV-B action. Therefore, as ABA (11) levels were also increased by the low UV-B treatment, such levels may account for increase of membrane-sterols in young tissues.

On the other hand, UV-B treatment somewhat promoted synthesis of α-tocopherol (5), γ-tocopherol (4) and phytol (6), all diterpenes derived of the plastidic methylerythritol phosphate (MEP) pathway (Lichtenthaler et al., 1997). These antioxidant compounds prevent photo-oxidative deterioration of unsaturated fatty acids, lipids and lipoproteins in the cell membrane of plants by detoxification of ROS (Fahrenholtz et al., 1974; Neely et al., 1988), but are also involved in the modulation of membrane fluidity and permeability (Fryer, 1992). It is well known that tocopherols can scavenge ROS directly by reactions through hydroxyl radicals avoiding propagation of lipid peroxidation in thylakoid membranes, and forming a first line of defense against membrane photo-degradation (Asada, 1994; Foyer et al., 1994). Therefore, they are essentials components for the plant protection against environmental stress and light-induced disorders (reviewed by Fryer, 1992). In our experiments, these diterpenes were even more abundant in mature leaves and when the grapevine plants were irradiated with high UV-B. The antioxidant function of tocopherols may contribute to avoid irreversible damages of membranes keeping them functional after the UV-B treatment (Munné-Bosch and Alegre, 2000). Experimental evidence has shown that tocopherols confer a protective effect to the photosynthetic apparatus under thermal and oxidative stress conditions (Copolovici et al., 2005; Delfine et al., 2000), acting as

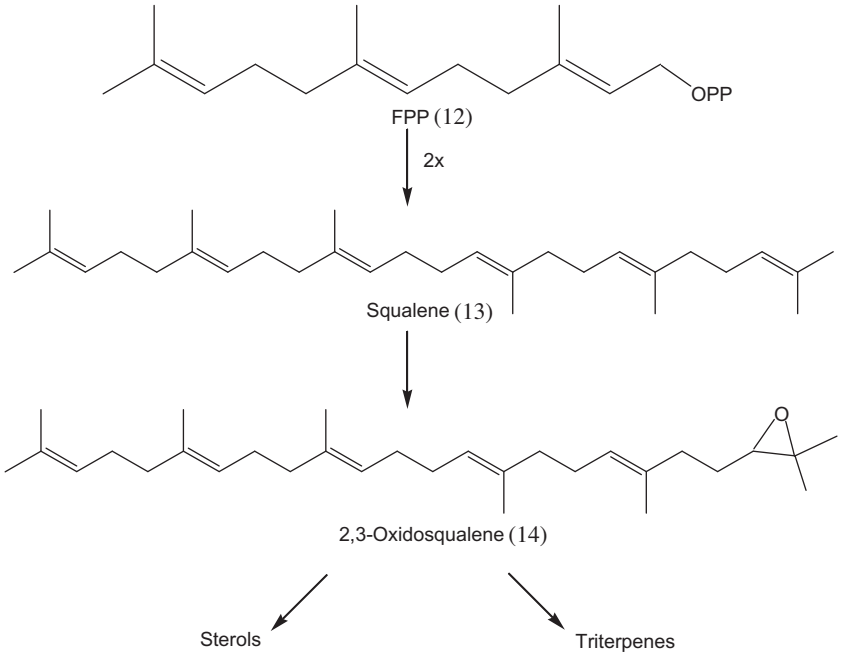

Fig. 8. A simplified scheme of potential fates of farnesyl diphosphate FPP (12).

antioxidants that protect the membranes against peroxidation and ROS (Loreto and Velikova, 2001; Loreto et al., 2001). Thus, the induction and accumulation of both tocopherols (4, 5) and phytol (6) could be interpreted as a mechanism to shorten duration of the "oxidative burst" and to protect grapevine tissues against high UV-B induced-ROS. These results are in agreement with the expression of genes involved in different antioxidant defense systems promoted by exposure of grape plants to high UV-B, such as genes encoding several heat shock proteins (with mitochondrial, chloroplastic, and cytoplasmic localization), different kind of lectins, and antioxidant enzymes (Pontin et al., 2010).

