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Corresponding Author: Professor Laura Rustioni,

Corresponding Author's Institution: Università del Salento

First Author: Laura Rustioni

Order of Authors: Laura Rustioni; Daniela Fracassetti; Bhakti Prinsi; Filippo Geuna; Alessandro Ancellotti; Valerio Fauda; Antonio Tirelli; Luca Espen; Osvaldo Failla

Abstract: Oxidations in grape berries are gaining major interest as they affect grape characteristics and quality. Considering berries, Reactive Oxygen Species are involved in the responses to both ripening process and stresses, including photooxidative sunburn. Redox metabolism involves a multitude of chemical and enzymatic reactions. In this study, four white grape cultivars were examined for natural ripening and photooxidative sunburn effects (obtained in artificial conditions) on berry pigmentation, chemical composition and enzymatic activity. The measured parameters included reflectance spectra, pigmentation (including berry browning), content of photosynthetic pigments, organic acid profiles, antioxidant activity, concentrations of antioxidants (total phenolics, ascorbic acid and reduced glutathione), enzymatic activities (guaiacol peroxidases, ascorbate peroxidase and catalase). The effects of the treatment (natural ripening and artificial photooxidative sunburn) on each considered parameter are described in the paper. Photooxidative sunburn strongly affected the contents of antioxidants and chlorophylls, increased the browning index and modulated the enzymatic activities investigated. Samples clearly clustered depending on the oxidation status. Furthermore, the PCA highlighted the similarities and differences in the responses to oxidative stress during ripening and photooxidative sunburn. PCA produced five functions with eigenvalues higher than 1, representing 87.03 % of the total variability. In particular, the scores of the function 1 discriminated the samples based on the oxidation status, while the function 2 separated the samples based on the sampling date, representing the physiological responses characteristic of ripening. Our work sheds light on this topic, and will allow a more conscious vineyard management, thus supporting the agricultural adaptation to climate changes.

Dear Editor Marcel Jansen,

This letter accompanies the submission of the manuscript titled: "Oxidations in white grape (Vitis vinifera L.) skins: comparison between ripening process and sunburn symptoms". After the accomplishment of the required English revision, the authors wish you could consider this article for publication in *Plant Physiology and Biochemistry*.

In this work, we compared the oxidative responses of white berry skin tissues to photo-oxidative sunburn and ripening processes. The measured parameters included reflectance spectra, pigmentation (including berry browning), content of photosynthetic pigments, organic acid profiles, antioxidant activity, concentrations of antioxidants (total phenolics, ascorbic acid and reduced glutathione), enzymatic activities (guaiacol peroxidases, ascorbate peroxidase and catalase).

The results reported in this work are original and are not published in or submitted elsewhere.

Best regards,

Laura Rustioni auro Autori

Dear Editor,

the paper has been revised following the reviewer' suggestions. The title and the text have been modified specifying the focus on photooxidation each time that the term "sunburn" was written.

We thank you and the reviewer for the time and attention dedicated to our manuscript

Best regards

Laura Rustioni

Oxidations in the berries affect grape characteristics and quality

Ripening and artificial sunburn effects were studied in 4 white grape cultivars

Chemical and enzymatic redox metabolisms were investigated

PCA <u>produced five functions with eigenvalues higher than 1highlighted similarities and differences in</u> oxidative stress responses

Function 1 is representative of sample oxidation and function 2 of natural ripening

Oxidations in white grape (*Vitis vinifera* L.) skins: comparison between ripening process and photooxidative sunburn symptoms

Laura Rustioni¹*, Daniela Fracassetti², Bhakti Prinsi³, Filippo Geuna³, Alessandro Ancelotti³, Valerio Fauda², Antonio Tirelli², Luca Espen³, Osvaldo Failla³

¹ Laboratorio di Coltivazioni Arboree, DiSTeBA (Dipartimento di Scienze e Tecnologie Biologiche e Ambientali), Università del Salento, Lecce, Italia

² Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via G. Celoria 2, 20133 Milan (Italy)

³ Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano Via G. Celoria 2, 20133 Milan (Italy)

