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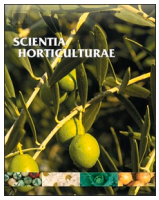

Epicuticular waxes: A natural packaging to deal with sunburn browning in white grapes

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ABSTRACT

Epicuticular waxes on grapevine berry cuticle provide protection of the inner tissues from biotic and abiotic stresses. However, little is known about their role in protecting grape epidermis from sunburn damages. This study investigated the effect of wax disruption in ten sun-exposed white skinned *Vitis vinifera* L. varieties. Browning symptom appearance was quantified one day and four days after wax disruption. It was also examined the content of the main photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) and total phenolics in the skins four days after the treatment.

The disruption of epicuticular waxes promoted grape sunburn: the skin browning intensity increased from 39.8 to 67.1 after one day and four days from the treatment, respectively. The loss of green color after wax disruption resulted from the degradation of chlorophyll *a*, since its content in the treated berries (9.01 μg g^{-1} of skin) was lower than that in the control berries (17.16 μg g^{-1}) after four days from the treatment. The co-occurrence of wax disruption in a sunlight excess environment caused also the photo-oxidation of carotenoids, which were higher in control berries (19.86 μg g^{-1}) than in the treated berries (14.51 μg g^{-1}). The browning intensity and the difference in the total phenolics of the skins between treated and control berries were significantly correlated, suggesting that the polymerization of phenolics into brown compounds after wax disruption could further enhance browning symptoms.

Therefore, epicuticular waxes could be considered as an important natural coating for grapes, providing defence against water loss and pest or pathogen attacks, but also effectively limiting sunburn browning and maintaining quality for both wine grapes and table grapes.

1. Introduction

Sunburn symptoms strongly affect grape quality and composition. Sunburn phenomena on grapes can be classified as sunburn browning (SB) or as sunburn necrosis (SN) (Gambetta et al., 2021). SB causes the appearance of yellow, brown or bronze spots on the sun-exposed skins of white grapes and it appears as a poor color development on the skins of black grapes (Krasnow et al., 2010). SN refers to necrotic spots on the fruit surface, ultimately leading in severe cases to entire berry cracking and shrivelling (Teker, 2023). Sunburn damages negatively affect grape quality and yield, with a consequent economic loss of up to 50 % crop value (Gambetta et al., 2021). Furthermore, wines obtained from sunburned grapes may have a more oxidative character (Rustioni et al., 2023), reducing upwards of 60 % their bottle price (Gambetta et al., 2022).

The occurrence of a berry to sunburn can be predicted from its developmental stage, its sun exposure and surface temperature, and the variety-specific susceptibility (Bahr et al., 2021). SB results when grape berries are exposed to excessive sunlight, while surface temperature plays a minor role (Rustioni et al., 2015a); SN, instead, is mainly a

Abbreviations: BII, browning intensity index; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; FCI, Folin-Ciocalteu index; IFW, intact fruit wax; PCR, polymerase chain reaction; R, reflectance spectra; DEW, disrupted epicuticular wax; ROS, reactive oxygen species; SB, sunburn browning; SN, sunburn necrosis; SSR, short sequence repeat; SW, skin weight; TSS, total soluble solids; *VIVC*, *Vitis* International Variety Catalogue.

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function of the surface temperature, which has to be higher than that necessary for SB to occur (Gambetta et al., 2021). Since global warming increases the frequency and intensity of heat waves (Perkins-Kirkpatrick and Lewis, 2020), sunburn damages to grapes will most likely increase in the next future (Santos et al., 2020). Sunburn is a complex disorder, involving different physiological mechanisms (Munné-Bosch and Vincent, 2019). Concerning SB, the modifications of the skin pigmentation are related to significant changes in the chemical composition (Vaughn and Duke, 1984). Different molecules are involved. Chlorophylls and carotenoids are degraded to avoid photosystems over-excitation and the accumulation of reactive oxygen species (ROS) (Gambetta et al., 2022; Rustioni et al., 2020). The high reactivity of ROS leads to the formation of brown pigments through the oxidative polymerization of phenolics (Pourcel et al., 2007). These brown pigments have important protective roles against further excessive radiation (Rustioni, 2017).

It is currently accepted that epicuticular waxes play a role as a protective screen against sunburn, as well (Gambetta et al., 2021). Plant cuticular waxes are a mixture of hydrophobic compounds coating the cuticle of all non-woody aerial plant organs (Lara et al., 2015). These waxes can be either intercalated within the cutin polymer (intracuticular waxes) or accumulated on the cuticle surface as wax crystals (epicuticular waxes) or films (Yeats and Rose, 2013). Besides their primary functions as a first line defence against pests and fungal pathogens and as a transpiration barrier (Jenks and Ashworth, 1999), epicuticular waxes on some species may provide photo-protection by scattering and reflecting incoming radiation (Shepherd and Griffiths, 2006). Rustioni et al. (2012) showed that grapevine berry epicuticular waxes increase the reflectance in the visible range, while the light scattering effect mainly provides for the opacification of the berries' surface. Waxy crystals display platelet-like morphology with either a 'broad-leaf'-like shape or a more 'spindly'-like shape in grapes suffering from water stress (Dimopoulos et al., 2020). Distribution and composition of epicuticular waxes can also differ among grapevine varieties (Yang et al., 2021). The chaotic organisation of the wax platelets may explain the light scattering effect of the berry surface (Rustioni et al., 2012). In sunburned grapes, the crystalline structure of the epicuticular waxes undergoes degradation to amorphous masses (Greer et al., 2006), perhaps affecting the putative protective role of this hydrophobic layer.

In our knowledge, few studies focused on the role of berry epicuticular waxes in preventing SB on grapes (Yang et al., 2021, 2023). In addition, an experiment to quantify this contribution has not been performed so far. In this study, the crystalline structure of white grape epicuticular waxes was disrupted. The aims were to: (1) evaluate berry SB one day and four days after wax disruption; (2) investigate biochemical changes in the berry skin after wax disruption; (3) explore whether wax disruption is involved in the correlations between grape SB and the loss of photosynthetic pigments and the oxidative polymerization of phenolics.

