Abscisic acid is involved in the response of grape (*Vitis vinifera* L.) cv. Malbec leaf tissues to ultraviolet-B radiation by enhancing ultraviolet-absorbing compounds, antioxidant enzymes and membrane sterols

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ABSTRACT

We investigated the interactions of abscisic acid (ABA) in the responses of grape leaf tissues to contrasting ultraviolet (UV) treatments. One-year-old field-grown plants of *Vitis vinifera* L. were exposed to photosynthetically active radiation (PAR) where solar UV-B was eliminated by using polyester filters, or where PAR was supplemented with UV-B irradiation. Treatments combinations included weekly foliar sprays of ABA or a water control. The levels of UV-B absorbing flavonols, quercetin and kaempferol were significantly decreased by filtering out UV-B, while applied ABA increased their content. Concentration of two hydroxycinnamic acids, caffeic and ferulic acids, were also increased by ABA, but not affected by plus UV-B (+UV-B) treatments. Levels of carotenoids and activities of the antioxidant enzymes, catalase, ascorbate peroxidase and peroxidase were elevated by +ABA treatments, but only if +UV-B was given. Cell membrane β-sitosterol was enhanced by ABA independently of +UV-B. Changes in photoprotective compounds, antioxidant enzymatic activities and sterols were correlated with lessened membrane harm by UV-B, as assessed by ion leakage. Oxidative damage expressed as malondialdehyde content was increased under +UV-B treatments. Our results suggest that the defence system of grape leaf tissues against UV-B is activated by UV-B irradiation with ABA acting downstream in the signalling pathway.

Key-words: oxidative damage; phenolic compounds.

INTRODUCTION

Solar ultraviolet (UV)-B radiation (wavelength range 280–315 nm) is mostly attenuated by stratospheric ozone and other atmospheric gases, so that only small amounts reach the earth’s surface. UV-B intensity varies over time and location, mainly because of changes in the solar angle and thickness of the ozone layer. Thus, as elevation increases the air mass decreases and there is a greater atmospheric transparency, especially with regard to shorter wavelength radiation, although local environmental conditions, such as clouds, can also modulate irradiation intensity (Madronich et al. 1995).

Even relatively small amounts of UV-B induce diverse morphological, physiological and biochemical responses in higher plants. UV-B is potentially harmful depending on the intensity, total dosage, plant species and the balance between UV-B and photosynthetically active radiation (PAR, 400–700 nm; Day 2001; Frohnmeyer & Staiger 2003; Kakani et al. 2003). However, experiments with unrealistic (i.e. different from that of natural environment) balances between UV-B radiation, UV-A radiation (315–400 nm) and PAR may exaggerate the effects of UV-B (Björn 1996; Caldwell & Flint 1997; Allen 1998). Also, it has been postulated that UV-A and visible light can induce both protective and repair mechanisms, thus reducing damage by UV-B (Jordan et al. 1992). Nevertheless, relatively high intensities of UV-B irradiation can result in overproduction of free radicals, namely reactive oxygen species (ROS), which cause oxidative damage to macromolecules such as DNA, proteins and lipids (Foyer, Lelandais & Kunert 1994). Lipid peroxidation, a widely used stress indicator, is promoted by UV-B and increases in membrane permeability are indicative of a disruption of membrane integrity (Dai et al. 1997; Alexieva et al. 2001). UV-B can also damage thylakoids and hamper synthesis of chlorophylls (Chl) and carotenoids (Day, Howells & Ruhland 1996). Plant growth and harvest yield can be reduced by ambient levels of UV-B, apparently as a result of UV-B-induced reduction in leaf expansion (Ballaré et al. 1996; Pinto et al. 1999). Notwithstanding the above, photosynthetic efficiency was found to be rather insensitive to solar UV-B under field conditions, or when plants are subjected to ‘realistic’ supplemental UV-B treatments (Searles, Caldwell & Winter 1995).
Moderate levels of supplemental UV-B can stimulate transcription of genes involved in protective responses (Brosché & Strid 2003 and references included therein), and relatively high levels of solar UV-B enhance the accumulation of UV-absorbing compounds, mainly flavonoids and related phenolics (Berli et al. 2008). UV-B is also known to trigger the expression of genes encoding phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). These are regulatory enzymes of the phenylpropanoid and flavonoid biosynthetic pathways (Jenkins, Fuglevand & Christie 1997; Jansen, Gaba & Greenberg 1998; Casati & Walbot 2003). These secondary metabolites accumulate in the vacuoles of epidermal cells and effectively absorb radiation from wavelengths in the UV-B range (Frohnmeyer & Staiger 2003). These are thus postulated to reduce UV-B transmittance and protect the photosynthetic apparatus in the leaf mesophyll (Burger & Edwards 1996; Mazza et al. 2000). Also, modifications in the plant’s architecture and morphology, such as increases in leaf thickness, number of epidermal cell layers, epicuticular wax and pubescence, are observed after UV-B irradiation (Semerdjieva et al. 2003).