*Correspondence to: Prof. Laura Rustioni, Università del Salento, Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Centro Ecotekne - via Provinciale Monteroni, 73100 Lecce. tel.: 0832 297076; email: laura.rustioni@unisalento.it

ABSTRACT

Oxidations in grape berries are gaining major interest as they affect grape characteristics and quality. Considering berries, Reactive Oxygen Species are involved in the responses to both ripening process and stresses, including photooxidative sunburn. Redox metabolism involves a multitude of chemical and enzymatic reactions. In this study, four white grape cultivars were examined for natural ripening and photooxidative sunburn effects (obtained in artificial conditions) on berry pigmentation, chemical composition and enzymatic activity. The measured parameters included reflectance spectra, pigmentation (including berry browning), content of photosynthetic pigments, organic acid profiles, antioxidant activity, concentrations of antioxidants (total phenolics, ascorbic acid and reduced glutathione), enzymatic activities (guaiacol peroxidases, ascorbate peroxidase and catalase). The effects of the treatment (natural ripening and artificial photooxidative sunburn) on each considered parameter are described in the paper. Photooxidative sunburn strongly affected the contents of antioxidants and chlorophylls, increased the browning index and modulated the enzymatic activities investigated. Samples clearly clustered depending on the oxidation status. Furthermore, the PCA highlighted the similarities and differences in the responses to oxidative stress during ripening and photooxidative sunburn. PCA produced five functions with eigenvalues higher than 1, representing 87.03 % of the total variability. In particular, the scores of the function 1 discriminated the samples based on the oxidation status, while the function 2 separated the samples based on the sampling date, representing the physiological responses characteristic of ripening. Our work sheds light on this topic, and will allow a more conscious vineyard management, thus supporting the agricultural adaptation to climate changes.

KEYWORDS: grape (*Vitis vinifera* L.); fruit ripening; sunburn; oxidative stress; reflectance spectroscopy; chemical composition; enzymatic activities

INTRODUCTION

It is well known that oxidations play important roles in plants. They are involved in physiological processes, such as ripening, as well as in both biotic and abiotic stress responses. Reactive oxygen species (ROS) are considered the main compounds responsible for the oxidative processes¹.

In plant cells, ROS are produced through two different mechanisms: excitation energy excess in the photosystems and byproducts of the oxidative metabolism in mitochondria, peroxisomes and chloroplasts²⁻ ⁵. To avoid cell damages, ROS can be stabilized or inactivated by antioxidants through electron transfer⁶.

The plant cells counteract the oxidative phenomena through enzymatic and non-enzymatic mechanisms. The most important enzymatic systems are superoxide dismutase (SOD)^{6,7}, catalase (CAT)^{8,9}, peroxidases, such as ascorbate peroxidase (APOX) and glutathione peroxidase (GPX)^{4,6,8-11}, ascorbate glutathione cycle^{8,12}, peroxiredoxins⁴, glutathione S-transferases¹³ and polyphenol oxidases¹⁴. Among the non-enzymatic systems, the most important are carotenoids^{3,6}, tocopherol^{4,9}, proline⁹, ascorbic acid¹⁰, glutathione⁶, lipid peroxidation¹⁵ and phenolics^{6,9,16}.

In the past, ROS were only considered toxic byproducts of the metabolisms. Nowadays, it is clear that they play fundamental roles in plants as signal molecules, controlling important processes such as growth, ripening and responses to biotic and abiotic stresses^{2,9,11,17}. Normally, production and detoxification of ROS are in equilibrium, but in case of stresses, an oxidative burst occurs¹⁸, leading to peroxidation of membrane lipid, disruptions of photosynthetic pigment, inactivation of the enzymes, especially those involved in the CO₂ fixation reactions, and cell death^{5,9,19}.

In grape berries, at veraison, a transcriptional reprogramming is triggered by internal and external stimuli. Internal stimuli are mainly hormones (especially abscisic acid and brassinosteroids in grape, a nonclimacteric fruit); however, an oxidative burst have been highlighted at the beginning of ripening in grape and also in other fruits (i.e. tomato), suggesting a role of ROS in the regulation of fruit development^{10,17,20,21}. During ripening, the photosynthetic activity in berries decreases, and this transitional phase can cause a disequilibrium among ROS formation and detoxification^{17,22}. At veraison and during ripening, antioxidant systems are over-activated in berry pulp and skin^{10,20,23,24}. Oxidations are also responsible for seed browning during fruit ripening^{14,25-30}.