2. Material and methods

2.1. Experimental site characterization

The experiment was conducted in a 13 year old private collection of *V. vinifera* L. germplasm. The collection was located in the Salento viticultural area (Apulia Region, Southern Italy). The vineyard was planted in a plain area (Latitude 40.35, Longitude 17.40, Elevation 25 m a.s.l.). Plants were spaced 2.2 m (inter-row) and 0.9 m (in-row), with a density of about 5000 plants ha^{-1}. Vines were trained at classic spur cordon; soil was managed by tillage, and the vineyard was equipped with a drip irrigation system. Weather conditions during the experimental campaign (August 18th-22nd, 2022) were characterised using data on temperature, precipitation, and incoming solar radiation collected from a conventional ground-based weather station located hundreds of metres away from the vineyard.

2.2. Plant material and genotyping

Ten white *V. vinifera* L. varieties of different geographical origins were randomly selected in the collection. The varieties were Attila, Chinuri, Chitiskvertskha Tetri, Diamant Muskat, Fiano, Italia, Jvari, Nosiola, Pampanaro and Stambolli. For each of these varieties, DNA was extracted from lyophilized tissues by grinding the vegetal material (shoot apexes) to a fine powder by a Tissue-Lyser II instrument (Qiagen, Hilden, Germany) and extracting the total DNA by the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA concentration and quality (e.g., 260/280 and 260/230 ratios) were measured by a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and by gel electrophoresis (1 % agarose). The varieties object of the study were genotyped by twelve SSR markers: the nine proposed as common grape markers for international use within the framework of the Grapegen06 European project named VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, VrZAG79 (Bowers et al., 1996, 1999; Scott et al., 2000; Sefc et al., 1999), plus VMC6E1, VMC6G1 (Crespan, 2003), and VMCNG4b9 (*Vitis* Microsatellite Consortium). The SSR analyses were performed following the protocol detailed in Migliaro et al. (2013) for ten SSR, while VVMD25 and VVMD32 were separately amplified with the same PCR conditions. PCR products (0.5 μL) were mixed with 9.35 μL of formamide and 0.15 μL of the GeneScan™ 500 LIZ Size Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Capillary electrophoresis was conducted in an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Allele calling was performed with GeneMapper software version 5.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a home-made bin set obtained from reference varieties. Allele sizes were recorded in bp and varieties showing a single peak at a given locus were considered as homozygous. Identifications were performed by comparing the obtained SSR profiles with the CREA Viticulture and Enology molecular database (which currently contains about 8000 unique profiles, and is constantly updated), literature information, and the *Vitis* International Variety Catalogue (Vitis International Variety Catalogue VIVC, 2023). Table S1, in Supplementary materials, reports the *VI*VC codes and the nuclear SSR profiles of the ten varieties analysed.

2.3. Experimental design

In the second half of August 2022, for each variety, six light-exposed grapes were selected. To allow the comparison of the responses to the same environmental conditions, the plants were treated and analysed on the same days, regardless of the phenological stage. However, in general, following the BBCH scale (Meier, 2001) adapted following Rustioni et al. (2014a), all the varieties used were in the middle of ripening (BBCH 86–88). On August 18th at 09.00 a.m. legal time, in half the grapes/variety, the epicuticular waxes were disrupted in five berries/-grape (disrupted epicuticular wax, DEW). 15 berries per variety were thus treated. The most exposed, opaque and normally sized berries were chosen for the treatment. The epicuticular waxes were mechanically disrupted through a slight rubbing of the berry surface with paper towels. The remaining three grapes per variety were kept with the epicuticular waxes, as a control (intact fruit wax, IFW).

2.4. Reflectance spectroscopy

On the day after the treatment, August 19th in the morning (day 1), reflectance spectra (R) from 400 nm to 700 nm (±10 nm accuracy) and the value of the RGB and CIELab coordinates were recorded five times for each treated grape (one measure from each of the 5 berries without epicuticular waxes) and three times for each control grape (in different berries). The same measurements were then repeated four days after the treatment, August 22nd in the morning (day 4). The RGB (Red, Green, Blue) color model describes components of a color in relation to the standardized reference wavelengths of monochromatic red, green, and

blue lights. Instead, the CIELAB (or CIEL*a*b*) color space is a three-dimensional color space consisting of three axes. L* expresses the human perceptual lightness of a surface and it is represented on a vertical axis with values from 0 (black) to 100 (white). The a* coordinate is the red/green axis (the more negative are the values, the greener are the hues; the more positive are the values, the redder are the hues). The b* coordinate is the yellow/blue axis, where positive and negative b* describe yellow and blue values, respectively (Ly et al., 2020). Overall, 480 reflectance spectra and color indexes were thus obtained. The R, RGB and CIELab values were measured using a portable colorimeter 'Spectro 1 PRO' (Variable Inc., Chattanooga, US) operating with a D65 10° light source (Domanda et al., 2023). Each record was directly saved in the related smartphone iOS app 'Specto by variable'. Browning intensity index (BII) was calculated following the formula proposed by Rustioni et al. (2014b), with minor modifications:

$$Browning\ intensity\ index = \frac{100R_{t680}}{R_{t490}} - \frac{100R_{c680}}{R_{c490}}$$

where R_{t680} = treated berries reflectance at 680 nm, R_{t490} = treated berries reflectance at 490 nm, R_{c680} = control berries reflectance at 680 nm, and R_{c490} = control berries reflectance at 490 nm. BII was obtained for each variety both in day 1 and in day 4. BII was calculated as the average difference between each R_{t680}/R_{t490} and the mean of R_{c680}/R_{c490}. In this work, BII mainly indicates a difference between DEW and IFW reflectance spectra. BII is related to the degradation of chlorophylls (whose maximum absorption wavelength in the red region is at ≈680) and the formation of brown compounds (whose maximum absorption wavelength is at 490 nm) in the grape skin (Rustioni et al., 2014b). Thus, the more positive is BII, the more severe are the browning symptoms in DEW skins.

