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UV screening by phenolics in berries of grapevine (*Vitis vinifera*)

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Abstract. The role of phenolics in UV-screening was investigated in berries of a white grape cultivar (*Vitis vinifera* L. cv. Bacchus). Fluorescence microscopy revealed accumulation of phenolics in the skin of berries and, by high performance liquid chromatography and mass spectrometry, flavonols and hydroxycinnamic acids were identified as the main groups of UV-absorbing phenolics. Relationships between natural radiation and the synthesis of phenolics were studied in plants that were cultivated in the absence of UV radiation in a greenhouse before outdoor exposure to three different light regimes: the entire solar spectrum, the solar spectrum minus UV-B radiation and only visible radiation. During six days of exposure, flavonol synthesis was significantly stimulated by natural UV, in particular UV-B, but concentrations of hydroxycinnamic acids decreased under all conditions. Direct comparison of fluorimetrically-determined skin absorbance with absorbance of extracted flavonols or hydroxycinnamic acids suggested that acclimation of UV screening depends almost exclusively on flavonol synthesis. While increased flavonol levels resulted in efficient UV-A shielding, UV-B shielding was incomplete, probably due to decreased levels of the UV-B-absorbing hydroxycinnamic acids during exposure.

Keywords: acclimation, caffeic acid, flavonoid, HPLC, kaempferol, microscopy, quercetin, UV-A, UV-B, viticulture.

Introduction

Plant photosynthesis is fuelled by the sun's visible radiation. When harvesting visible radiation, plants are also exposed to ultraviolet (UV) radiation in the wavelength range from approximately 290–400 nm. Within this range, radiation belonging to the UV-B band (280–315 nm) shows a particularly high potential to damage lipids, nucleic acids and proteins (Jordan 1996; Vass 1997; Hollósy 2002). During the extreme summer of 1998, grapes harvested in German vineyards for the nationally important white-wine industry were severely browned and shriveled (Mohr 1998; Mohr and Düring 2000): this phenomenon was attributed to overheating of the berries (*cf*. Müller-Thurgau 1883; Pool 1988; Schultz *et al*. 1999). While there have been many studies of the effects of UV radiation on leaves (Searles *et al*. 2001) there has been little or no investigation of the effects of UV, especially UV-B, radiation on fruit including grape berries. In this paper, therefore, we decided to extend our previous studies on grapevine leaves (Kolb *et al*. 2001; Pfündel 2003) to grape berries and we found that different responses to UV exposure exist between these two grapevine tissues which appear relevant to the fruit damage observed in German vineyards in 1998.

One of the general strategies against UV damage observed in most groups of photosynthetic organisms is to screen out UV radiation with UV-absorbing compounds (Cockell and Knowland 1999; Rozema *et al*. 2002). In leaves of many higher plants, phenylpropanoid derivatives serve as UV screens: the hydroxycinnamic acids, exhibiting a C_6-C_3 carbon skeleton, and the flavonoids, having a C_{15} backbone (Cockell and Knowland 1999; Winkel-Shirley 2001). These phenolics can be accumulated in the epidermis to shield the photosynthetic mesophyll tissue underneath (Caldwell *et al*. 1983; Tevini *et al*. 1991; Bornman and Teramura 1993; Jordan 1996). Within a leaf, however, epidermal UV-screening is variable and appears to be increased in response to high UV-B intensities (Robberecht and Caldwell 1978; Robberecht *et al*. 1980; Caldwell *et al*. 1983; Flint *et al*. 1985).

The hypothesis that UV-B radiation improves UV-screening by stimulating synthesis of UV screens has been supported by an action spectrum for flavonoid synthesis in

Abbreviations used: *F*, chlorophyll fluorescence; UV-A, radiation from 315–400 nm; UV-B, radiation from 280–315 nm; V, exposure to visible radiation; VA, exposure to visible plus UV-A; VAB, exposure to visible plus UV-A plus UV-B radiation.

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parsley cell cultures, which peaked at 295 nm (Beggs and Wellmann 1994). Also, exposure to artificial UV radiation increased flavonoid concentrations in leaves (Tevini *et al*. 1991; Olsson *et al*. 1998). It is important to note that these findings were obtained under radiation conditions which were largely unrelated to the natural situation, in which UV-B radiation is always accompanied by much higher intensities of both UV-A (315–400 nm) and visible radiation (Green 1983). Hence, effects of artificial UV-B need to be confirmed outdoors (Caldwell *et al*. 1994; Fiscus and Booker 1995; Deckmyn and Impens 1997). Therefore, it has been essential to show in UV-B exclusion experiments in the field that indeed current levels of UV-B radiation stimulate flavonoid synthesis (Lingakumar *et al*. 1999; Mazza *et al*. 1999; Kolb *et al*. 2001).

Increasing flavonoid levels in the leaf, however, do not necessarily correspond with improved epidermal UV screening because considerable fractions of flavonoids are located in the mesophyll in some plant species (Burchard *et al*. 2000; Rozema *et al*. 2002; Semerdjieva *et al*. 2003). Studies combining flavonoid quantification with measurements of epidermal transmission for UV radiation, however, have indicated that flavonoid levels increased by UV radiation result in improved UV screening (Burchard *et al*. 2000; Mazza *et al*. 2000; Bilger *et al*. 2001; Kolb *et al*. 2001; Markstädter *et al*. 2001; Krause *et al*. 2003). This is consistent with the view that natural UV-B intensities reduce epidermal UV transmittance by stimulating synthesis of epidermal flavonoids.

Flavonoids, especially those exhibiting an *ortho*-dihydroxyl grouping, in addition to their role in UV screening, are also potent antioxidants (Torel *et al*. 1986; Husain *et al*. 1987; De Beer *et al*. 2002; Yamasaki *et al*. 2003). The antioxidant action of flavonoids might play a role in UV stress management because UV-B radiation specifically induced synthesis of *ortho*-dihydroxyl flavonoids (Markham *et al*. 1998*a*; Olsson *et al*. 1998; Hofmann *et al*. 2003). Indeed, the combination of UV absorption with antioxidative function enhances the roles of flavonoids engaged in UV screening because reactive oxygen species created by UV-excited flavonoids could be efficiently quenched (Cockell and Knowland 1999).