Oxidations play a central role in sunburn symptoms, as well³¹. Excessive photosynthetically active radiation could produce damages to photosystems as a result of ROS production³². Thus, photooxidative sunburn lead to symptomatic pigmentations due to chlorophyll loss and brown pigment accumulation^{19,33-36}. Under controlled conditions, it is possible to sunder the effects of excessive radiations and temperatures (or other meteorological and physiological parameters), simply keeping the detached berries in conditioned rooms with an artificial light system for few hours¹⁹. Of course, this simplification is not completely representative of the complexity of the vineyard conditions, but it allows to focus the attention on the effects of specific parameters.

Currently, great attention is given to climatic changes, which could cause metabolic dysfunctions in berries, by modifying the environmental conditions for plant growth³⁷⁻⁴⁰. This could lead to a different modulation

of the ripening processes, as well as it could impose more stressful conditions. This topic is of particular interest in the Mediterranean basin.

The aim of this research is to compare photooxidative sunburn and ripening processes, highlighting differences and similarities concerning the biochemical changes involved in the oxidative responses in grape skin.

1. MATERIALS AND METHODS

1.1. PLANT MATERIAL AND EXPERIMENTAL DESIGN

The experiment was carried out in vintage 2015. Chardonnay, Muscat blanc à petits grains, Riesling and Cortese grapes have been selected for the study. All of them were grown in the same germplasm collection vineyard, in Oltrepò pavese (longitude 9°05', latitude 44°58', elevation 144m a.s.l.) on a hilly terrace. Soil is typical clay (Udic Paleustalfs fine silty, mixed, superactive, mesic following the USDA soil taxonomy ⁴¹). Adopting Koeppen's classification ⁴², the experimental site has a mesothermal climate with transitional characteristics between Oceanic and Mediterranean types. Plants were spaced by 2.5 m (interrow) x 1 m (intrarow), with a density of about 4000 plants/ha, and trained to Guyot system (winter pruning: two-bud spur and 10–12 bud cane).

48 samples were taken: for each cultivar, 3 replicates of shaded bunches (3 bunches/replicate) were selected in 3 BBCH-phenological phases, and samples collected at stage 'bunch closure' were divided in two parts (one representing the control T0 samples and the other undergone artificial photooxidative sunburn). Bunches were collected in BBCH 79 – bunch-closure – 16^{th} July (T0), BBCH 85 – post-veraison – 6^{th} August (T1) and BBCH 89 – berries ripe for harvest – 27^{th} August (T2) ⁴³. A sub-set of green berries collected at BBCH 79, were treated four hours in order to cause photooxidative sunburn (S). Two LED lighting systems (version LumiGrowTM Pro 325, LumiGrow, Novato, CA, USA) were used to obtain a light intensity (1788 ±226 µm of photons m⁻²s⁻¹) similar to the midday conditions in a late spring sunny day in the local vineyards, as described by Rustioni et al.¹⁹. The treatments were carried out in an air conditioned dark room kept at 24°C, however, an increase in the temperature in grape berries should be expected due to irradiance, reaching about 38°C¹⁹. The photo-oxidation of green berries produced the classical brownish areas on the exposed berry surfaces.

Reflectance spectra were collected on fresh materials. Entire grape skins (T0, T1 and T2) or symptomatic portions of them (S) were separated and immediately frozen in liquid nitrogen. Chemical and enzymatic analyses were carried out on sub-samples of the skin powder mixture of each biological replicate. Samples were kept at -80°C until analyses, and extractions were made by adding solvents directly on frozen powders to avoid oxidations.