2.5. Spectrophotometric assays

2.5.1. Preparation of the grape skin extracts

On August 23rd in the morning, the grapes were collected and processed within a few hours from their harvesting. Five berries for each control grape and the five berries without epicuticular waxes for each treated grape were peeled. The skins obtained were weighed and then extracted with 5 mL of 85 % acetone. Skin extracts were kept at −20 °C until analyses. For each grape, the remaining berries were crushed by hand and the total soluble solids (TSS) (°Brix) of the grape juices were determined by refractometer (MA882, Milwaukee Instruments Inc., US).

2.5.2. Determination of chlorophylls and carotenoids

Photosynthetic pigments were quantified by recording the absorbance of the skin extracts at 440.5, 644 and 662 nm using an UV–Vis Spectrophotometer (UV-1900i, Shimadzu Europa GmbH, DE). Chlorophyll *a*, chlorophyll *b* and carotenoids were calculated following the formulas proposed by Holm (1954), reported by Dinu et al. (2022), and adapted to the extraction procedures:

$$Chlorophyll\ a\ \left(\mu g\ g^{-1}\ of\ skin\right) = \frac{(A662\ \times\ 9.78) - (A644\ \times\ 0.99)}{SW} \times 5$$

$$Chlorophyll\ b\ \left(\mu g\ g^{-1}\ of\ skin\right) = \frac{(A644\ \times\ 21.40) - (A662\ \times\ 4.65)}{SW} \times 5$$

$$Carotenoids\left(\mu g\, g^{-1}\, of\, skin\right) = \frac{(A440.5 \times 4.69) - [0.267 \times (Chl\, a + Chl\, b)]}{SW} \times 5$$

where An = absorbance at n wavelength, Chl *a* = chlorophyll *a*, Chl *b* = chlorophyll *b*, SW = skin weight (g).

For each variety, delta IFW-DEW for chlorophyll *a* was calculated as the difference between the mean of IFW chlorophyll *a* values and each DEW chlorophyll *a* value. The same delta was also calculated for chlorophyll *b* and carotenoids.

2.5.3. Total phenolic index

Total phenolic index was determined for each extract following the method proposed by OIV (2023), with minor modifications. In a 10 mL flask, 5 mL of water were added to 0.5 mL of undiluted skin extract. Then, 0.5 mL of Folin-Ciocalteu reagent were added. Lastly, 2 mL of 20 % (*m/v*) Na_2CO_3 were added and the flask was filled up to 10 mL with distilled water. After 30 min of reaction, the absorbance was read at 750 nm (using a blank made in the same way, but with water instead of the skin extract). Total phenolic index was expressed as Folin-Ciocalteu Index (FCI) g^{-1} of skin by the formula:

$$FCI\ g^{-1}\ of\ skin = \frac{A750\ \times\ 4}{SW}$$

where A_{750} = absorbance at 750 nm, SW = skin weight (g).

For each variety, delta IFW-DEW for FCI was calculated as the difference between the mean of IFW FCI values and each DEW FCI value.

2.6. Statistical analyses

Statistical analyses were performed by RStudio (Posit PBC, Boston, UK) and SPSS® statistical software (IBM Corporation, Armond, US). One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was carried out for the assessment of significant differences related to the BII differences between day 1 and day 4 ($p < 0.05$). Factorial ANOVA with Bonferroni post-hoc test was carried out to determine the significant differences related to IFW/DEW for the same day ($p < 0.05$). BII accession means were separated for each day by one-way ANOVA with Bonferroni post-hoc test ($p < 0.05$). BII average difference between day 1 and day 4 for each accession was tested by paired *t*-test at a significant level of $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***). One-way ANOVA with Bonferroni post-hoc test was applied to determine significant differences ($p = 0.05$) in the TSS (°Brix) values and the spectrophotometric indexes using the accession as factor. Significance of the differences between IFW and DEW for the spectrophotometric assays was quantified by unpaired *t*-test at a significant level of $p < 0.05$ (*) or $p < 0.01$ (**). Correlations between BII and the spectrophotometric assays were performed by the Spearman's rank test (Spearman's R) per $p = 0.05$.

3. Results

3.1. Climate conditions

Fig. 1 reports the main meteorological conditions that occurred at the measurement site during the experimental campaign (August 18th-22nd, 2022). The weather was characterised by maximum temperatures above 30 °C, as typical during summertime, due to persistent clear-sky conditions. In particular, the first two days were hot and wet (average temperature and relative humidity of 28 °C and 72 %, respectively), while the last two were slightly cooler and drier (26.3 °C and 60 %). Therefore, this period was considered representative of typical sunny summertime conditions in the Mediterranean region, and thus suitable to evaluate the sunburn susceptibility.

3.2. Grapevine genotyping

The molecular analyses were performed on the ten grapevine samples and the identification results with the SSR data are summarised in Table S1.