While flavonoid-dependent screening includes the UV-B and frequently exhibits its absorption maximum well in the UV-A range (315–400 nm), UV-shielding by hydroxycinnamic acids is confined to UV-B and the short-wavelength UV-A (see e.g. Kolb *et al*. 2001). The degree to which hydroxycinnamic acids support flavonoid screening, however, differs markedly between species; they contribute minimally to UV-B screening in leaves of *Vicia faba* (Markstädter *et al*. 2001), but considerably in *Arabidopsis thaliana* and grapevine (Landry *et al*. 1995; Sheahan 1996; Kolb *et al*. 2001). Further, in primary leaves of rye (*Secale cereale*), the importance of hydroxycinnamic acids for UV screening depends on the developmental stage (Burchard *et al*. 2000). Also, depending on the species studied, hydroxycinnamic acids either are largely unaffected by radiation conditions (Tevini *et al*. 1991; Burchard *et al*. 2000) or they are considerably increased by high visible radiation (Kolb *et al*. 2001). It remains to be established if high levels of hydroxycinnamic acids confer elevated UV-B resistance of leaves.

Despite the considerable progress made in understanding general properties and heterogeneity of UV screening in leaves, information on UV screening in fruits is sparse. Since both leaves and fruits are exposed to UV radiation, one might assume that UV shielding is also present in fruits. However, anatomy, histology and physiology differ markedly between fruit and leaf and, therefore, the extent to which features of UV screening are shared between the two plant organs is unknown. Recently, we studied UV shielding in grapevine leaves in great detail (Kolb *et al*. 2001). In this paper, we investigate white grape berries to discover if UV screening ability is identical in the fruit as in leaves and found that similarities exist for flavonols but not for hydroxycinnamic acids; the possible consequences of these organ-specific differences on UV-B screening are discussed.

Materials and methods

Plants and radiation exposure

Three-year old grafted vines (*Vitis vinifera* L. cv. Bacchus) were grown in pots of commercially-available soil (Einheitserde Typ T, Einheitserde Werkverband, Sinntal-Jossa, Germany) in a shaded glasshouse as described by Kolb *et al*. (2001). Three groups of eight vines representing 23–27 bunches were exposed to three different radiation regimes in the field in July 2001 (location: 49.8° N, 9.9° E; altitude: 200 m). The sugar contents of berries (data not shown) indicated that the grapes investigated had already passed veraison and thus, had entered the final phase of ripening (*cf.* Currle *et al*. 1983).

The vines were placed in three different types of growth boxes constructed with roofs and walls of foils that transmitted (1) natural radiation, or (2) natural radiation with UV-B (280–315 nm) screened out, or (3) natural radiation with UV-A (315–400 nm) plus the UV-B spectral range screened out. These three regimes are denoted 'VAB', 'VA', and 'V', respectively. Spectral data of these three radiation regimes have been reported by Kolb *et al*. (2001). During the 6-d exposure interval, mostly cloudless conditions prevailed and the ELDONET dosimeter located 80 km away at the University of Erlangen (Lebert *et al*. 2002) reported daily doses of 6.4 MJ m^{-2}, 1.5 MJ m^{-2} and 45 kJ m^{-2} for the visible, UV-A and UV-B spectral range, respectively. By comparison, UV radiation in the greenhouse was virtually absent, and visible light intensity was 15% of the outdoor intensity.

In general, exposed grapes from the upper third of bunches were examined, and 2 d before exposure, any leaves that shaded bunches were removed. Initial conditions were established 1 d before exposure. At 2100 h on each day of exposure one grape from each bunch was selected for measurements of epidermal UV-A transmittance using a portable UV-A-PAM chlorophyll fluorimeter (Gademann Messgeräte, Würzburg, Germany). At days 3 and 6, one grape of one randomly selected bunch per plant was cut off at the peduncle and stored for at least 1 h in a dark and moist container before measurements of skin transmittance for UV-A and UV-B radiation with a Xe-PAM

fluorimeter (Walz, Effeltrich, Germany). The berry caps of 2 mm thickness used for these measurements were subsequently frozen in liquid nitrogen and stored at –80°C for chromatographic analysis.

Epidermal UV-A screening of radiation-exposed *v.* non-exposed sides of grapes was studied with plants exposed in our foil boxes during late June and early July 2000 under mostly cloudless conditions, and also with vineyard-grown vines during August 2002. In both experiments, only berries that had been acclimated for 3 weeks were investigated. Acclimation in the vineyard was started by mounting filter foils above the bunch zone and removing any leaves that shaded bunches. During vineyard exposure, weather conditions fluctuated and radiation doses below 50% of the maximum values reported above occurred during 6 d of the experiment.

UV screening

Epidermal transmittance for UV radiation was determined with an Xe-PAM fluorimeter (Walz) as described earlier (Bilger *et al*. 1997; Kolb *et al*. 2001). The outer side of berry caps was exposed at an angle of 45° to both the excitation source and to the fluorescence detector. Chlorophyll fluorescence at the F_0 level was elicited by UV-B ($F_{UV\text{-}B}$: 314 nm, bandwidth 24 nm), UV-A ($F_{UV\text{-}A}$: 360 nm, bandwidth 28 nm) or blue-green radiation (F_{BG}: 490 nm, bandwidth 165 nm), and the fluorescence at wavelengths > 690 nm was measured. In a similar manner to leaf experiments (Markstädter *et al*. 2001; Kolb *et al*. 2001), transmittance of UV-B and UV-A radiation through the grape skin, denoted $T_{UV\text{-}B}$ and $T_{UV\text{-}A}$, respectively, was estimated by normalizing quotients of fluorescence excited from the grape surface to fluorescence quotients obtained from the opposite side of berry caps (mesophyll tissue):

$$T_{UV\text{-}B} = 100(F_{UV\text{-}B}/F_{BG})/(F_{Mes,UV\text{-}B}/F_{Mes,BG}) \quad (1)$$

$$T_{UV\text{-}A} = 100(F_{UV\text{-}A}/F_{BG})/(F_{Mes,UV\text{-}A}/F_{Mes,BG}) \quad (2)$$

where $F_{Mes,UV\text{-}B}$, $F_{Mes,UV\text{-}A}$, and $F_{Mes,BG}$ denote the fluorescence from the naked berry pulp excited by UV-B, UV-A or blue-green radiation, respectively. During berry acclimation, only minor variations in $F_{Mes,UV\text{-}A}/F_{Mes,BG}$ and $F_{Mes,UV\text{-}B}/F_{Mes,BG}$ were observed (data not shown). Hence, changes in $F_{UV\text{-}A}/F_{BG}$ and $F_{UV\text{-}B}/F_{BG}$ are expected to be mostly independent of changes in spectral properties of chlorophyll but mainly reflect variations of skin UV transmittance. Skin absorbance was derived from transmittance using the exponential relationship between the two parameters.