To cope with increased ROS, such as superoxide radicals (O$_2^-$), hydroxyl radicals (·OH), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (O$_2$) and nitric oxide (NO·), it appears that plant cells have developed an efficient antioxidant defence system. This system involves both enzymatic and non-enzymatic mechanisms. The former are represented by antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX) and glutathione reductase (GR) (Karabal, Yücel & Ökte 2003), along with increases in antioxidant molecules as tocopherols, ascorbic acid, glutathione and carotenoids. Carotenoids play an important role in the light-harvesting complex, not only extending the range of light absorbed by the photosynthetic apparatus, but also by photoprotecting the photosystems (Ort 2001). They quench triplet state Chl molecules and scavenge singlet oxygen and other toxic ROS that are formed within the chloroplast (Ong & Tee 1992), thus dissipating the excess of excitation energy under stress conditions (Young 1991).

Abscisic acid (ABA) is a phytohormone associated with the plant’s responses to a range of abiotic stresses as drought, high temperature, chilling and salinity, and increases in ABA levels are postulated to regulate adaptation to these environmental stresses (Zhu 2002; Assmann 2005). However, studies dealing with the interaction between ABA and UV-B are scarce, particularly in regard to the question of whether ABA can mediate the plant’s tolerance to enhanced UV-B radiation (Duan et al. 2008). It has been observed that ABA controls stomatal closure of guard cells (Leung & Giraudat 1998), including grape (Stoll, Loveys & Dry 2000). In grape applied ABA reduces vegetative growth (Dry, Loveys & Düring 2000), but in Vitis paraguayensis ABA enhances dry matter accumulation (Sansberro, Mroginski & Bottini 2004). In wheat ABA increases leaf carotenoid content and allocation of carbohydrate in grains (Travaglia et al. 2007). In grape ABA application was also shown to induce synthesis of polyphenols (Jeong et al. 2004), and result in sugar accumulation in the berries (Pan et al. 2005), as well as increased fruit yield (Quiroga et al. 2009). There is also evidence that ABA induces accumulation of ROS in plant cells as second messengers for the activation of defensive responses (Sakamoto, Matsuda & Iba 2008). This ABA signal also induces the expression of genes encoding SOD, CAT and APX (Jiang & Zhang 2001) as well as the enhancement of the non-enzymatic defence systems (antioxidant molecules, Jiang & Zhang 2002).

Malbec is the cultivar of choice for most Argentine red wines and in the Mendoza region its cultivation extends from altitudes of 500 m to more than 1500 m. Such variations in altitude account for different fluence rates and dosages of UV-B reaching vineyard plants (Berli et al. 2008).

This paper presents results that support the hypothesis that ABA is causally involved in protective responses by leaf grape plant tissues to UV-B irradiation and that ABA does this by enhancing both the enzymatic and non-enzymatic response systems.

**MATERIAL AND METHODS**

**Plant material and treatments**

The experiment was carried out at Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina (33°0′S, 68°52′W) at an altitude of 940 m. One-year-old plants of a selected clone of *Vitis vinifera* L. cv. Malbec were cultivated under a UV protection cover (low-density polyethylene; 100 μm). After 3 months under the above conditions, the leaves were removed and the plants were pruned to the fifth bud from the base of the shoot. They were located under field conditions and allowed to break buds and grow for 1 month in a completely randomized block design, in a 2 x 2 factorial arrangement of treatments with five blocks (experimental unit two plants).

**Minus UV-B treatments**

Solar UV-B radiation was filtered to produce a minus UV-B (−UV-B) treatment, by using a canopy of 100 μm clear polyester (PE) filters (Oeste Aislante, Buenos Aires, Argentina). This PE filter absorbed more than 95% of UV-B, affecting ca. 30% of UV-A and 15% of PAR.