Fiano, Pampanaro, Nosiola and Italia are known Italian varieties registered in the Italian Catalogue of Grapevine Varieties (Italian Catalogue of Grapevine Varieties-Catalogo Nazionale delle Varietà di Vite, 2023). Fiano is an ancient southern Italian grape with its documented history stretching back to the Roman Empire and, today, it is Campania that claims the grape as its own. Pampanaro is a little-known native and traditional autochthonous grape of Lazio, while Nosiola is a grape

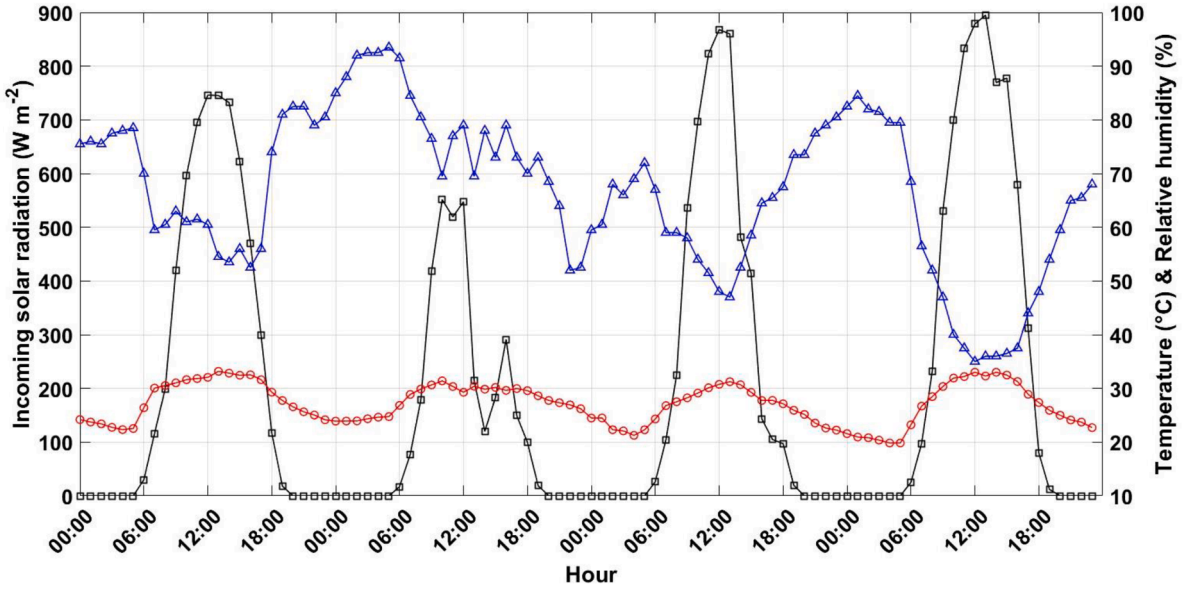

Fig. 1. Main meteorological parameters at the measurement site: Incoming solar radiation (black line), air temperature (red line) and relative humidity (blue line), during the measurement period, August 18th-22nd, 2022.

variety native of the Trento area, located in Trentino Alto Adige, northeastern Italy. Italia is a fairly popular Italian seeded white table grape variety.

Chitiskvertskha Tetri, Jvari and Chinuri are indigenous grape varieties from Georgia and Jvari is characterised by functionally female flowers. This trait is considered ancestral and related to *Vitis vinifera* domestication (Fechter et al., 2012).

Attila variety belongs to table grape breeding program in Hungary for new table grape varieties resistant to downy mildew, powdery mildew and grey rot, with high cold hardiness (Pernesz, 2004).

Diamant muskat is a French variety derived from the cross Riesling Weiss x Muscat St. Laurent. No more information is available in literature about this grapevine variety.

Stambolli is a variety from Albania not counted in the *V*IVC and no information is provided from literature. Because an accession with the same name and SSR profile is stored in the CREA-germplasm collection of Conegliano, we can assume the true-to-type of the variety based on the microsatellite fingerprinting profile, aware of the fact that further morphological observations are necessary to complete the passport data of the variety.

3.3. Reflectance spectroscopy

Table 1 reports BII and components L*a* and RGB at day 1 and day 4 for the two treatments IFW and DEW. Within 24 h from the disruption of epicuticular waxes (day 1), BII average was positive. L*, a* and RGB color indexes were found to be significantly different between the IFW and DEW: L*, R, G and B were higher in IFW berries; only a* was higher in DEW berries. Instead, no significant difference was produced for component b* by factorial ANOVA (data not shown). After 96 h from the disruption of epicuticular waxes (day 4), BII average was significantly higher than BII at day 1. L*, a* and RGB color indexes were all significantly different between the IFW and DEW, being only a* higher in DEW berries. Again, no significant difference was produced for component b* by factorial ANOVA (data not shown).

Chinuri was the less browned variety after 24 h from the disruption of epicuticular waxes, followed by Attila (Table 2). Diamant Muskat had the highest BII, followed by Pampanaro (Fig. 2b). After 96 h from wax disruption, Chinuri still was the most tolerant variety to browning damages, followed by Chitiskvertskha Tetri. There were no significant differences in BII average between day 1 and day 4 for these two varieties, as well as for the other Georgian variety Jvari. It is worth noting that Attila showed a boost of sunburn symptoms from day 1 to day 4, which can also be noted by its highly significant BII average difference between the two days. On the contrary, Diamant Muskat did not undergo any further browning on day 4. Therefore, the BII average difference for Diamant Muskat was not significant. Pampanaro had the highest BII in day 4 (Fig. 2c) and it was the variety with the highest susceptibility to browning after 4 days.

Table 1
BII and components of the CIELab color space and RGB color model for IFW and DEW berries at day 1 and day 4. BII, browning intensity index; L*, lightness; + a* (positive), red values; R, red component; G, green component; B, blue component. IFW, intact fruit wax; DEW, disrupted epicuticular wax. Average values ± standard error over ten varieties are reported. Different capital letters in BII row mean significant differences related to day 1/day 4. Different lowercase letters mean significant differences related to IFW/DEW for the same day. One-way ANOVA with Bonferroni post-hoc test was carried out for BII differences. Factorial ANOVA with Bonferroni post-hoc test was carried out for L*, a*, R, G, B.