Epidermal UV-A transmittance was also estimated using a newly developed portable UV-A-PAM fluorimeter (Gademann Messgeräte) which excites F_0 chlorophyll fluorescence by diodes emitting radiation at 375 nm (10 nm bandwidth) and at 470 nm (25 nm bandwidth). Fluorescence was detected at wavelengths > 650 nm. Here, the ratio of fluorescence excited at 375 and 470 nm was used to estimate UV screening. Instead of authentic mesophyll, a fluorescence standard (Walz) with emission properties roughly comparable to mesophyll tissue was employed to estimate epidermal transmittance. Because of the different UV-A excitation wavelengths, UV-A transmittance measured with the Xe-PAM fluorimeter was lower but proportional to that obtained with the UV-A-PAM instrument (data not shown). In this work, UV-A-PAM results were corrected by an experimentally determined proportionality factor to yield values which are directly comparable to Xe-PAM data.

HPLC

Berry caps were freeze-dried and homogenised to fine powder in a 5-mL Teflon sample flask of a Mikro-Dismembrator II equipped with an agate grinding ball (B. Braun Melsungen, Melsungen, Germany). After adding 250 µL of extraction medium (50% (v/v) aqueous methanol containing 0.01% (w/v) phosphoric acid and 30 µg mL^{-1} quercetin as internal standard) the flask was shaken for 30 s at low speed. The extract was collected, and the flask and grinding ball were washed twice with 250 µL of extraction medium. The pooled extracts and washings were centrifuged for 10 min at 20 000 *g* at 4°C. The resulting pellet was extracted twice more at room temperature with 125 µL of extraction medium and the pooled supernatants were clarified for chromatography by centrifugation (5 min at 20 000 *g* at 4°C).

Phenolics were analysed on a LiChrospher-100 RP18 column (see Kolb *et al*. 2001). Elution started with a linear decrease of solvent A (0.01% w/v H_3PO_4) from 80% (v/v) to 66% (v/v) over a period of 7 min, followed by isocratic elution for 5 min. Solvent B was methanol (0.1 % w/v:H_3PO_4 (9:1 v/v). A decrease to 56% (v/v) of solvent A then occurred within 2 min, to 40% (v/v) during a further 18 min, and then to 35% during another 3 min. Finally, 100% solvent B was reached during a 2 min gradient followed by isocratic elution for 5 min.

Chromatograms were recorded at 314 and 360 nm which represent the maxima of the UV-B and UV-A excitation windows of the Xe-PAM fluorimeter, respectively. Concentrations of hydroxycinnamic acids were derived from the absorbance at 314 nm using the linear relationships between pure phenolic compounds and absorbance (Kolb *et al*. 2001); quercetins and kaempferols were determined at 360 nm using calibration lines (r^2>0.99) established with quercetin-3-*O*-glucoside (Extrasynthèse, Genay, France) and kaempferol-3-*O*-glucoside (Roth, Karlsruhe, Germany), respectively.

Molar concentrations (cP) of a phenolic compound (P) were normalized to sample dry weight (dw) using the quercetin added to the extraction solvent as internal standard (free quercetin has not been detected in grapes):

$$cP = A_{P,LC}(\lambda)F_P(\lambda)[A_{Std}(\lambda)/A_{Std,LC}(\lambda)](V/dw), \quad (3)$$

where $A_{P,LC}(\lambda)$ and $F_P(\lambda)$ represent the area of the HPLC peak of P at wavelength λ and the HPLC calibration factor at λ for P, respectively. The peak areas of the quercetin standard in chromatograms of the pure extraction solvent and of grape extracts are designated $A_{Std}(\lambda)$ and $A_{Std,LC}(\lambda)$, respectively. The parameter V signifies the total volume used for extraction of one berry cap (i.e. 1 mL).

For mass spectrometry, the analytical system consisted of an Agilent 1100 Series HPLC (Agilent, Waldbronn, Germany) and a 1100 Series LC/MSD trap mass spectrometer (Type SL, Agilent). The system was operated by the ChemStation software version 4.1 for LC and LC/MS (Agilent). Separations was on a ZORBAX Eclipse XDB-C8 column (150 × 4.6 mm, 5 µm particle size; Agilent).

Elution started with a linear decrease of solvent A (0.3% v/v formic acid) from 85% (v/v) to 65% (v/v) over a period of 20 min. Solvent B was acetonitrile. All UV-absorbing substances were effectively eluted during this initial gradient, and no additional UV-absorbing compounds were eluted with subsequent decrease to 15% of solvent A over a further 20 min. The flow rate was 0.5 mL min^{-1} and only HPLC grade chemicals obtained from Sigma (Praha, Czech Republic) were used.

Mass spectra were acquired with the Agilent Ion Trap SL mass spectrometer equipped with an electrospray ionisation source (ESI) operated in the positive mode. The ESI parameters were: spray needle voltage, 4.5 kV; nebulizer, N_2 at 50 psi; drying gas, N_2 delivered at 10 L min^{-1}; drying temperature, 325°C; ESI capillary voltage, 230 V. The scan range at full scan mode was 50–2200 m/z and scan speed 13 000 m/z per sec. Characteristic ions were used for peak assignment.