**Plus UV-B treatments**

Solar UV-B radiation was supplemented with an additional dose of 15 μW cm$^{-2}$, over a 5 h period (from 1100 to 1600 h) centred on solar noon, using two UV-B fluorescent lamps (TL 100 W/01; Philips, Nieuwegein, the Netherlands), suspended above the plants. These lamps emitted UV-B at a narrow spectrum of 310–315 nm and a maximum at 311 nm. This supplemental UV-B treatment was performed in order
to mimic the UV-B irradiance received by vineyards at ca. 1500 m, the altitude at which the most highly regarded vineyards are located in the Mendoza region. To minimize differences between the –UV-B and plus UV-B (+UV-B) treatments with regard to wind, temperature or humidity under plastic sheeting, we placed a 40 μm low-density polyethylene (PET) cover over the +UV-B-treated plants. This low-density PET transmitted most of the solar radiation (ca. 75% of UV-B, 80% of UV-A and 85% of PAR).

ABA treatment
Starting from bud-break a 1 mM solution of ±-cis, trans abscisic acid (90%, Kelinon Agrochemical Co., Beijing, China) containing 0.1% Triton X-100 and a minimum amount of 96% aqueous ethanol (to initially dissolve the ABA, ca. 10 μL mg⁻¹) was sprayed weekly onto the leaves to run-off (five applications). Sprays were accomplished in the evening to minimize photodegradation. This ABA dose was chosen according to previous work with a range of species (Sansberro et al. 2004; Travaglia et al. 2007) including grape (Quiroga et al. 2009).

Control treatment
A solution containing distilled water plus 0.1% Triton X-100 and a minimum amount of ethanol as described above was sprayed weekly as described above.

The two oldest (1-month-old and fully expanded) leaves from the base of the shoot of each plant were collected at midday the day after the last ABA application. One was cooled with ice and immediately processed to assess protein content, antioxidant enzymatic activity and membrane integrity. The other leaf was quickly frozen at -20 °C for subsequent evaluation of photosynthetic and photoprotective pigments, phenolic compounds, sterols and lipid peroxidation.

Light measurements
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 and UV-B, respectively. Figure 1 shows the solar radiation (PAR and UV-B) received on a typical sunny summer day at elevations of 1450 and 940 m, plus the UV-B supplemental irradiation to which the plants of the +UV-B treatment were submitted. However, the UV-B fluence rates and doses did vary somewhat among the experiments, depending on the meteorological conditions (data not shown).

ABA quantification
Each sample, consisting of 200 mg fresh weight (FW) of grape leaf (1 month old and fully expanded), was homogenized in a mortar with liquid nitrogen and extracted with 2 mL of 80:19:1 (v/v) methanol : twice-distilled water : acetic acid at 4 °C. After 12 h, 40 ng of hexa-deuterated ([2H₆]-ABA (a gift from Professor R.P. Pharis, University of Calgary, Canada) dissolved in 2 μL of methanol was added for ABA quantification and allowed 1 h equilibration of the isotopes. Then, the sample was centrifuged 10 min at 9300 g and the supernatant evaporated in a rotavapour with vacuum at 35 °C. The aqueous residue was adjusted to pH 3.0 with acetic acid and partitioned four times with equal volumes of ethyl acetate saturated with 1% acetic acid. After solvent evaporation in vacuum at 35 °C, the residue was dissolved in 1 mL of water at pH 3.0 (1% acetic acid) and passed through Sep-Pak C18 reversed phase (500 mg of material) cartridge (Waters Associates, Milford, MA, USA). This elution was performed at a flow rate of 0.2 mL min⁻¹ using the following gradient: 1 mL each one of twice-distilled water pH 3.0, hexane and 80% aqueous methanol in 1% acetic acid. The entire eluate was collected, and after solvent evaporation in vacuum at 35 °C, the residue was
was dissolved in 1 mL of water at pH 3.0. This solution was transferred to Oasis WAX (weak anion exchanger, 60 mg of material) cartridges (Waters Associates) in a gradient of 1 mL of each of 5% NH₄OH in methanol, 5% formic acid in methanol and finally 2% formic acid in methanol. The acidic eluate (which contains the ABA) was evaporated at 35 °C and vacuum and then converted to the methyl-ester (Me) derivatives with 10–20 μL of methanol plus 50–100 μL of fresh ethereal CH₂N₂ (30 min at room temperature). After solvents had been eliminated under a gentle flow of N₂ at room temperature, Me samples were dissolved in ethyl acetate, and 1 μL was injected split–splitless into a HP-5 cross-linked methyl silicone capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) fitted in a capillary gas chromatograph-electron impact mass spectrometer (GC/MS) (Clarus 500, PerkinElmer, Shelton, CT, USA). The GC column was eluted with He (1 mL min⁻¹). The GC temperature programme was 100 °C to 260 °C at 20 °C min⁻¹, then 10 min at 260 °C. The mass spectrometer was operated with electron impact ionization energy of 70 eV. The injector temperature was 230 °C, ion source temperature was 260 °C and the interface temperature was 280 °C. After performing selected ion monitoring (SIM) the amount of ABA was calculated by comparison of the peak areas of the major ions selected ion monitoring (SIM) the amount of ABA was and the interface temperature was 280 °C. After performing temperature was 230 °C, ion source temperature was 260 °C then 10 min at 260 °C. The mass spectrometer was operated under a gentle N₂ stream. The solid residue was diluted under a gentle flow of N₂ at room temperature. Absorbance of the extracts was read with 0.6 mL of acidified ethyl acetate. Then, extracts were pooled, dried by filtering through a glass conic mini-column packed with NaSO₄, and evaporated to dryness under a gentle N₂ stream. The solid residue was diluted with 30 μL of dry pyridine (Flucka, Steinheim, Germany) and 70 μL of N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) 1% TCMSi (Sigma Chem. Co., St. Louis, MO, USA). After 75 min at 70 °C an aliquot of 1 μL of derived extract was injected and analysed by GC/MS. The GC/MS analysis was carried out with a PerkinElmer Model Clarus 500 with the same conditions as for ABA determinations, except that the oven temperature programme was: initial temperature at 80 °C for 1 min, then from 80 to 250 °C at a rate of 20 °C min⁻¹, and held for 1 min, then augmented at 6 °C min⁻¹ to 300 °C, and held for 2 min, and finally increased at 20 °C min⁻¹ to 320 °C, and held for 24 min. The identity of each phenolic and sterol compounds was established by retention times on capillary GC (see Fig. 3) and the full mass spectra of silylated standards performed in the range from 120 to 700 m/z (quercetin, kaempferol, caffeic acid, p-coumaric acid and ferulic acid; Sigma Chem. Co.) and from the National Institute of Standards and Technology (NIST) library (sterols). The Total Ion Chromatogram of quercetin (the most abundant metabolite studied) was used as a reference compound for quantification. A six-point calibration curve was obtained for quercetin, the standard solutions contained 15% (v/v) methanol and were subjected to the same extraction-derivation procedure described for the samples. The quantification was performed by relating the peak areas of the identified compounds in each sample and was calculated from the calibration plot.