Among the terpenes identified and showing high variation under UV-B were the sesquiterpene E-nerolidol (7) and the monoterpenes α-pinene (8), terpinolene (9) and carene (10). These metabolites are produced by plants at low concentrations and their production was increased in the presence of UV-B, more associated with high UV-B and in young leaves. However, in the case of the monoterpenes, they were not detected in control plants suggesting that they were synthesized *de novo* as a result of UV-B elicitation. In this sense, in previous work, it was found that this radiation regulated expression of genes encoding TPS (Pontin et al., 2010). These substances are highly lipophylic and possess good antioxidative capacity in the lipophilic test systems. Graßmann et al. (2001, 2003) demonstrated that terpinolene (9) protects low-density lipoprotein from oxidation.

It is well-known that in vegetative tissues ABA (11) levels increase when plants are exposed to adverse environmental conditions, such as drought, salt and temperature stress (Crozier et al., 2000). In the present experiments, and in agreement with previous results (Berli et al., 2010) ABA (11) levels increased in response to UV-B following a dose–response behavior according to the fluence rate of UV-B and in the young leaves. However, no increase in expression of genes involved in ABA (11) synthesis was previously found (Pontin et al., 2010). Thus, increased ABA (11) levels could be explained by hydrolysis of glycosylated ABA from vacuoles in plants exposed to UV-B, since ABA (11) levels in UV-B treated tissues experiment a sharp decrease 24 h post irradiation, even respect to non-irradiated controls. In young leaves, ABA (11) levels decreased 21.2-fold when submitted to high UV-B and 9.4-fold in low UV-B irradiation, regarding the values obtained immediately after the irradiation treatments had finished. Furthermore, in mature leaves they decreased 5.7-fold in high UV-B and 8.3-fold at low UV-B irradiation. Such a decay in ABA (11) levels below the controls could be explained by an increase in its turnover, which correlate fairly well with the induction of a gene encoding ABA 8′-hydroxylase in UV-B treated plants (the major ABA catabolic pathway in higher plants; Pontin et al., 2010).

Several experiments have shown that volatile isoprenoids protect leaves against abiotic stresses as burst of heat (Delfine et al., 2000; Loreto et al., 1998; Sharkey and Singsaas, 1995; Singsaas et al., 1997) and ozone (Loreto et al., 2001), suggesting that isoprenoids are effective antioxidants in leaves. Also, they are proposed to act as chloroplast membranes stabilizers, a role that has been assigned to other isoprenoids, for example xanthophylls and α-tocopherol (5) (Havaux, 1998; Sharkey, 1996). Many volatile plant monoterpenes and sesquiterpenes combine rapidly with ROS (Calogirou et al., 1999), and their emission is stimulated by high light and temperature conditions (Delfine et al., 2000; Duhl et al., 2008). Thus, these compounds might also be involved in resistance to abiotic stress. In this study, treated plants showed that E-nerolidol (7), pinene (8), terpinolene (9) and carene (10) increased either under high UV-B as well as low UV-B irradiation when compared to control plants. These results are comparable to those obtained by Chapman (2009) in lemon verbena (*Aloysia citriodora*), where higher leaf concentrations of monoterpenes were found when grown without protection to UV-B exposure as compared to plants with UV-B exclusion. In the present work, maximum accumulation of both mono and sesquiterpenes was observed in plants treated with high UV-B. Thus, induction of these compounds could be interpreted as a mechanism in order to protect grapevine plants against UV-B-induced oxidative damage. Our results suggest that endogenous terpenes may have an important antioxidant role in plants since their levels are increased as the tissues are exposed to high UV-B irradiation.