Microscopy

Caps from freshly-harvested grape berries were embedded in Jung Tissue Freezing Medium (Leica Microsystems, Bensheim, Germany). The specimen were frozen and cross sections of 50 µm thickness were prepared using a cryomicrotome (CM1900, Leica Microsystems)

operated at –14°C. Samples were examined in 0.5% (w/v) ammonia to elicit blue-green fluorescence from phenolics (Hutzler *et al*. 1998). Transmission and fluorescence images were obtained using a Leica DMR microscope equipped with the Leica fluorescence cube D that excites between 355 and 425 nm and detects fluorescence at wavelengths > 470 nm. Images were recorded with a Leica DC500 digital camera system controlled by the Leica IM1000 Image Manager version 1.20.

Statistics

Data were analysed by Two Way Analysis of Variance (Sigma Stat for Windows Version 2.03, SPSS, München, Germany) with radiation and time as the treatment factors in acclimation experiments, and radiation and grape orientation as the treatment factors when side-specific effects of radiation were considered. In the case of statistical significance, groups of data were compared pairwise using the Student–Newman–Keuls method. Linear regression analysis was performed with Sigma Stat for Windows. Statistically significant differences were concluded for P values < 0.05 and the term 'significant' is thus defined throughout the text.

Results

Light microscopy shows that the grape skin consists of several cell layers (Fig. 1*A*) as described previously (Alleweldt *et al*. 1981). Green fluorescence was particularly intense in the skin. demonstrating that phenolics are especially accumulated in this tissue (Fig. 1*B*). The green fluorescence was unevenly distributed in the skin, which

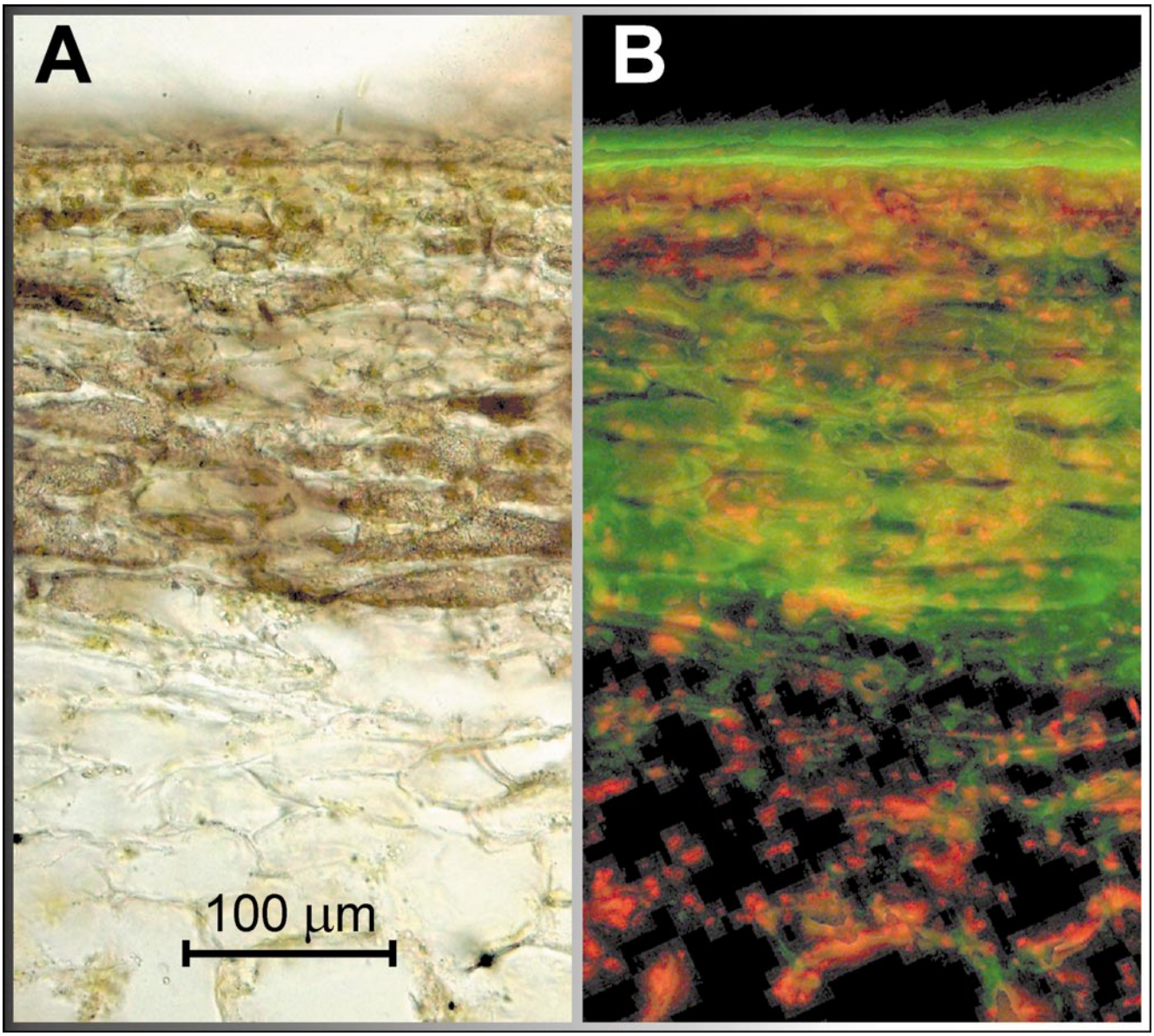

Fig. 1. Micrographs of a cross-section of outer grape tissue. The figure shows a light transmission image (*A*) and a fluorescence image (*B*) of a cross section through the radiation-exposed skin region of a grape berry. The specimen was treated with ammonia to enhance green fluorescence from phenolic compounds. The red fluorescence arises from chlorophyll.

supports the view that phenolics are mostly confined to vacuoles (Day *et al*. 1993; Hutzler *et al*. 1998). Photosynthetic pigment-protein complexes, as indicated by red chlorophyll fluorescence, are present in both skin and pulp.