**Total UV-absorbing phenolic compounds and anthocyanins (photoprotective pigments)**

Two leaf discs (1 cm² each) were placed in 2 mL of 99:1 (v/v) methanol : HCl and allowed to extract 72 h in darkness and at room temperature. Absorbance of the extracts was read at 305 or 546 nm for determinations of total UV-absorbing compounds or anthocyanins, respectively, according to Mazza et al. (1999), with a Cary-50 UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA, USA) with 1 and 10 mm optical path cells.

**Characterization and quantification of phenolic compounds and sterols**

The extraction and derivation procedure was performed according to Minuti, Pellegrino & Tesei (2006), with modifications as follows. Grape leaves were frozen in liquid N₂ and ground to a fine powder using a mortar and pestle. Twenty milligrams of frozen leaf powder was placed in a centrifuge tube and sonicated (200 W, Cleanson CS-1106, Buenos Aires, Argentina) with 0.6 mL acidified ethyl acetate (0.2% of 37% HCl v/v) and centrifuged for 5 min at 9300 g. Supernatant was collected and the pellets extracted twice with 0.6 mL of acidified ethyl acetate. Then, extracts were pooled, dried by filtering through a glass conic mini-column packed with NaSO₄, and evaporated to dryness under a gentle N₂ stream. The solid residue was diluted with 0.2% of 37% HCl v/v) and centrifuged for 5 min at 9300 g. Supernatant was collected and the pellets extracted twice with 0.6 mL of acidified ethyl acetate. Then, extracts were pooled, dried by filtering through a glass conic mini-column packed with NaSO₄, and evaporated to dryness under a gentle N₂ stream. The solid residue was diluted

**Photosynthetic pigments**

For determinations of Chl a, Chl b and carotenoids, one leaf disc (1 cm²) was placed in 5 mL of dimethyl sulphoxide (DMSO) and allowed to extract for 45 min in darkness at 70 °C. Absorbance was read at 665, 649 and 480 nm against a blank of reagents, according to Chapelle, Kim & McMurtrey (1992) and the formulas of Wellburn (1994) as follows: Chl a = 12.19 OD₆₆₅ - 3.45 OD₆₄₉; Chl b = 21.99 OD₆₄₉ - 5.32 OD₅₄₉; carotenoids = (1000 OD₄₃₀ - 2.14 Chl a - 70.16 Chl b)/220; Total Chl = Chl a + Chl b.