4. Conclusions

The results obtained, along with others from a previous paper (Pontin et al., 2010) suggest that UV-B modulates the metabolism of terpenes leading to specific responses according to fluence rate of UV-B. Relatively low UV-B irradiation induces acclimation responses consisting in *de novo* synthesis of terpenes (sterols) related with membrane stability, and sesquiterpenes involved in defense against abiotic (like UV-B) and biotic stresses. While relatively high irradiance induces mechanisms of defense against the oxidative damage, diterpenes with antioxidant properties and ABA (11). Therefore, it can be accepted the hypothesis that high UV-B irradiation promoted in grapevine leaves production of plastidic terpenes via the methylerytritol phosphate (MEP; Lichtenthaler et al., 1997) pathway as a way to cope with ROS, while low UV-B induce synthesis of enzymes of terpene cytosolic MAV pathway leading to production of sterols and triterpenes involved in adaptation to stress.

5. Experimental

The general procedures to obtain plant material and the experimental design and treatments were previously reported in Pontin et al. (2010).

5.1. Plant material

Plant material was obtained from a virus-free vineyard of *Vitis vinifera* L. cv. Malbec; three-node wood cuttings were collected and treated with 100 mg L^{-1} indole-3-butyric acid (IBA, Sigma Chem. Co., St. Louis, MO, USA) in order to promote root development. Then explants were planted in 2.5 L plastic pots filled with hydrated-perlite and maintained under greenhouse conditions. From three-month old plants, uninodal cuttings were taken to obtain *in vitro* cultured plants. Shoots were surface sterilized by immersion in EtOH–H_2O (7:3, v/v) for 3 min followed by treatment with 1.5% NaOCl for 10 min with occasional swirling, and washing three times with sterilized distilled H_2O for 5 min. Explants (1–1.5 cm length) with one axillary bud were cultivated in glass tubes containing 20 mL of solid MS medium plus salts and vitamins, 30 g L^{-1} sucrose, 1 mg L^{-1} 6-benzylaminopurine (BAP), and 7.5 g L^{-1} agar. *In vitro* shoot tips were sub-cultured to fresh medium at 6 weeks intervals into half-strength MS micro- and macro-nutrients, excluding FeEDTA (0.1 mM Na_2EDTA + 0.1 mM $FeSO_4$) and supplemented with full-strength MS vitamins, 30 g L^{-1} sucrose, 0.5 μM 1-naphthaleneacetic acid and 7.5 g L^{-1} agar. Explants (one per glass flask, 12 cm height × 6.5 cm diameter) were incubated at 25 ± 2 °C and a photosynthetically active radiation (PAR) of 80 μmol m^{-2} s^{-1} provided by cool fluorescent tubes with a 16/8 h photoperiod. Flask tops were covered with low-density polyethylene, which transmitted most of the PAR.

5.2. UV-B treatment

UV-B treatments were carried out with 45 d old *in vitro* grown plants, having six fully expanded leaves, and in the same controlled growth chambers described above. For different light treatments,

supplemental UV-B was given using a TL 100W/01 tube (311 and 313 nm spectrum peaking; Philips, Eindhoven, The Netherlands) suspended 40 cm above the flasks. A 4.75 kJ m^{-2} d^{-1} total effective dose of UV-B normalized at 311 nm was provided in two different irradiation treatments: "low UV-B" (8.25 μW cm^{-2} irradiance during the 16 h per day photoperiod) and "high UV-B" (33 μW cm^{-2} irradiance during the last 4 h of the 16 h per day photoperiod). For controls, UV-B was filtered by a clear polyester (100 μm, Oeste Aislante, Buenos Aires, Argentina), which absorbs more than 95% of UV-B, and reduced 15% of PAR. PAR was measured with a Li-250 light meter with a Li-190 quantum sensor (Li-COR Inc., Lincoln, NE, USA) and UV-B irradiance was controlled at the top of the flask with a PMA2200 radiometer with a PMA2102 UV-B detector (Solar Light Company Inc., Glenside, PA, USA). After the treatments, three fully expanded apical leaves (young) and two basal leaves (mature) were harvested, weighed and used for the different analytical determinations. Three independent biological replicates ($n = 3$) were used for terpene analysis and TPS activity assays.