Chromatograms of UV-absorbing phenolics obtained with berries grown in the greenhouse and after 6 d of acclimation to VAB conditions are shown in Fig. 2. Compared with the data from greenhouse-grown berries, exposure to VAB conditions reduced heights of peaks labelled 1–3, but new peaks 4–7 and B1–B4 appeared. The latter four peaks were observed in grape berries but not in grape leaves. The spectral properties and retention time of the substances giving rise to peaks 1–7 matched those of major phenolics in grape leaves (see Kolb *et al*. 2001). We conclude, from comparison with our previous work, that the phenolics producing peaks 1–3 are hydroxycinnamic acids,

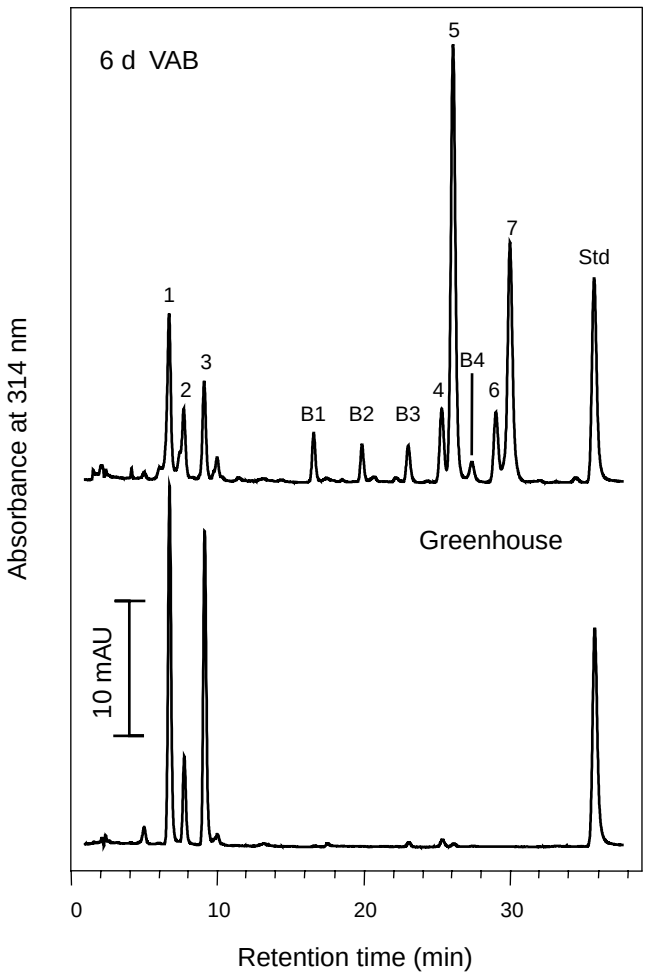

Fig. 2. Chromatography of phenolic compounds. Chromatograms show unprocessed absorbance at 314 nm *vs* retention times of methanolic extracts from a green-house grown grape berry (Greenhouse), and from a grape berry exposed for 6 d to VAB conditions (6 d VAB). Peaks labelled 1–7 were also observed in grape leaves but peaks B1–B4 were only found in grape berries (see text). 'Std' denotes the internal standard, free quercetin.

namely, *trans*-caffeic acid, *cis*-coumaric acid and *trans*-coumaric acid. Further, peaks 4–7 are produced by the flavonols quercetin (peaks 4 and 5) and kaempferol (peaks 6 and 7).

This was confirmed by mass spectroscopy; peak 1 yielded m/z ratios of 163, 313 and 335 corresponding to free caffeic acid minus H_2O $[M+H–H_2O]^+$, caffeoyl-tartaric acid (caftaric acid) $[M+H]^+$, and caftaric acid plus Na^+ $[M+Na]^+$. Peaks 2 and 3, which are separated in Fig. 2 but not in the HPLC method used for mass spectroscopy, exhibited m/z ratios of 147, 279, 297 and 319 being consistent with $[M+H–H_2O]^+$ of free coumaric acid, $[M+H]^+$ of coumaroyl-tartaric acid (coutaric acid), $[M+H–H_2O]^+$ of coutaric acid, and $[M+Na]^+$ of coutaric acid, respectively. Major ions of peak 4 exhibited m/z ratios of 303 and 611 which agree with $[M+H]^+$ ions of free quercetin and quercetin-deoxyhexose-hexose, respectively. That peak 5 includes quercetin-hexose is derived by m/z ratios of 465 and 303. Peak 5 also exhibited ions with m/z ratio of 479 suggesting the presence of a quercetin-3-hexuronide. Major m/z values of peak 6 were 287, 449, 471 being consistent with $[M+H]^+$ of kaempferol, $[M+H]^+$ of kaempferol hexoside and $[M+Na]^+$ of kaempferol hexoside, respectively. In addition to the latter three m/z values, peak 7 exhibited an m/z ratio of 463 indicating the presence of a kaempferol hexuronid.

We were unable to identify minor peaks B1–B4 which exhibited UV spectral properties similar to the flavonoid naringenin (not shown). As these four peaks remained minor components under all conditions accounting, on average, for less than 7% and 3% of the chromatographically-detected absorbance at 314 nm and 360 nm, respectively (data not shown), further studies focused only on hydroxycinnamic acids and flavonols. Exposure to outdoor conditions decreased concentrations of caffeic and coumaric acids in a similar fashion but differences between radiation regimes were not significant (Figs 3*A*, *B*). In obvious contrast, quercetin and kaempferol increased during outdoor exposure and concentrations of both flavonols differed significantly between treatments: highest and lowest concentrations were observed under the VAB and V conditions, respectively (Figs 3*C*, *D*).

Fluorimetrically determined transmittance for UV-B and UV-A radiation of berry skins is depicted in Fig. 4. Three days of exposure to VA and VAB conditions, but not to V conditions, decreased UV-B transmittance significantly (Fig. 4*A*). Transmittance of UV-A radiation was significantly decreased by all exposure conditions but the initial decrease was most pronounced under VAB and least under V conditions; significant differences between all three outdoor conditions existed at day 3 (Fig. 4*B*). That acclimation of UV-A transmittance was mostly confined to exposed sides was demonstrated using the UV-A-PAM technique (Fig. 4*C*). After 3 weeks of acclimation to V conditions, exposed sides of grapes exhibited lower UV-A transmittance

than non-exposed sides; however, VA and VAB conditions decreased UV-A transmittance of radiation-exposed sides of grapes significantly more than was observed under V conditions. The differences between exposed sides of grapes under VA or VAB regimes, and also the differences between non-exposed sides under all three radiation regimes, were not significant.