**Membrane integrity**

Ten leaf discs (1 cm² each) were washed three times with twice-distilled water to remove surface-adsorbed electrolytes, and then placed in vials containing 30 mL of twice-distilled water. They were incubated in darkness for 24 h at 8 °C. The electrical conductivity of the solution (Cₑ) was measured, after equilibration at 25 °C, with a pH/CON 510 meter (Oakton Instruments, Vernon Hills, IL, USA). Samples were then heated for 1 h at 80 °C, cooled and kept in darkness for 16 h at 8 °C. Then, the electrical conductivity of heat-killed tissues (Cₑ) was measured at 25 °C. Membrane integrity was calculated according to Vásquez-Tello et al. (1990) as follows: relative membrane integrity = [1 – (Cₑ/Cₑ)] × 100.

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**Lipid peroxidation**

In order to evaluate oxidative damage as lipid peroxidation, malondialdehyde (MDA) content was measured following the procedure described by Beligni & Lamattina (2002). Seventy-five milligrams of leaf tissue was suspended in 1.5 mL of stock solution [15% (w/v) trichloroacetic acid (TCA), 0.5% (w/v) thiobarbituric acid (TBA) and 0.25% (w/v) hydrochloric acid]. The mixture was stirred vigorously and incubated at 95 °C for 60 min. After centrifugation at 9300 g for 10 min, the supernatant was collected. Absorbance of the extracts was measured at 535 nm with 1 mm optical path cell. The concentration was calculated considering MDA molar extinction coefficient = 1.56 × 10⁵ M⁻¹ cm⁻¹.

**Protein content and antioxidant enzyme activity**

Samples of 250 mg fresh weight leaf were homogenized using a mortar and pestle with 5 mL of extraction solution (100 mm potassium phosphate buffer pH 7.5; 0.1% Triton X-100; 1 mm EDTA; 0.5 mm ascorbic acid) at 5 °C and 0.25 g of insoluble polyvinylpyrrolidone (PVPP), and centrifuged for 5 min at 9300 g. Supernatant was collected and frozen at −20 °C until assayed for proteins and antioxidants. Protein content was determined according to Bradford (1976) with bovine serum albumin (BSA) as standard, measuring the absorbance at 595 nm.

The CAT activity was assayed by monitoring the consumption of H₂O₂ at 240 nm in 2.5 mL reaction mixture containing 50 mm potassium phosphate buffer (pH 7.5) and 75 μL of sample. The reaction was started by adding 100 μL of 34.8 mm H₂O₂. The mixture was stirred vigorously and incubated at 95 °C for 60 min. After centrifugation at 9300 g for 10 min, the supernatant was collected. Absorbance of the extracts was measured at 535 nm with 1 mm optical path cell. The concentration was calculated considering MDA molar extinction coefficient = 1.56 × 10⁵ M⁻¹ cm⁻¹.

The APX activity was measured by the decrease in absorbance of ascorbate at 290 nm in 2.5 mL reaction mixture containing 100 mm potassium phosphate buffer (pH 7.0), 1 mm EDTA, 0.5 mm ascorbic acid and 1 mm H₂O₂. The reaction was started by adding 40 μL of the sample and changes in absorbance were followed for 60 s according to Barka (2001).

The POX activity was assayed by monitoring the oxidation of guaiacol to tetraguaiacol at 470 nm in 2.5 mL reaction mixture containing 50 nm potassium phosphate buffer (pH 6.0), 1.2 mm H₂O₂ and 2 mm guaiacol. The reaction was started by adding 100 μL of the sample and changes in absorbance were followed for 60 s as stated by Zhang & Kirkham (1994).

**Statistical analysis**

Statistical analyses were performed as a randomized block with a 2 × 2 factorial arrangement of treatments with the software Statgraphics Centurion XV version 15.0.10 (Statpoint Technologies Inc., Warrenton, VA, USA). The effect of UV-B, ABA application and their interaction were determined by multifactorial ANOVA.

**RESULTS**

UV-B increased the content of ABA (as assessed by GC/MS/SIM quantification) in grape leaves 2.7-fold, ABA treatment 3.6-fold, and combined effect of UV-B and ABA 12.5-fold (Fig. 2a).