5.3. ABA (11) quantification

Samples of 100 mg leaf fr. wt were homogenized in a mortar with liquid nitrogen and 2 mL of MeOH:twice-distilled H_2O:H_3PO_4 (80:19:1, v/v/v). The extract was maintained over-night at 4 °C, and then centrifuged 10 min at 10,000*g*. The supernatant was added with [$^2H_6$]-ABA (100 ng; gift of J.D. Cohen, Department of Horticulture, University of Minnesota, Saint Paul, MN, USA) dissolved in MeOH (100 μL) as internal standard, and allowed 1 h in darkness and 4 °C for isotope equilibration. The solvent was evaporated in a Speed Vac at room temperature (Eppendorf-Concentrator Plus; Westbury, NY, USA). The residue was dissolved in 3 mL of twice-distilled H_2O pH 3 (1% H_3PO_4) and passed through a Sep-Pack C18 reversed phase cartridge (500 mg of material, Waters Associates, Milford, MA, USA). This elution was performed using *n*-hexane (2 mL) and MeOH (2 mL):twice distilled H_2O:H_3PO_4 (80:19:1, v/v/v). The last fraction was collected and after solvent evaporation in vacuo at room temperature, the residue was dissolved in twice-distilled H_2O (1 mL) at pH 7.0 (1% NH_4OH) and transferred to Oasis WAX (weak anion exchanger) cartridges (60 mg of material; Waters Associates). Then column was washed with 5% NH_4OH in MeOH (2 mL), MeOH (2 mL) and 3.5% H–CO_2H in MeOH (3 mL). The acidic eluate (which contained ABA) was evaporated at 35 °C and then converted to methyl-ester derivatives with MeOH (3 μL) plus fresh ethereal CH_2N_2 (5 μL) (30 min at room temperature). After organic solvents had been eliminated in a vacuum concentrator, samples were dissolved in *n*-hexane (50 μL), and 1 μL was injected split–splitless into a Perkin–Elmer Elite-5MS, crosslinked methyl silicone capillary column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness) fitted in a capillary gas chromatograph–electron impact mass spectrometer (GC–EIMS; Clarus 500, PerkinElmer, Shelton, CT, USA). The GC column was eluted with He (0.7 mL min^{-1}). The GC temperature program was 100–200 °C at 20 °C min^{-1}, then augmented to 280 °C at 4 °C min^{-1} and held for 15 min. The mass spectrometer was operated with electron impact ionization energy of 70 eV. The injector temperature was 230 °C, ion source temperature was 120 °C and the interface temperature was 150 °C. After performing selected ion monitoring (SIM) the amount of ABA was calculated by comparison of the peak areas of the major ions for the methyl-ester derivative of the deuterated internal standard [$^2H_6$]-ABA(194/166) relative to its non-labeled counterpart (190/162).

5.4. Diterpenes and triterpenes derivatives quantification

Samples of leaf fr. wt (100 mg) were ground to a fine powder using a mortar and pestle, and then macerated with $CHCl_3$:MeOH (2 mL, 1:1, v/v). The suspension was transferred to glass vials with Teflon-coated screw caps and allowed to extract over-night in darkness at 4 °C. The macerated residue was shaken and centrifuged 15 min at 10,000*g*. The supernatant was collected and evaporated to dryness in Speed Vac at room temperature. The residue was dissolved with *n*-hexane (500 μL) of and then, 1 μL was injected in split–splitless mode in a GC–EIMS system for analysis of terpenes. The analysis was carried out with the same column and equipment described above. The oven temperature program was: initial temperature at 60 °C for 1 min followed by an increase of 10 °C min^{-1} to 280 °C and held at 280 °C for 22 min. To estimate the concentration of the different metabolites, 50 ng μL^{-1} of *n*-hexadecane as internal standard (Supelco, Bellefonte, PA, USA) was added to each sample. The identities of compounds were confirmed by comparison of their retention times and full scan mass spectra with those of authentic standards, and with mass spectra of the National Institute of Standards and Technology (NIST) library.