1.2. REFLECTANCE SPECTROSCOPY

At least 20 reflectance spectra were collected for each sample replication, for a total of 1200 spectra analyzed. Detections were obtained by using a Jaz System spectrometer (Ocean Optics, B.V., Dunedin, USA), completed with a channel with a DPU module and an ILX511b detector, an OFLV-3 filter, an L2 lens, and a 50 µm slit as installed options. A reflection probe QR600-7-VIS125, consisting of a tight bundle of seven optical fibers (600 µm in diameter), in a stainless-steel ferrule (six illumination fibers around one read fiber), was coupled to the spectrophotometer. A probe holder was included to ensure the analytical reproducibility: the distance between the sample surface and the probe was fixed at 12 mm. The instrument was set up with a near infrared– visible (NIR–vis) light source (Ocean Optics), 4095 power setting and an integration time automatically corrected by the instrument. Collected spectra ranged between 341 and 1025 nm and had a spectral resolution of about 0.3 nm. In this work, the visible spectral changes (400–750 nm) are presented and discussed. Each spectrum was set to be the average of 15 spectra, which were directly calculated by the instrument. The spectra were converted into percentage of

reflectance (R) after a calibration with a blank, obtained using a polytetrafluoroethylene (PTFE) diffuse reflectance standard (Ocean Optics B.V.). To underline the reflectance variations among different wavelengths, each spectra value was raised to the fourth power. These spectra were used for the calculation of indexes. The browning intensity index (BII) was calculated according to Rustioni et al.³⁴ by using the following formula:

$$BII = \frac{100R_{t678}}{R_{t490}} - \frac{100R_{T0678}}{R_{T0490}}$$

where R_{tn} = reflectance of T1, T2 or S spectrum at n wavelength, and R_{T0n} = reflectance of the average T0 spectrum of the corresponding cultivar at n wavelength.

Chlorophyll indexes were calculated by using the algorithms proposed by Merzlyak et al.⁴⁴ (ICI) and Rocchi et al.⁴⁵ (IChl_{tot}; IChl_a; IChl_b):

$$\begin{split} & IChI = (R_{800}/R_{678}); \\ & IChI_{tot} = IChI_a + IChI_b; \\ & IChI_a = log \left[(R_{800}/R_{675}) - (R_{800}/R_{660}) \right]; \\ & IChI_b = log \left[(R_{800}/R_{650}) - (R_{800}/R_{630}) \right]. \\ & \text{where } R_n = \text{reflectance at n wavelength.} \end{split}$$

For the description of the spectrum modifications during ripening or as a consequence of photooxidative sunburn, the reflectance spectra were converted to approximate the (quasi)linear relationship between pigment content and optical reflectance-based indices using reciprocal values. A normalization to chlorophyll red maximum (678 nm) was then performed. Finally, the difference (*delta spectra*) between each T1, T2 or S spectrum *vs.* the average T0 spectrum of each cultivar was calculated, as already proposed by Rustioni et al.³⁴.

1.3. SPECTROPHOTOMETRIC ASSAYS

1.3.1. Chlorophylls and carotenoids

Photosynthetic pigments were quantified adapting the method proposed by Lichtenthaler⁴⁶: 100 mg of skin powder were extracted for 3 hours, in dark conditions, in 1.5 ml of ethanol 95% (calcium carbonate salt was added to prevent degradation). After centrifugation at 10000 rpm for 10 minutes at 4°C (Eppendorf 5810 Centrifuge), the absorbance at 470, 490, 508, 648.6, 664.2 and 750 nm was recorded. Chlorophylls (*a*, *b* and total) and carotenoids (including xanthophyll) were calculated following the formulas proposed by Lichtenthaler⁴⁶ and adapted to the extraction procedures:

$$Chlorophyll a = \frac{(13.36 A_{664.2} - 5.19 A_{648.6}) * 8.1}{DW} * \frac{1.5}{8000}$$

$$Chlorophyll b = \frac{(27.43 A_{648.6} - 8.12 A_{664.2}) * 8.1}{DW} * \frac{1.5}{8000}$$

$$Total Chlorophylls = \frac{(5.24 A_{664.2} + 22.24 A_{648.6}) * 8.1}{DW} * \frac{1.5}{8000}$$

$$Carotenoids = \frac{(4.785 A_{470} + 3.657 A_{664.2} + 12.76 A_{648.6}) * 8.1}{DW} * \frac{1.5}{8000}$$

where A_n = Absorbance at n wavelength, and DW = dry weight

The ratios among chlorophyll *a* and *b*, as well as among chlorophylls and carotenoids, were also calculated.