The relationship between fluorimetrically determined UV absorbance of grape skins and UV absorbance of extracted phenolics per dry weight is depicted in Fig. 5. Positive linear correlations existed between absorbance of skins and extracted flavonoids in both the UV-B and UV-A spectral range (Figs 5*A*, *B*). In contrast, variations in skin absorbance were independent of absorbance of hydroxycinnamic acids in the UV-B range and exhibited only a weak negative relationship in the UV-A range (Figs 5*C*, *D*).

Discussion

The present work introduces data on acclimation and capacity of UV screening in white grape berries. To our knowledge, this is the first report analysing actual UV-transmittance and its relation to concentration of phenolic sunscreens in fruits. For our studies, we chose grape berries, to compare characteristics of UV screening in fruits with

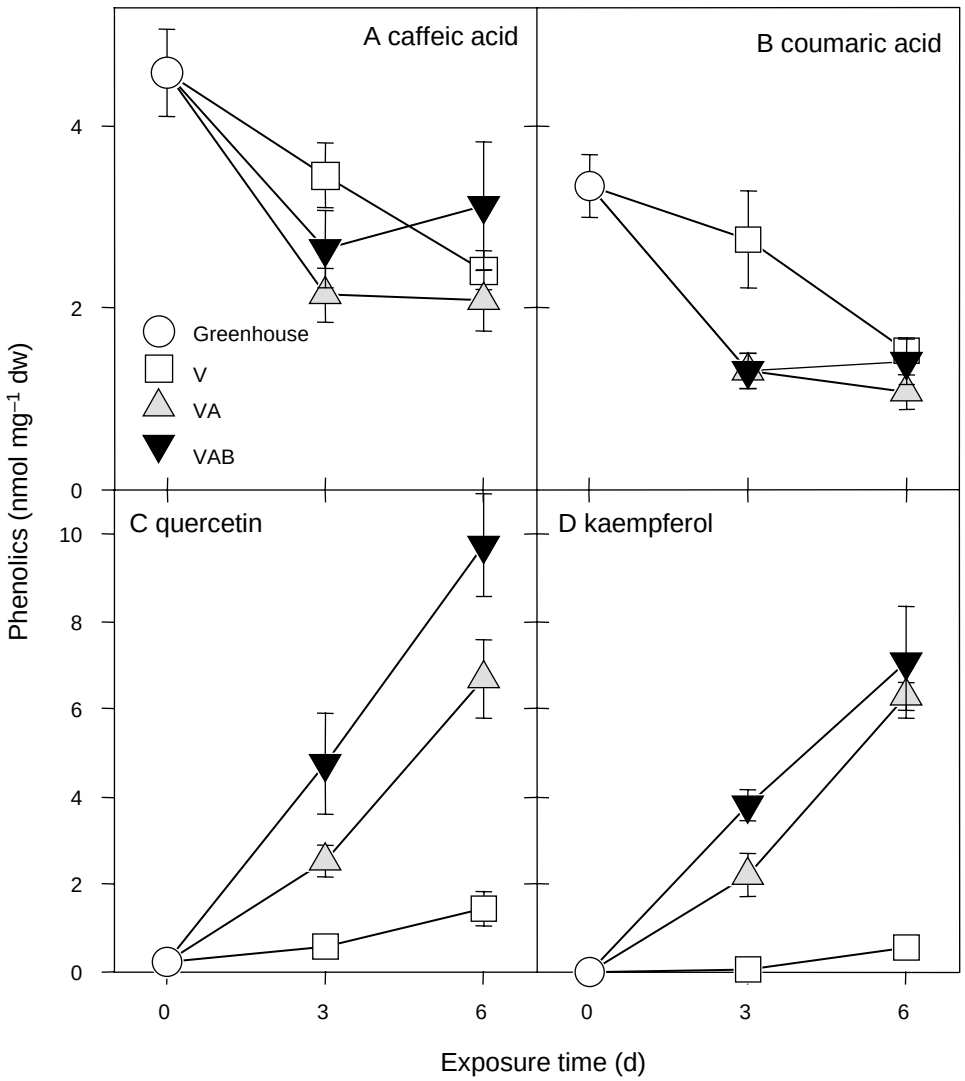

Fig. 3. Effects of outdoor exposure on concentration of phenolics. Concentrations of phenolic compounds normalized to the dry weight of berry caps of radiation-exposed sides of grape are shown. (*A*) Caffeic acid (peak 1 in Fig. 2), (*B*) coumaric acid (peaks 2 and 3 in Fig. 2), (*C*) quercetin (peaks 4 and 5 in Fig. 2), and (*D*) quercetin (peaks 6 and 7 in Fig. 2). In this and also in subsequent figures, open circles, open squares, grey upright triangles and closed inverted triangles represent data from greenhouse-grown grapes measured before outdoor exposure, and from grapes exposed to V, VA, and VAB, conditions, respectively. Bars indicate standard errors of means (n = 7 and 20 for each outdoor condition and for the greenhouse, respectively). Where small standard errors occur, bars are sometimes hidden by symbols.

those of leaves in a species that we have characterised in detail (Kolb *et al*. 2001).

The characteristics of the peripheral tissues of grape berries and grape leaves differ: berries exhibit a multi-layered skin (Fig. 1*A*) but leaves of grapevine and of many flowering plants species possess a single-layered epidermis (Caldwell *et al*. 1983; Kolb *et al*. 2001). Figure 1*B* shows that differences in histology are paralleled by differences in distribution of phenolics; in berries, fluorescence microscopy detected an accumulation of phenolics within the entire skin region, which also exhibited chlorophyll fluorescence, but phenolics are mostly confined to the chlorophyll-free epidermis in grape leaves (Kolb *et al*. 2001). This suggests that different forms of UV shielding may also occur in the grape berry and grape leaf. In the leaf, a UV filter is mounted in front of photosynthetic tissue but in berries phenolic UV screening appears to be situated within peripheral photosynthetic tissue.