Color attributes	Day 1		Day 4	
	IFW	DEW	IFW	DEW
BII	39.75 ± 2.26^{B}		67.13 ± 3.15^{A}	
L*	50.08 ± 1.16^{a}	44.58 ± 0.82^{b}	54.56 ± 0.67^{a}	43.92 ± 0.79^{b}
a*	4.60 ± 0.27^{b}	7.38 ± 0.28^{a}	2.88 ± 0.22^{b}	7.56 ± 0.27^{a}
R	122.96 ± 2.96^{a}	114.46 ± 2.01^{b}	134.61 ± 1.65^{a}	116.54 ± 1.86^{b}
G	117.68 ± 2.98^{a}	102.28 ± 2.09^{b}	129.26 ± 1.75^{a}	100.16 ± 1.99^{b}
B	128.31 ± 2.46^{a}	113.27 ± 1.94^{b}	132.19 ± 2.04^{a}	103.58 ± 2.31^{b}

Table 2
Browning intensity index (BII) at different stages (day 1 and day 4) for each variety considered in this work. Average values ± standard error are reported. Within each day, different letters represent significantly different means by one-way ANOVA with post-hoc test Bonferroni ($p < 0.05$). BII average difference between day 1 and day 4 was tested by paired *t*-test at a significant level of $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

Accession	BII (Day 1)	BII (day 4)	BII average difference
Diamant Muskat	61.06 ± 7.91^{a}	65.59 ± 6.99^{abc}	4.54
Pampanaro	59.23 ± 6.48^{ab}	98.39 ± 9.13^{a}	39.16**
Italia	48.63 ± 7.11^{abc}	86.93 ± 9.58^{ab}	38.29***
Nosiola	43.75 ± 5.84^{abc}	80.54 ± 11.47^{ab}	36.80**
Jvari	40.09 ± 6.45^{abc}	64.48 ± 10.40^{abc}	24.39
Stambolli	37.92 ± 4.97^{abc}	61.83 ± 5.38^{bc}	23.90**
Fiano	32.41 ± 4.89^{abc}	78.40 ± 11.35^{abc}	46.00***
Chitiskvertskha Tetri	31.81 ± 8.48^{abc}	44.57 ± 8.88^{cd}	12.76
Attila	25.31 ± 4.45^{bc}	67.28 ± 6.80^{abc}	41.97***
Chinuri	17.33 ± 6.90^{c}	23.29 ± 3.20^{d}	5.96

3.4. Spectrophotometric assays

Table 3 reports TSS (°Brix) in the grape musts and chlorophyll *a*, chlorophyll *b*, carotenoid contents and FCI in IFW and DEW skin extracts for all the varieties considered in this work. Chitiskvertskha Tetri and Jvari had the highest TSS, while Italia the lowest TSS. Chinuri and Attila were the varieties with the lowest varietal (IFW) content of chlorophyll *a* and carotenoids in the skin.

Table 4 reports the spectrophotometric indexes (chlorophyll *a*, chlorophyll *b*, carotenoids and FCI) for IFW and DEW skin extracts. Average differences were all negative, but only those for chlorophyll *a* and carotenoids were significant.

Table 5 shows the correlations between BII in day 1 and day 4 with deltas IFW-DEW for chlorophyll *a*, chlorophyll *b*, carotenoids and FCI. On day 1, all the correlations were positive and significant. On day 4, all the correlations were still positive, but only those between BII and carotenoids and FCI were significant.

4. Discussion

4.1. Reflectance spectroscopy

Epicuticular waxes play an important role in quality maintenance of grapes. This work aimed to investigate the berry response to SB after wax disruption. The degree of SB for berries was evaluated with the BII (Rustioni et al., 2014b). As shown in Table 1 and Fig. 2b, just 24 h after the disruption of epicuticular waxes, browning symptoms appeared in DEW berries. Epicuticular waxes are able to reduce light exposure levels in the underlying epidermal tissues by scattering and reflecting visible and UV radiation (Shepherd and Griffiths, 2006). The lower level of light reaching the chloroplasts in waxy berries may induce less production of ROS and, consequently, reduce the formation of oxidised phenolic forms and complex brown polymers (Felicetti and Schrader, 2008). In fact, Yang et al. (2023), working on *V. vinifera* table grape variety Zuijinxiang, found that berries without epicuticular waxes (treated by an arabic gum aqueous solution) had higher hydrogen peroxide (H_2O_2) content than the IFW berries during post-harvest storage of grapes for one month at 4 °C. In our experimental plan, however, berries were not treated chemically in order to remove waxes. Indeed, just a slight rubbing of the skins ultimately lead to the berry browning. It was formerly reported by Rustioni et al. (2012) that the light-scattering effect of epicuticular waxes, which provides photo-protection for the epidermis, can be explained by the crystalline form and the chaotic orientation of the wax platelets. Waxy crystals can degrade into amorphous structures under either high heat stress conditions (approximately at 50 °C, as demonstrated by Khanal et al. (2013)) or mechanical action, as in this case. Plate-like wax crystals reflect and scatter a higher proportion of light than amorphous waxes (Gambetta et al., 2021). The positive values of BII in Table 1 demonstrate that amorphous waxes lose their role as a protective screen against sunburn.

Yang et al. (2021) showed that the removal of epicuticular wax accelerated the morphological changes of both white and red table grape berries. Nevertheless, this work focused just on white grapes to study how the disruption of epicuticular waxes affects the color of the berries. In red grapes, in fact, it is more difficult to distinguish the appearance of brown hue, because anthocyanins strongly absorb at similar wavelengths (Rustioni et al., 2015b). Furthermore, anthocyanins play an antioxidant role by scavenging ROS (Castañeda-Ovando et al., 2009), thus hampering the formation of brown pigments in the grape skin. Instead, the modification in berry colour after the disruption of epicuticular waxes is much more evident in white *V. vinifera* varieties, which are unable to biosynthesize anthocyanins (Kobayashi et al., 2004). Indeed, the browning of the berry exocarp was already noted for all the accessions in day 1, although with different intensity among them (Table 2).