No statistical difference was observed in the Chl content, both total (Table 1) or Chl a and Chl b (data not shown), and in carotenoids content. Simultaneous action of UV-B and applied ABA showed the highest carotenoids content, although only significant differences were obtained at P ≤ 0.1 for UV-B treatments (Table 1). The content of total UV-absorbing compounds was increased by UV-B and applied ABA with a significant interaction between UV-B and ABA (Table 1) meaning that ABA effects are UV-B dependent. Anthocyanin levels were also increased by interactive action of UV-B and applied ABA (Table 1). Figure 2b shows the different leaf pigmentation for each treatment.

The UV-absorbing compounds characterized by GC/MS were flavonols (quercetin and kaempferol) and the hydroxycinnamic acids (caffeic acid, ferulic acid and p-coumaric acid). Figure 3 shows the GC/MS profiles of the flavonols and sterols. The quercetin concentration was approximately 20-fold higher than kaempferol, and the...
levels of both were significantly increased by UV-B and applied ABA treatment (Table 2). There was a significant interaction between UV-B and ABA in kaempferol concentration, indicating that ABA promotion of kaempferol is UV-B dependent. These results also correlate well with the spectrophotometric determinations of total UV-absorbing compounds (Table 1). In samples of all treatments the concentration of caffeic acid was approximately fivefold higher than that of p-coumaric acid, and 20-fold higher than that of ferulic acid (Table 2). Caffeic acid and ferulic acid were significantly increased by exogenously applied ABA, although there was no significant effect of ABA application on the p-coumaric acid concentration. None of the hydroxycinnamic acids was affected by filtering out UV-B.

The activity of CAT was significantly increased by applied ABA, but there was no significant effect of filtering out UV-B (Table 3). The activity of APX was promoted by UV-B (relative to the treatment where UV-B was filtered out) and there was a significant interaction between UV-B and ABA. The activity of POX was also affected by the interaction between UV-B and ABA. This suggests that ABA promotion of APX and POX activities is UV-B dependent.

The sterols β-sitosterol and stigmasterol were identified by capillary GC/MS analysis and in all treatments the β-sitosterol content was approximately fivefold higher than that of stigmasterol. Membrane integrity and β-sitosterol content were significantly improved by ABA application, but were not affected by filtering out UV-B (Table 4). Oxidative damage, as assessed by MDA concentration, was significantly decreased by filtering out UV-B. Stigmasterol content was not affected by any of the treatments (data not shown).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total Chl</th>
<th>Carotenoids</th>
<th>UV-absorbing compounds</th>
<th>Anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>+UV-B</td>
<td>ABA</td>
<td>30.293 ± 2.860</td>
<td>6.862 ± 0.727</td>
<td>0.631 ± 0.050</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>25.599 ± 1.720</td>
<td>5.847 ± 0.443</td>
<td>0.527 ± 0.006</td>
</tr>
<tr>
<td>−UV-B</td>
<td>ABA</td>
<td>28.586 ± 2.915</td>
<td>5.515 ± 0.397</td>
<td>0.339 ± 0.007</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>26.576 ± 3.106</td>
<td>5.297 ± 0.445</td>
<td>0.336 ± 0.018</td>
</tr>
</tbody>
</table>

Table 1. Total chlorophylls (Chl) (μg cm⁻² leaf), carotenoids (μg cm⁻² leaf), total ultraviolet (UV)-absorbing compounds (OD₃65 cm⁻² leaf) and anthocyanins (OD₅46 cm⁻² leaf) in grape leaves of plus UV-B (+UV-B) and minus UV-B (−UV-B) treatments either sprayed or not (control) with abscisic acid (ABA).
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**DISCUSSION**

Although UV-B represents only a small portion of solar radiation that reaches the earth surface, its impact on biological processes is considered of great importance (Björn, Widell & Wang 2002; Jordan 2002). That is, exhaustive research has shown that ROS production is triggered in several cell compartments when plant tissues are exposed to UV-B, and as a result oxidative damage of proteins and cell membranes may occur (Scandalios 1993). Our study was performed in a natural environment, and while an increase in MDA content was observed, which reflects lipid peroxidation and therefore some level of stress, membrane damage and photooxidation of Chl in leaves exposed to UV-B irradiances were not observed. The maintenance of Chl levels in the UV-B treatments was rather unexpected, as Pfundel (2003) reported that UV-B was responsible for photosystem II (PSII) inhibition (i.e. pigment bleaching and inhibition of overall photosynthesis). However, in Pfundel’s experiments the grape leaves had been adapted to growth in darkness prior to UV-B treatments. Similar inhibitory effects had been also reported in maize tissues along with decreases in photosynthetic pigments, gas exchange, Rubisco activity and total sugar content (Correia et al. 2005), although these UV-B effects were obtained after nitrogen deficiency. Thus, the different results found in previous studies compared with our results may indicate different experimental conditions and/or genetic differences and/or differences in developmental stages or tissues.