The principal phenolic groups in berries, hydroxycinnamic acids and flavonols have been identified (Fig. 2). Identification of individual compounds using mass spectrometry agreed with earlier data from white grape berries (Okamura and Watanabe 1981; Singleton and Trousdale 1983; Vrhovsek 1998; Lu and Foo 1999). Levels of hydroxycinnamic acids decreased during acclimation to all three radiation regimes (Figs 3*A*, *B*), which is in sharp contrast to stimulation of hydroxycinnamic acids observed in grape leaves under similar experimental conditions (Kolb *et al*. 2001). Similar to leaves, UV radiation and, in particular that of the UV-B range, elicited in berries an increase of the B-ring *ortho*-dihydroxylated quercetin and the B-ring *mono*-hydroxylated kaempferol (Figs 3*C*, *D*). Also, like grape leaves, concentration ratios of quercetin to kaempferol in berries were much smaller when UV was present during exposure than under V conditions (Fig. 3). Obviously, grapevine differs from *Marchantia, Petunia* and *Oryza* species in which UV exposure increased ratios of *ortho*-dihydroxylated to *mono*-hydroxylated flavonoids with only moderate effects on total flavonoid levels and, hence, on flavonoid-dependent UV absorption (Markham *et al*. 1998*a*, *b*; Ryan *et al*. 1998). As antioxidative capacity is increased in *ortho*-dihydroxylated compared to *mono*-hydroxylated flavonoids (see Introduction), radical scavenging might represent a primary function of flavonoids in UV protection in the latter species. In grapevine, UV-induced *de novo* synthesis of quercetin and kaempferol, however, clearly supports the function of flavonols in UV screening. Clearly, decreasing levels of hydroxycinnamic acids during exposure (Figs 3*A*, *B*) are not consistent with an important UV-protective role of hydroxycinnamic acids in berries.

The function of flavonols in UV screening in berries was confirmed by data on skin transmittance for UV radiation. Taking into account that concentration of phenolics is related to their absorbance, which according to Beer's law is exponentially linked to transmittance, the behaviour of UV-A transmittance corresponded well with changes of flavonol concentrations (compare Figs 3*C*, *D* with Fig. 4*B*). That changes in UV-B transmittance (Fig. 4*A*) were smaller than changes in UV-A transmittance is explained, in part, by less effective absorbance of flavonols in the UV-B compared

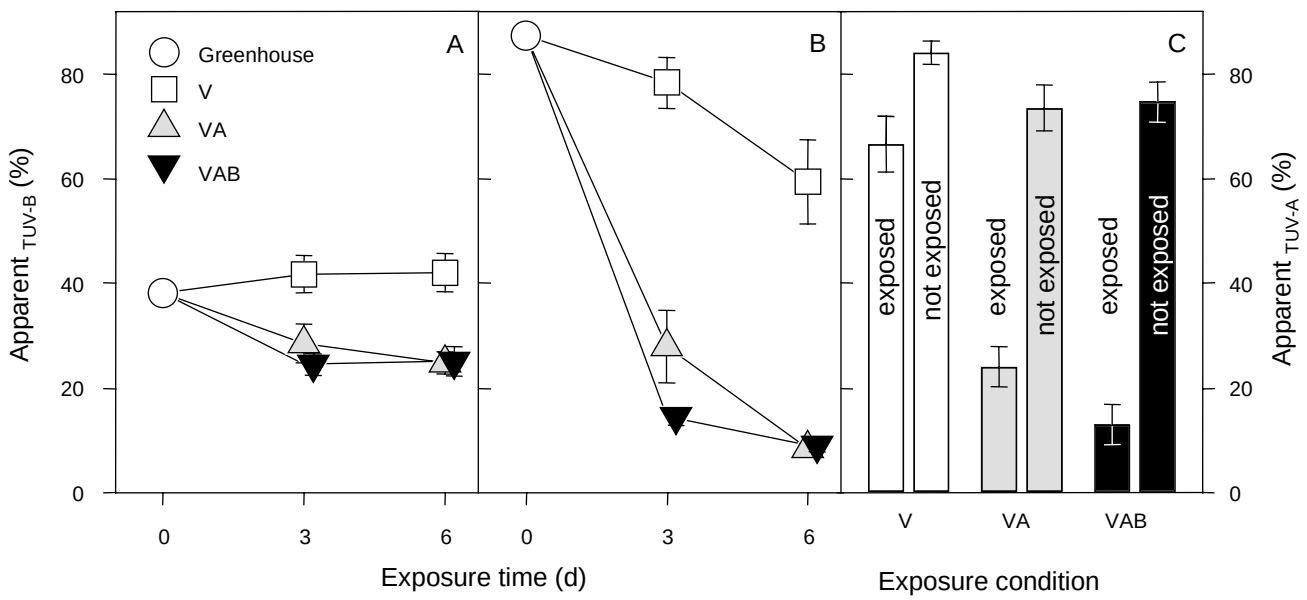

Fig. 4. UV transmittance of grape skin. Effects of outdoor exposure on apparent transmittances as determined with an Xe-PAM fluorimeter of grape skins are shown for UV-B (*A*) and UV-A radiation (*B*). UV-A transmittance, determined with a UV-A-PAM fluorimeter, from exposed sides ('exposed') and the corresponding sides oriented towards the centre of the bunch ('non exposed') are shown (*C*). Berries were exposed for 3 weeks to V, VA, and VAB conditions.

to UV-A regions. Also, the specific decreases in (flavonol-dominated) UV-A transmittance on exposed berry sides (Fig. 4*C*) resembled the behaviour of grape leaves which exhibit lower UV-A transmittance of adaxial compared to abaxial sides (unpublished results). Generally, therefore, the role of flavonols in UV screening appears to be similar in berries and leaves of grapevine.