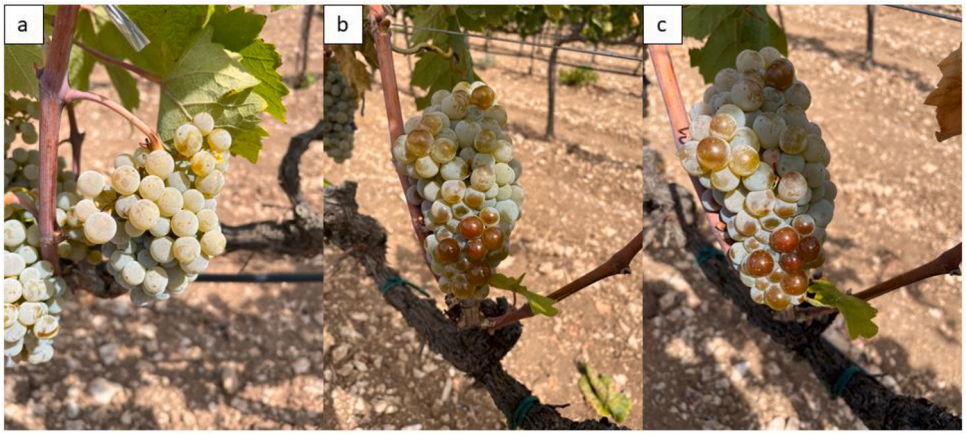

Fig. 2. Grapes of Pampanaro at different stages: (a) IFW day 4; (b) DEW day 1; (c) DEW day 4.

Table 3
Total soluble solids (TSS) (°Brix) in the grape musts and chlorophyll *a*, chlorophyll *b*, carotenoids (μg g^{-1} of skin) and FCI (g^{-1} of skin) in IFW (intact fruit wax) and DEW (disrupted epicuticular wax) skin extracts. Average values ± standard error for each variety are reported. Different letters in each column represent significantly different means by one-way ANOVA with post-hoc test Bonferroni ($p < 0.05$). TSS (°Brix) values represent the mean of six replicates (IFW + DEW), with the exception of the variety Jvari (four replicates). Spectrophotometric analyses represent the mean of three replicates.

Accession	TSS (°Brix)	IFW				DEW			
		Chlorophyll a	Chlorophyll b	Carotenoids	FCI	Chlorophyll a	Chlorophyll b	Carotenoids	FCI
Chitiskvertskha Tetri	24.78 ± 0.92^{a}	17.17 ± 5.46^{bc}	15.55 ± 9.17^{a}	10.26 ± 10.60^{c}	5.23 ± 0.60^{bc}	10.88 ± 0.91^{ab}	6.90 ± 0.22^{bc}	20.68 ± 1.28^{a}	9.45 ± 1.76^{a}
Jvari	21.95 ± 1.69^{ab}	16.78 ± 7.08^{bc}	18.11 ± 9.61^{a}	14.39 ± 4.47^{c}	7.43 ± 0.85^{ab}	9.73 ± 1.96^{ab}	5.74 ± 0.80^{bc}	16.40 ± 3.61^{ab}	10.33 ± 2.73^{a}
Fiano	20.40 ± 0.28^{bc}	14.27 ± 1.77^{bc}	7.27 ± 1.62^{a}	13.84 ± 0.94^{c}	2.03 ± 0.29^{c}	10.49 ± 1.05^{ab}	7.63 ± 0.64^{bc}	15.28 ± 1.17^{ab}	0.45 ± 0.15^{b}
Attila	20.13 ± 0.30^{bc}	6.11 ± 0.15^{c}	6.94 ± 0.80^{a}	14.15 ± 0.44^{c}	2.50 ± 0.40^{bc}	12.42 ± 1.40^{a}	21.62 ± 3.57^{a}	8.71 ± 2.00^{b}	2.05 ± 0.64^{b}
Chinuri	19.87 ± 0.46^{bcd}	7.41 ± 1.02^{c}	3.92 ± 0.79^{a}	12.10 ± 0.90^{c}	3.56 ± 0.41^{bc}	9.97 ± 1.10^{ab}	6.02 ± 0.40^{bc}	15.31 ± 1.09^{ab}	5.72 ± 0.70^{ab}
Stambolli	19.58 ± 0.25^{bcd}	13.39 ± 1.20^{bc}	7.08 ± 1.21^{a}	18.44 ± 1.26^{bc}	1.84 ± 0.40^{c}	6.85 ± 0.57^{ab}	5.80 ± 0.31^{bc}	12.95 ± 0.51^{ab}	3.01 ± 0.39^{b}
Pampanaro	18.28 ± 0.86^{bcd}	42.66 ± 5.87^{a}	19.60 ± 3.86^{a}	44.37 ± 3.05^{a}	11.08 ± 1.14^{a}	5.03 ± 0.68^{b}	2.58 ± 0.18^{c}	13.98 ± 2.32^{ab}	2.08 ± 0.20^{b}
Diamant Muskat	17.83 ± 0.51^{cd}	30.78 ± 2.02^{ab}	15.33 ± 1.58^{a}	37.23 ± 2.47^{ab}	12.28 ± 2.51^{a}	5.99 ± 0.97^{ab}	4.66 ± 1.17^{c}	12.85 ± 0.83^{ab}	1.67 ± 0.18^{b}
Nosiola	17.42 ± 0.95^{cd}	12.25 ± 2.72^{bc}	6.55 ± 2.72^{a}	18.06 ± 2.65^{bc}	1.49 ± 0.72^{c}	7.20 ± 1.57^{ab}	3.27 ± 0.42^{c}	15.09 ± 2.18^{ab}	1.33 ± 0.52^{b}
Italia	16.67 ± 0.42^{d}	10.80 ± 1.71^{c}	8.27 ± 0.84^{a}	15.78 ± 0.93^{bc}	2.37 ± 0.53^{bc}	11.50 ± 2.32^{ab}	13.56 ± 2.87^{ab}	13.85 ± 1.88^{ab}	3.08 ± 0.52^{b}

Table 4
Chlorophyll *a*, chlorophyll *b*, carotenoids (μg g^{-1} of skin) and FCI (g^{-1} of skin) in IFW (intact fruit wax) and DEW (disrupted epicuticular wax) berry skin extracts. Average values ± standard error over ten varieties are reported. Different letters in row represent significantly different means by unpaired *t*-test at a significant level of $p < 0.05$ (*) or $p < 0.01$ (**).