1.3.2. Total phenolic content

Total phenolics were quantified following the method proposed by Di Stefano et al.⁴⁷, with minor modifications: 50 mg of skin powder were extracted by 1.5 ml of hydrochloric ethanol, a solution containing ethanol:water:hydrochloric acid 37% 70:29:1 (v/v). The extraction lasted 24 hours at room temperature under continuous shaking. Then, samples were centrifuged at 10000 rpm for 10 minutes at 4°C. In a 10 ml flask, 2.5 ml of water were added to 0.5 ml of diluted extract. Then, 0.5 ml of Folin Ciocalteu reagent were added. After 3-5 minutes incubation, 2 ml of 10% Na₂CO₃ were added, and the flask was filled up to 10 ml with water. After 90 minutes of reaction, the absorbance was read at 700 nm (using a blank made in the same way, but with water instead of the tissue extract).

The total phenolic compounds were calculated as catechin (mg/l) by the formula:

catechin (mg/l) = 186.5 * A₇₀₀ * d

where A₇₀₀ = absorbance at 700 nm and d = sample dilution

1.3.3. Antioxidant activity - DPPH assay

The analysis of the antioxidant capacity of grape skin samples was carried out by employing the DPPH assay, following the method of Brand-Williams et al.⁴⁸ with some modifications⁴⁹. The DPPH solution was diluted in methanol to obtain 1.00 \pm 0.03 absorbance units at 515 nm. The grape skin samples were suspended (20 g/L) in 70 % methanol (v/v), sonicated for 5 min and centrifuged at 14000 rpm for 10 min at 10°C (benchtop centrifuge, Hettich, Tuttlingen, Germany). The supernatant was recovered and diluted up to 8 times by performing 3 dilution steps 1:2 each. The DPPH solution (2.94 mL) was placed in a cuvette and 60 µL of sample were added. The DPPH assay was carried out for all the diluted samples. The absorbance at 515 nm was measured after 50 min incubation at 20±1 °C. A calibration curve was prepared by adding increasing concentrations of Trolox, from 0.25 to 5 mmol; each concentration was assayed in triplicate. Results were expressed as mmol Trolox per 100 g of fresh grape skin. Each grape skin extract was analyzed in triplicate.

1.4. HPLC ANALYSES

1.4.1. Preparation of grape skin extract

For the quantification of organic acids, ascorbic acid and reduced glutatione (GSH), a grape skin extract was prepared as reported by Pons et al.⁵⁰ with some modifications. Fifty mg of grape skin were suspended in 1.5 mL of hydrochloric acid 0.1 mol containing EDTA 300 mg/L and sodium fluoride 200 mg/L, in order to inhibit the polyphenol oxidase activity⁵¹. The suspension was sonicated for 5 min and centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was collected, filtered with PVDF filters 0.22 μ m (Millipore, Billerica, MA, USA) and immediately analyzed for the quantification of organic acids (Paragraph 2.4.2) and ascorbic acid (Paragraph 2.4.3). The derivatization with *p*-benzoquinone was carried out prior HPLC analysis for the assessment of GSH (Paragraph 2.4.4).

1.4.2. Determination of organic acids

The content of tartaric acid, malic acid, citric acid and succinic acid was determined as described by Fracassetti et al.⁵². An Acquity HClass UPLC (Waters, Milford, MA, USA) system equipped with a photo diode array detector 2996 (Waters) was used. Chromatographic separations were performed with a Hypersil BDS C8 250 x 4.6 mm, 5 μ m particle size (Alltech, Deerfield, IL, USA). The separation was carried out in isocratic condition using sulphuric acid 0.01 N as eluting solvent. The flow rate was 0.8 mL/min, and the column

temperature was 25°C. Calibration curves were obtained for tartaric acid, malic acid, citric acid and succinic acid concentrations in the range from 0.1 g/L to 5 g/L. Quantification was performed according to the external standard method. Data acquisition and processing were carried out by Empower 2 software (Waters) at 210 nm.