The increase in UV-absorbing compounds, including anthocyanins, is another response that is postulated to protect cell membranes, as the increased levels of phenolic compounds, as assessed by gas chromatograph-electron impact mass spectrometer (GC/MS) analyses, in mg g⁻¹ fresh leaf of plus ultraviolet (UV)-B (+UV-B) and minus UV-B (−UV-B) treatments either sprayed or not (control) with abscisic acid (ABA) (ABA mediates grape response to solar UV-B).

Table 2. Content of quercetin, kaempferol, caffeic acid, ferulic acid and p-coumaric acid, as assessed by gas chromatograph-electron impact mass spectrometer (GC/MS) analyses, in mg g⁻¹ fresh leaf of plus ultraviolet (UV)-B (+UV-B) and minus UV-B (−UV-B) treatments either sprayed or not (control) with abscisic acid (ABA)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Quercetin</th>
<th>Kaempferol</th>
<th>Caffeic acid</th>
<th>Ferulic acid</th>
<th>P-coumaric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>+UV-B</td>
<td>ABA</td>
<td>2.006 ± 0.229</td>
<td>0.120 ± 0.010</td>
<td>0.630 ± 0.085</td>
<td>0.025 ± 0.002</td>
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<tr>
<td></td>
<td>Control</td>
<td>1.476 ± 0.183</td>
<td>0.053 ± 0.008</td>
<td>0.298 ± 0.067</td>
<td>0.019 ± 0.001</td>
</tr>
<tr>
<td>−UV-B</td>
<td>ABA</td>
<td>0.940 ± 0.084</td>
<td>0.036 ± 0.002</td>
<td>0.437 ± 0.074</td>
<td>0.027 ± 0.002</td>
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<tr>
<td></td>
<td>Control</td>
<td>0.700 ± 0.047</td>
<td>0.032 ± 0.004</td>
<td>0.285 ± 0.039</td>
<td>0.022 ± 0.002</td>
</tr>
</tbody>
</table>

Table 3. Catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) activities (nm min⁻¹ mg⁻¹ protein) in grape leaves of plus ultraviolet (UV)-B (+UV-B) and minus UV-B (−UV-B) treatments either sprayed or not (control) with abscisic acid (ABA)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CAT</th>
<th>APX</th>
<th>POX</th>
</tr>
</thead>
<tbody>
<tr>
<td>+UV-B</td>
<td>ABA</td>
<td>84.979 ± 6.625</td>
<td>314.725 ± 29.763</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>46.405 ± 14.297</td>
<td>213.761 ± 15.953</td>
</tr>
<tr>
<td>−UV-B</td>
<td>ABA</td>
<td>62.880 ± 5.453</td>
<td>181.594 ± 12.512</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>44.047 ± 7.416</td>
<td>217.294 ± 35.875</td>
</tr>
</tbody>
</table>

Table 4. Membrane integrity (in %), and β-sitosterol (mg g⁻¹ fresh leaf) and malondialdehyde (MDA) (ng g⁻¹ fresh leaf) content in grape leaves of plus ultraviolet (UV)-B (+UV-B) and minus UV-B (−UV-B) treatments either sprayed or not (control) with abscisic acid (ABA)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Membrane integrity</th>
<th>β-Sitosterol</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>+UV-B</td>
<td>ABA</td>
<td>96.776 ± 0.529</td>
<td>0.3840 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>95.392 ± 0.769</td>
<td>0.277 ± 0.020</td>
</tr>
<tr>
<td>−UV-B</td>
<td>ABA</td>
<td>96.909 ± 0.562</td>
<td>0.380 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>95.328 ± 0.704</td>
<td>0.256 ± 0.014</td>
</tr>
</tbody>
</table>