Spectrophotometric analysis	IFW	DEW	Average difference
Chlorophyll *a*	17.16 ± 2.20^{a}	9.01 ± 0.57^{b}	−8.16**
Chlorophyll *b*	10.86 ± 1.56^{a}	7.78 ± 1.09^{a}	−3.08
Carotenoids	19.86 ± 2.27^{a}	14.51 ± 0.73^{b}	−5.35*
FCI	4.98 ± 0.75^{a}	3.92 ± 0.67^{a}	−1.06

The L* and a* values are shown in Table 1. Just after one day, DEW berries exhibited a lower L* value than that of IFW. Therefore, wax disruption reduced L* value. The reduction in L* value for DEW berries compared to IFW fruits can be explained by both the disruption of epicuticular wax crystals, whose optical properties were described above, and the formation of brown compounds in the exocarp, which are able to absorb in the visible photosynthetic active radiation (Rustioni, 2017). On the contrary, a* coordinate, albeit positive in both DEW and IFW berries, was much higher in DEW berries than in IFW ones, indicating a loss of green color in the DEW berry skins due to the wax disruption. Similar results for L* and a* were obtained also by Yang et al. (2023): they found L* was higher in IFW berries than in the berries without epicuticular waxes, while a* was the opposite. The differences in RGB color space between IFW and DEW (Table 1) confirmed how wax disruption modified the skin pigmentation of grapes.

Yang et al. (2021, 2023) reported that the removal of epicuticular wax increased berry weight loss during storage at either 20 °C or 4 °C. Wax removal accelerated cuticular transpiration, which in turn is the main cause of the berry weight loss by veraison when stomata become nonfunctional (Swift et al., 1973; Zhang and Keller, 2015). In addition, wax removal promoted cell membrane leakages and cell wall metabolism, ultimately decreasing the fruit firmness (Chu et al., 2018; Yang et al., 2023). Water loss and the fruit softening upon wax removal might aggravate browning intensity during storage. In this work, pre-harvest exposure to radiation for three days significantly increased BII from 39.8 to 67.1 (Table 1).

However, for some varieties, such as the Georgian varieties Chinuri and Chitiskvertskha Tetri, BII did not produce any significant difference between day 1 and day 4 (Table 2). Intriguingly, Chinuri and Chitiskvertskha Tetri were also the least sunburned accessions after four days. Sargolzaei et al. (2021) suggested for Georgian varieties a relatively low susceptibility to sunburn with respect to the general *V. vinifera* species, and, concerning the variety Rkatsiteli, they suggest that it could be due to its relatively low phenolic content in the skin. However, this mechanism does not seem to fit here for Chinuri and Chitiskvertskha Tetri because their varietal (IFW) phenolic content in the skin (FCI) was similar to that obtained for Italia (Table 3), which instead was one of the highest BII in both days. Thus, we should hypothesise that other

Table 5
Correlations between BII (day 1 and day 4) and delta IFW-DEW spectrophotometric assays (significance code: *, $p < 0.05$; **, $p < 0.01$).

		Delta chlorophyll *a*	Delta chlorophyll *b*	Delta carotenoids	Delta FCI
BII day 1	Spearman's R	.475**	.421*	.378*	.397*
	Sig. (2-code)	.008	.020	.039	.030
	N	30	30	30	30
BII day 4	Spearman's R	.168	.176	.471**	.457*
	Sig. (2-code)	.376	.351	.009	.011
	N	30	30	30	30

mechanisms could be also involved in the tolerance of Georgian grapes to sunburn. In our experimental conditions, considering that sunburn susceptibility decreases during berry development (Rustioni et al., 2015a), it is worth to notice that Chinuri and Chitiskvertskha Tetri might have suffered fewer browning damages because their grapes were some of the ripest among all (look at the TSS and the IFW content of chlorophyll *a* and carotenoids in Table 3). Anyway, an in-depth classification of the grapevine germplasm accessions to browning susceptibility is beyond the scope of this paper and needs to be developed in a proper study.

4.2. Spectrophotometric assays

The hypothesis that the increased a* value in DEW berries might be due to the chlorophyll degradation was investigated by performing chlorophyll *a* and chlorophyll *b* spectrophotometric assays on the IFW and DEW berry skin extracts. In fact, the chlorophyll *a* content of DEW extracts was lower than that of IFW extracts (Table 4), proving that wax disruption accelerated chlorophyll *a* degradation. When ROS production exceeds ROS scavenging in chloroplasts because of stressful conditions, as in the case of the disruption of epicuticular waxes in a radiation excess environment, plants need to minimise ROS production (Foyer and Shigeoka, 2011). Since photosynthesis is an important source of ROS (Foyer and Noctor, 2003), one well established way to maintain cellular redox homeostasis is to adjust photosynthesis by promptly reducing chlorophyll concentration (Rustioni et al., 2015a). Yang et al. (2023) found a much lower chlorophyll content in grapes without epicuticular waxes than in the IFW grapes just after one week of post-harvest storage at 4 °C. Furthermore, in their study, berries without epicuticular waxes had a higher expression of several genes involved in chlorophyll catabolism pathway than IFW grapes. In our work, however, no significant difference was found for chlorophyll *b*, although its content was higher in IFW extracts (Table 4). The different degradation behaviour between chlorophyll *a* and *b* is rather interesting, since Rustioni et al. (2015a) showed that photo-oxidative sunburn produced stronger degradation of chlorophyll *b* with respect to chlorophyll *a*. Nevertheless, it should be taken into account the different environmental conditions between the two studies and the high variability used in this work, suggesting different varietal responses in chlorophyll *a*/chlorophyll *b* degradation ratio to radiation excess.