5.5. Monoterpenes and E-nerolidol determinations

Samples of $CHCl_3$:MeOH (500 μL, 1:1, v/v) extract of above was placed in glass vials with Teflon-coated screw caps (1.5 mL), added with 500 μL of *n*-hexane, shaken and left over-night in darkness at 4 °C. From the hexane extract, an aliquot of 1 μL was injected in split–splitless mode in the GC–EIMS system, with the same conditions as for sterols and tocopherols determinations, except that the oven temperature program was: initial temperature at 45 °C for 1 min, followed by an increase of 2 °C min^{-1} to 130 °C, then from 130 to 250 °C at a rate of 20 °C min^{-1} and held for 10 min at 250 °C. The ionization potential was 70 eV and a range of 40–500 amu was scanned. Compounds were identified by comparison of retention times with a set of authentic standards, E-nerolidol (7), pinene (8), terpinolene (9), carene (10) obtained from Fluka (Sigma–Aldrich, Steinheim, Switzerland), and peak areas were referred to the standard *n*-hexadecane for quantification.

5.6. Terpene synthase activity measurements

Samples of leaf fr. wt. (100 mg) were homogenized in a cold-ice mortar with pestle and 1 M potassium phosphate buffer (800 μL, pH 6.5–7), 20% (w/v) glycerol, 10 mM sodium metabisulfite, 10 mM ascorbic acid, 15 mM $MgCl_2$, 0.5% PVP (insoluble polyvinyl polypirrolidone, Sigma Chem. Co., St. Louis, MO, USA, MW 40.000) and 1.47 mM 2-β mercapthoethanol. Each total protein homogenate was centrifuged 5 min at 14,000*g* and supernatant (10 μL) was incubated with 0.2 μM of radioactive *trans, trans*-farnesyl pyro-phosphate triammonium salt (40 μL, [1-3H]-FPP, specific activity 20.5 Ci mM^{-1}, Perkin Elmer, Boston, MA, USA), FPP (12) (0.14 μM of Sigma Chem. Co., St. Louis, MO, USA) as carrier, and reaction buffer containing 250 mM Tris:HCl (pH 6.5–7), 50 mM $MgCl_2$. The mixture was incubated at 30 °C and after 20 min the reaction was stopped with H_3PO_4 (10 μL, 85%). The reaction products were partitioned with *n*-hexane (150 μL) and reacted with silica gel powder (5 mg, 240–300 Mesh, Sigma Chem. Co., St. Louis, MO, USA). An aliquot of *n*-hexane (50 μL) was located in a scintillation vial with Fluka cocktail (4 mL) (Sigma Chem. Co., St. Louis, MO, USA) and radioactivity was determined by using a Tricarb liquid scintillation analyzer (Perkin Elmer, Illinois, USA). TPS activity was expressed as nmol [^{3}H]-FPP transformed mg of protein^{-1} h^{-1} according to Vögeli and Chapell (1988). The protein concentration of the extract was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (Bio-Rad Laboratories, Philadelphia, PA, USA) as standard.

5.7. Statistical analysis

Statistical evaluations were performed using the software Statgraphics Centurion XVI version 15.0.10 (Statpoint Technologies Inc., Warrenton, VA, USA). Significance of differences was conducted with LSD of Fisher test. Differences were considered significant at a probability level of $P \leqslant 0.05$. The effect of UV-B, leaf ontogeny and their interaction were determinate by multifactorial ANOVA. Results are reported as a mean of three independent replicated assays, and each experiment was repeated three times. Principal component analysis (PCA) was applied to the data set, and performed with the *InfoStat* software (*InfoStat version 2008*. Grupo InfoStat, Argentina). The results of this analysis are then presented as a two-dimensional graphical display of the data (*Biplots*).

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