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compounds are correlated with a more efficient absorption of harmful UV-B (Frohnmeyer & Staiger 2003; Merzlyak et al. 2008). Such phenolics transform short-wave, high-energy and highly destructive radiation into longer-wavelength light and these wavelengths are less destructive to the cellular leaf structures, including the photosynthetic apparatus (Bilger, Johnsen & Schreiber 2001). Additionally, phenolic compounds may enhance protection against oxidative stress, as they possess chemical structures capable of scavenging free radicals; for example, they have also been found to be effective antioxidants (Blokhina, Virolainen & Fagerstedt 2002). In many higher plant tissues, two classes of phenolics appear to be involved in epidermal UV screening: the hydroxycinnamic acids and the more complex flavonoids. These compounds vary from species to species and according to developmental stages and tissues. Our results with grape leaves showed that the UV-absorbing compounds found in the leaf tissue were flavonols (quercetin and kaempferol) and hydroxycinnamic acids (caffeic acid, p-coumaric acid and ferulic acid). However, only quercetin and kaempferol were significantly enhanced by +UV-B treatments. Quercetin is a more efficient ROS-scavenger than kaempferol, due to its increased number of hydroxyl groups; moreover, we found that quercetin’s concentration in grape leaf tissues was approximately 20-fold higher than kaempferol. Such results are consistent with the finding of increased expression of genes involved in the biosynthesis of flavonols and phenylpropanoids in grape plants submitted to UV-B radiation (Pontin et al. 2008). The accumulation of phenolic compounds that selectively and passively absorb UV radiation in the epidermis probably represents the most cost-effective strategy for adaptation in the case of regular and prolonged exposure to elevated doses of UV-B, in that it should reduce the need for maintaining continuously active avoidance and repair processes (Cockell & Knowland 1999; Liakoura, Bornman & Karabourniotis 2003). Kolb et al. (2001) also found enhanced synthesis of flavonoids, specifically the flavonols quercetin and kaempferol in grape leaves exposed to UV-B. Núñez-Olivera et al. (2006) obtained an increase in the concentrations of Chl and carotenoids and a reduction in UV-absorbing compounds in grape plants of the cultivar Tempranillo grown under UV-B exclusion. It is worth noting, however, that solar UV-B seems to cause some damage in Tempranillo grapevines (purportedly susceptible). This did not happen with Malbec (more tolerant), given that our +UV-B treatments maintained Chl levels and actually increased carotenoids levels.

Antioxidant enzymatic activity could partially explain why membrane integrity was not affected by +UV-B when ABA was applied, as the activities of CAT, APX and POX were higher with this treatment. In effect, it has been reported that POX is induced by light in grapevine (Ros Barceló et al. 2003) and ABA may induce the biosynthesis of these enzymes (Jiang & Zhang 2001). While ABA can increase ROS production and induce the activities of SOD, CAT, APX and GR in plants (Jiang & Zhang 2001; Sakamoto et al. 2008), these studies considered that ABA is mainly an enhancer of oxidative damage to plants. In contrast, our results show that increased CAT activity was caused by ABA treatment independently of UV-B, while the activities of APX and POX were increased by ABA when +UV-B was present.

Cell membrane integrity, evaluated by its electrolyte leakage, may be a good indicator of stress tolerance in plants (Vásquez-Tello et al. 1990). In our experiments a positive correlation was obtained between this variable and the defence system induced by ABA; for example, membrane integrity was increased after ABA application with or without the use of +UV-B treatments. This enhancement in membrane integrity was associated with an increase in the membrane content of β-sitosterol. Plant membranes include several sterols in their structure, where sitosterol usually predominates (Hartmann 1998; Nomura et al. 1999) and our results confirm this conclusion.

Overall, our results strongly suggest that ABA is involved in stress tolerance of grape leaves and that they function by enhancing the ability of epidermal tissues to filter out UV-B. That is, they increase levels of antioxidant enzymes and enhance the sterol-structural defence, although these responses are, in some cases, also dependent on the co-presence of high levels of UV-B irradiation. In other words, the antioxidant defence system in grape leaf tissues is initially activated by UV-B irradiation and ABA often acts downstream in the signalling pathway (although some responses are activated by ABA alone). The first line of defence likely includes elevated levels of flavonols and hydroxycinnamic acids; these filter part of the UV-B. We found that quercetin and kaempferol were increased by +UV-B treatments, with an additional increase being seen when ABA was also co-applied with the +UV-B treatments. In contrast, increases in caffeic acid and ferulic acid concentration were triggered only by ABA. It is postulated that a part of the UV-B-origin energy excess is dissipated by carotenoids. In our experiments carotenoids levels were enhanced by +UV-B treatments alone, although one has to remember that +UV-B treatment also increased endogenous ABA concentrations in the leaf tissue.

The antioxidant enzymatic system (CAT, APX and POX) is also postulated to contribute to the attenuation of UV-B-induced stress situation, and its activity is enhanced by exogenously applied ABA, but only under the +UV-B treatments (APX and POX). Finally, an extra structural resistance to UV-B-induced damages includes an enhancement of membrane sterols that, in our study, are exclusively augmented by ABA but not by UV-B itself.

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