The change trend of carotenoids was similar to that of chlorophyll *a*, with the content in IFW extracts being higher than that in DEW extracts (Table 4). Carotenoids are pigments associated with both photosystem I and photosystem II. They serve as “energy knob”, either collecting light and transferring its energy to the reaction center when light intensity is low, or quenching chlorophyll excitation energy when light intensity is too high (Yaroshevich et al., 2015). However, if the excited states of chlorophylls are not efficiently quenched, singlet oxygen ($^1O_2$) arises (Dogra and Kim, 2020). Effectively, superoxide anion content ($\cdot O_2^-$), which can directly come from the monovalent reduction of $^1O_2$, was much higher in DEW berries than in IFW ones at the end of one month storage (Yang et al., 2023). Carotenoids can scavenge $^1O_2$ in order to prevent photosystem II damage, but this scavenging leads to their oxidative modification into aldehydes and endoperoxides (Ramel et al., 2012). Thus, the higher light exposure in the chloroplasts of DEW berries could determine a loss of carotenoids as a consequence of the scavenging activity of these pigments.

Total phenolic contents (FCI) of IFW and DEW skin extracts were statistically similar, though FCI value of IFW was higher than that of DEW (Table 4). Phenolics are widely spread substances in plants, one of their main functions being the antioxidant activity against ROS (Close and McArthur, 2002). The metabolism of phenolics in the skin as a response to wax disruption is quite complex: on one hand, phenolic compounds undergo oxidation by scavenging ROS, on the other hand ROS burst in plant tissues can act as a messenger for phenolic biosynthesis (Razem and Bernards, 2003). Therefore, even if grapes are directly exposed to sunlight and oxygen, just a few days of exposure to radiation after wax disruption might be a really short time to discover any clear difference in the FCI between IFW and DEW extracts. Yang et al. (2023), during one month of post-harvest storage period at 4 °C, did not find any significant difference in FCI between IFW berries and berries without epicuticular waxes within the first week. In the second week of storage, FCI started to decline both in the IFW berries and the berries without epicuticular waxes but the latter possessed lower polyphenol content, as a probable consequence of more oxidations in those berries. The same trend was observed in the third week. However, during the fourth and last week of storage, FCI in the berries without epicuticular waxes remained stable and higher than in IFW berries, which instead kept decreasing. According to the authors, the upregulation of polyphenolic biosynthesis by wax removal explained the observed FCI difference between berries without epicuticular waxes and IFW berries in the last experimental week. Nevertheless, we are aware that biochemical changes in the skin after wax disruption during either pre-harvest exposure to light radiation or post-harvest storage at 4 °C could differ, especially for a non-climacteric fruit like grapes (Robinson and Davies, 2000). The co-occurring effect of a prolonged pre-harvest exposure to light radiation and wax disruption on the accumulation dynamic of phenolics in the grape skin needs to be specifically investigated.

Table 5 reports the correlation between the sunburn symptoms at either day 1 or day 4 and the deltas between IFW and DEW calculated for each spectrophotometric index. A regression between BII and chlorophyll loss due to light exposure was already reported by Rustioni et al. (2015a). However, the positive and significant correlations at day 1 between BII and both deltas for chlorophyll *a* and chlorophyll *b* stress the importance of chlorophyll degradation after wax disruption in the sunburn symptom appearance. It has been shown that pheophorbide and pheophytin, which are some of the initial products of chlorophyll *a* catabolism, contribute to fruit browning (Heaton and Marangoni, 1996). Besides, chlorophyll loss is a consequence of the accumulation of ROS, which are involved in the formation of phenolic polymers and brown compounds. Indeed, the browning appearance of fruits is well correlated also with the oxidation of carotenoids and the polyphenolic compounds by polyphenol oxidases (Min et al., 2017). Anyway, chlorophylls did not correlate significantly with BII at day 4, while delta carotenoids and delta FCI did so (Table 5). SB late development is thus independent from any further chlorophyll senescence, whereas it keeps relating to the oxidation of carotenoids and polyphenols. While chlorophyll breakdown is fast and it ends with the formation of a final colorless compound (Hörtensteiner and Kräutler, 2011); phenolic polymers, instead, are more easily oxidised than their corresponding original phenols (Rustioni, 2017). The increased antioxidant capacity of phenolic polymers explains why SB can rise up over time (look at BII in Table 1), even after the depletion of chlorophylls.

5. Conclusions

Sunburn damages in grapes cause significant losses in quality and yield of both wine grapes and table grapes. Little is known about the protective role of grape epicuticular waxes to resist photo-oxidative stress. In this study, we investigated the effect of epicuticular waxes disruption on sunburn development of ten white skinned *V. vinifera* L. varieties. The disruption of epicuticular waxes in sun-exposed grapes promoted skin browning intensity and overall a modification of the exocarp pigmentation. The loss of green color after wax disruption mainly resulted from the degradation of chlorophylls. Chlorophyll senescence is one of the first symptoms of fruit quality deterioration and it is a marker of oxidative process in skin cells. Among these oxidative reactions, the loss of carotenoids and the polymerization of phenolics into brown compounds lead to a further increase of browning intensity, even in a short-time distance from wax disruption. Therefore, epicuticular waxes could be considered as an important coating for grapes,

providing defense against water loss and pest or pathogen attacks, but also effectively limiting sunburn browning.

CRediT authorship contribution statement

Corrado Domanda: Methodology, Formal analysis, Investigation, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Vito Michele Paradiso:** Methodology, Resources, Data curation, Writing – review & editing, Supervision. **Daniele Migliaro:** Methodology, Formal analysis, Resources, Investigation, Data curation, Writing – original draft. **Gianluca Pappaccogli:** Methodology, Formal analysis, Data curation, Resources, Investigation, Writing – original draft. **Osvaldo Failla:** Resources, Data curation, Writing – review & editing, Supervision. **Laura Rustioni:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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