To further elucidate the role of flavonols in berries, skin absorbance was plotted against corresponding absorbance of extracted flavonols or hydroxycinnamic acids (Fig. 5). In the UV-B and UV-A spectral range, only flavonols and not hydroxycinnamic acids exhibited a significant positive correlation with skin absorbance. The coefficient of determination was much better for UV-A data than for UV-B data (Figs 5*A*, *B*), which is in agreement with high absorbance and a predominant role of flavonols for UV-A screening rather than for UV-B screening to which hydroxycinnamic acids might contribute. In fact, the regression line of the plot of UV-B *v.* flavonol absorbance extrapolated to a positive ordinate intercept (Fig. 5*A*); this suggests that hydroxycinnamic acids add an offset to variable flavonoid-dependent UV-B screening. We deduce from these data, and also from decreasing concentrations of hydroxycinnamic acids during acclimation to outdoor conditions (Fig. 3), that in contrast to grape leaves, acclimation of UV shielding in berries results mostly from flavonol formation.

This results in conspicuous differences in the concentration ratios of hydroxycinnamic acids to flavonols between berry and leaf. After 6 d of acclimation to VA or VAB conditions, berries exhibited ratios close to 0.25 but 6 d of exposure VA or VAB conditions yielded ratios of 0.85 and 0.48 in leaves, respectively. These data were derived from Fig. 3 and from Kolb *et al*. (2001), respectively. Under the same conditions, UV-B transmittance was greater than 20%

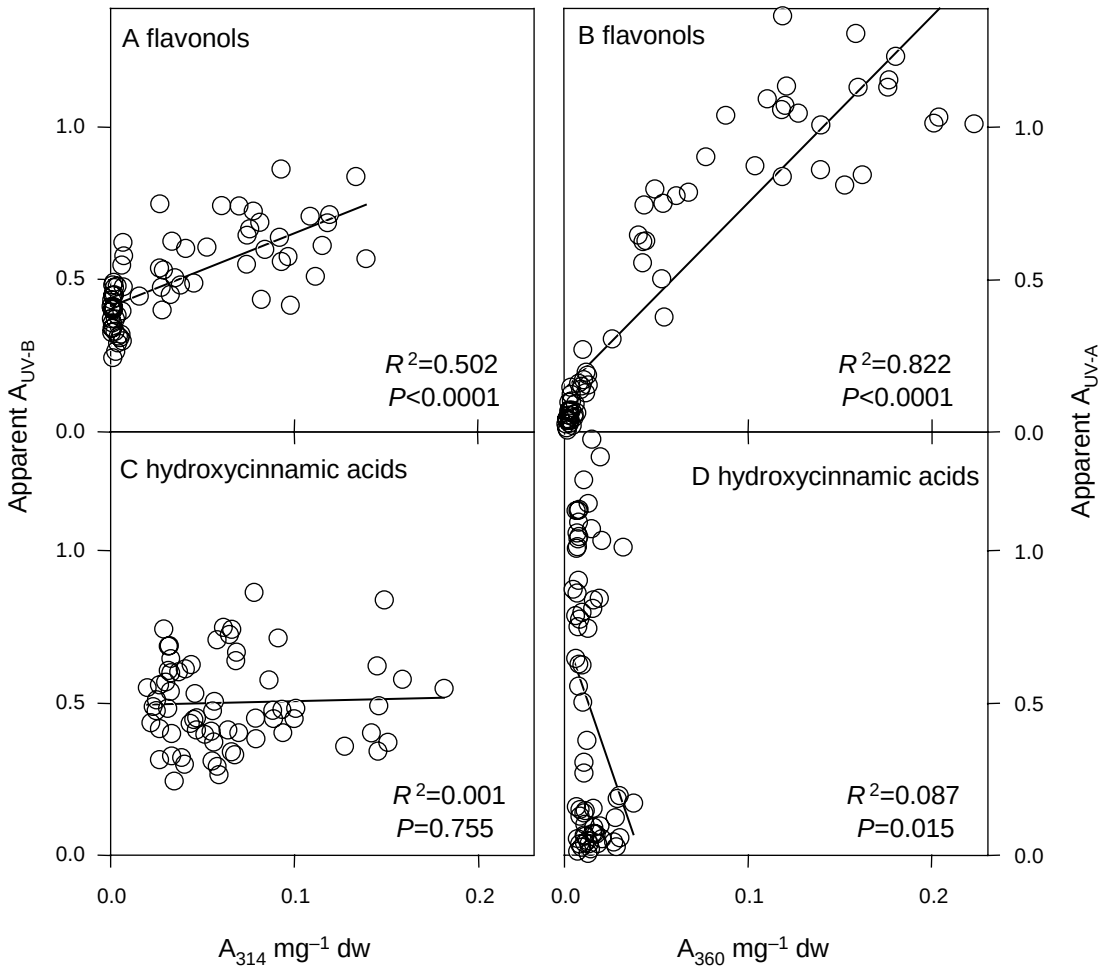

Fig. 5. Relationship between apparent UV absorbance of grape skin and UV absorbance of extracted phenolics. The figure compares fluorimetrically-estimated absorbance of grape skins in the UV with UV absorbance of phenolics extracted from these skins. Skin absorbance was derived from fluorimetrically-determined transmittance according to Beer's law. (*A*) and (*C*) Skin absorbance in the UV-B *v.* absorbance of flavonols and hydroxycinnamic acids at 314 nm, respectively; (*B*) and (*D*) skin absorbance in the UV-A *v.* absorbance of flavonols and hydroxycinnamic acids at 360 nm, respectively. Lines result from linear regressions for which coefficients of determination (r^2) and values of *P* are given.

in berries (Fig. 4) but below 5% in grape leaves while UV-A transmittance was comparable for berry and leaf (Fig. 4 and unpublished results). Therefore, it is not the different distribution of phenolics (Fig. 1; Kolb *et al*. 2001) but rather the lack of formation of hydroxycinnamic acids in response to UV-B stress that appears to be responsible for less efficient UV-B screening in berries than in leaves.

It is known that sudden exposure of previously shaded grape berries, for example by either summer pruning or leaf removal, can cause sunburn characterised by browning and shrivelling of the berries (Pool 1988). This phenomenon, which was massively demonstrated in German vineyards in the hot summer of 1998, was previously attributed to overheating of the berries (see Introduction section). Our data, however, clearly demonstrate incomplete UV-B screening in such grape berries and strongly suggest that sunburn damage of grape berries is initiated by UV-B radiation. Consequently, we are now further investigating the effects of natural UV-B radiation on sunburn in grape berries in the vineyard and possible methods to minimize such damage.

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