1.4.3. Determination of ascorbic acid

Quantification of ascorbic acid (AA) was carried out by UPLC-UV⁵³. The separation was performed with a Waters Acquity HClass UPLC (Waters) equipped with a photodiode array detector Waters 2996 (Waters) and a C18 column (Nova-Pak 150 x 3.9 mm, 4 μ m, Waters). The chromatographic separation was carried out with an isocratic elution running acetate buffer 50 mmol/L, at pH 4.5 amended with 5% methanol (v/v) for 15 min, followed by column washing (100% methanol for 2 min) and column conditioning (4 min). The flow rate was 0.9 ml/min. Column temperature was 25°C and the injection volume was 20 μ l. Chromatographic data were registered from 230 nm to 500 nm and processed at 261 nm by Empower 2 software (Waters).

1.4.4. Determination of reduced glutathione

Reduced glutathione (GSH) was quantified by derivatization with *p*-benzoquinone (pBQ)⁵⁴. Briefly, the extract (500 μ L) was added with pBQ 0.4 mmol dissolved in methanol (50 μ L). After mixing for 1 min, 500 μ L of 3-mercapto propanoic acid (0.5 mmol in citrate buffer 0.3 mol, pH 3.5) were added. GSH adducts with pBQ were determined by UPLC-UV following the separation conditions described by Fracassetti and Tirelli⁵⁴. Besides GSH, the method also allows the quantification of cysteine.

1.5. ENZYMATIC ACTIVITIES

Frozen samples (0.5 g) were finely powdered in liquid N₂ using a pestle and mortar, and then homogenized in six volumes of cold (-20°C) acetone. Samples were washed twice on Whatman 41 filter paper (Whatman International Ltd) with 3 ml of cold acetone and dried under vacuum. The acetone powder was then suspended in 3 volumes of extraction buffer [40 mM phosphate buffer (pH 7.2) containing 50 mM KCl, 50 mM MgCl₂, 1 mM ascorbic acid, 5mM mercaptoethanol, 0.5% NP-40, 2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride]. The extract was centrifuged at 14000 g for 20 min, then both protein content and enzymatic activities were measured on the obtained supernatant. Protein contents were quantified by the BioRad protein microassay procedure (Bio-Rad Laboratories).

Guaiacol peroxidases activity (GPX) was evaluated measuring the oxidation of guaiacol as previously described by Pandolfini et al.⁵⁵, while the activities of ascorbate peroxidase (APX) and catalase (CAT) were measured according to Ozden et al.⁵⁶.

1.6. DATA ELABORATION

The study was focused on the coherent effects among cultivars, considering that one year of data collection in only one site is not sufficient to clearly describe the differential responses of each cultivar taking into account the plasticity of the grape varieties. Thus, to highlight the treatment effects (ripening vs. photooxidative sunburn) on grape berries, the responses of individual cultivars are not discussed in detail, but these results are available in supplementary materials. Furthermore, results have been normalized to underline the coherence of the effects of oxidations among the different cultivars. Data were elaborated by using SPSS statistical software (version PASW Statistics 21, SPSS Inc. Chicago, Illinois). To normalize the data variability across the different cultivars, results were expressed as percentage variation from the average TO value of each studied variety (with the exception of the BII, because it already considers the variation with respect to the TO average spectrum of each cultivar). One-way ANOVA was carried out in order to determine statistical differences (p < 0.05) due to the ripening and/or photooxidative sunburn treatment.

Moreover, the Principal Component Analysis (PCA) was done to cluster the varieties and sampling points in relation to the parameters investigated.

2. RESULTS AND DISCUSSION

2.1. REFLECTANCE SPECTROSCOPY

Figure 1 represents the variations of the spectra with respect to the TO average spectrum within each cultivar (delta spectra), reported as average of all the four cultivars examined. The spectral modifications related to ripening and photooxidative sunburn clearly appear. The characteristic bands of chlorophylls a and b appear as minimum pick at \approx 678 nm and shoulder \approx 650 nm, respectively. Due to the normalization choice (678 nm), at this wavelength the *delta spectra* value is 0; however, the spectral minimum clearly underlines the degradation of chlorophylls. Similar results are shown in Rustioni et al.³⁴. The negative values between 650 and 678 nm at ripening (T2) indicate a lower degradation of chlorophyll b with respect to chlorophyll a during ripening (confirmed also by the intermediate degradation proportion between the bands of the two chlorophylls in the T1 spectrum). For that reason, it seems that chlorophylls a and b undergo differential degradations during ripening and photooxidative sunburn. The relative minimum observed around 490 nm in T2 could be also ascribed to a major effect of the chlorophyll degradation (blue absorption) with respect to the accumulation of brown pigments. Considering photo-oxidation, Rustioni et al.³⁴ found an absorption maximum at \approx 490 nm, while, in the present study, spectra of samples S show only a shoulder at this wavelength. Concerning sunburn photooxidation, the main peak maximum is around 525 nm. However, this band is broad, with different shoulders, one of them at ≈550 nm (maximum in T1 and T2). Similar results were described in Rustioni et al.⁵⁷, in relation to white berry color modifications during ripening, and in Rustioni et al.²⁹ and VanderWeide³⁰, concerning grape seed natural and artificial ripening. Absorption of low energetic visible wavelength is possible due to highly delocalized π -conjugated systems. For example, considering phenolic compounds, absorption maxima at high visible wavelengths (\approx 530 nm) are usually ascribed to anthocyanins and it is possible thanks to the HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) delocalization among the 3 flavonoid π conjugated rings⁵⁸. It is possible to hypothesize that the breadth of the band shown in the S delta spectrum (Fig. 1) is indicative of the variability among reaction products obtainable by phenolic free radical oxidations. In less stressing conditions, such as natural exposition of nearly ripen bunches at the end of August, the incipient symptoms are related to low oxidative phenomena (small oxidative polymers with narrow π -conjugated systems)³⁴. More intense (artificial oxidation of berries rich in chlorophyll) or prolonged oxidations could produce larger oxidized polymers with larger π-conjugated systems (with absorption spectra more similar to anthocyanins). Free radical scavenging occurs by hydrogen and electron donations. The phenolxyl radicals produced through phenolic antioxidative reactions are potential prooxidant when their lifetime is prolonged⁵⁹. On the other hand, the antioxidant activity of phenolics is related to the stability of the resulting phenoxyl radical, due to the resonance stabilization of π -conjugated systems⁶⁰. The larger π -conjugated systems of oxidized brown pigments could have an improved antioxidant power (becoming favorable free radical scavengers), resulting in the characteristic oxidative bursts and producing larger and more conjugated polymers.



Figure 1: Treatment spectral variations with respect to the TO average spectrum of the related cultivar (normalized at 678 nm). The line thickness is representative of the error bars (95% CI).

2.2. CHEMICAL COMPOSITIONS AND ENZYMATIC ACTIVITIES

To compare the effects induced by ripening and photooxidative sunburn among grape berries of different cultivars, and hence characterized by individual chemical composition, the changes observed were expressed as variation with respect to the average value at TO (normalized at 100), within each cultivar (Table 1). Reflectance indexes already underwent various normalizations during data processing and, thus, they are used in their original values.

Normalized data were subjected to ANOVA, to highlight the explained and unexplained variance. We found a dominant effect of the treatment (T0; T1; T2 and S) for each parameter measured (Fig. 2). Only in four cases out of 22, the treatment did not reach the highest fraction of the explained variance. However, only concerning chlorophylls/carotenoids ratio, succinic, tartaric and citric acids and GPX the treatment did not produce significant differences, while, concerning ascorbic acid, despite the strong effect of the cultivar, the treatment effect was significant.

Overall, these results highlighted a very similar trend among all the cultivars, confirming the reliability of analyzing data as the average value of all of them, in order to better highlight the common responses during ripening and photooxidative sunburn (Table 1).





Figure 2: Explained and unexplained variance of the normalized data subjected to ANOVA. In the graph, italic numbers on the top of the bars indicate the significance p-values of the treatment effect for each parameter considered.

Table 1: Reflectance indexes (Table 1a), spectrophotometric analyses (Table 1b), HPLC quantifications (Table 1c) and enzymatic activities (Table 1d) in grape berry skins at different ripening stages (T0, T1, and T2) and after the photooxidative sunburn treatment (S). Results are expressed as relative increase with respect to the average T0 value (normalized at 100), calculated within each variety and averaged among all of them (with the exception Browning Intensity Index, which already represents the relative increase of brown pigments with respect to the T0 and other spectrophotometric indexes due to the presence of negative values). Average values and standard errors are reported. Different letters represent significantly different means (p < 0.05)

	Т0	T1	T2	S
Browning Intensity Index	-0.13 ± 0.22^{a}	3.20 ± 1.07^{b}	4.14 ± 1.98^{b}	36.26 ± 3.12 ^c
IChl	$10.29 \pm 0.98^{\circ}$	4.96 ± 0.32^{b}	4.64 ± 0.27^{b}	$2.36 \pm 0.27^{\circ}$
IChI _{Tot}	0.63 ± 0.08 ^ª	-0.19 ± 0.06^{b}	-0.19 ± 0.06^{b}	$-1.50 \pm 0.12^{\circ}$
IChl _a	0.42 ± 0.04^{a}	$0.06 \pm 0.03^{\circ}$	0.15 ± 0.02^{b}	-0.63 ± 0.07^{d}
IChl _b	$0.21 \pm 0.04^{\rm a}$	-0.02 ± 0.19^{b}	$-0.34 \pm 0.03^{\circ}$	-1.04 ± 0.07^{d}

Table 1a – **REFLECTANCE INDEXES**

Table 1b - SPECTROPHOTOMETRIC ANALYSES

	Т0	T1	T2	S
Total Chlorophylls	100.00 ± 4.54^{a}	60.85 ± 7.74 ^b	32.82 ± 4.36 ^c	15.05 ± 2.32 ^d
Chlorophyll a	$100.00 \pm 4.60^{\circ}$	66.94 ± 8.42 ^b	36.56 ± 4.64 ^c	13.70 ± 2.55 ^d
Chlorophyll b	100.00 ± 4.57ª	49.44 ± 6.52 ^b	25.88 ± 3.87 ^c	17.57 ± 2.18 ^c
Carotenoids	100.00 ± 3.43 ^a	66.21 ± 6.27 ^b	44.68 ± 4.52 ^c	18.94 ± 3.61^{d}
Chlorophyll a/Chlorophyll b	100.00 ± 1.37^{b}	137.67 ± 3.22 ^a	144.98 ± 4.99^{a}	73.84 ± 7.16 ^c
Clorophylls/Carotenoids	100.00 ± 2.22 ^ª	88.95 ± 3.99 ^a	71.37 ± 2.24 ^a	75.12 ± 6.63 ^a
Antioxidant activity	100.00 ± 2.01 ^ª	63,269 ± 2.26 ^b	59,065 ± 3.80 ^b	26,336 ± 8.83 ^c
Total Phenolic content	100.00 ± 3.48^{a}	72.614 ± 3.94 ^b	60.282 ± 4.16^{bc}	51,132 ± 5.55 ^c

Table 1c - HPLC QUANTIFICATIONS

	то	T1	T2	S
Tartaric acid	100.000 ± 3.89^{a}	$78,23 \pm 6.22^{\circ}$	87.90 ± 7.39 ^a	105.58 ± 9.89 ^a
Malic acid	100.000 ± 4.67^{b}	93.43 ± 9.57 ^b	93,90 ± 7.59 ^b	168,46 ± 14.77 ^ª

Citric acid Succinic acid	$100.000 \pm 7.18^{\circ}$ $100.000 \pm 6.18^{\circ}$	97.95 ± 12.27° 101.78 ± 11.16°	106.59 ± 6.46° 101.58 ± 6.58°	137.27 ± 13.03° 88.59 ± 6.95 ^a
Ascorbic acid	100.000 ± 8.21^{b}	130.57 ± 62.68 ^b	389.27 ± 212.31 ^ª	0 (n.d.) ^c
Glutathione	100.000 ± 3.96^{b}	140.63 ± 9.81 ^b	197.26 ± 23.32 ^ª	0 (n.d.) ^c
able 1d - ENZYMATIC ACTIVI	TIES			
able 1d - ENZYMATIC ACTIVI	TIES TO	T1	T2	S
able 1d - ENZYMATIC ACTIVI Guaiacol peroxidases (GPX)	TIES T0 100,000 ± 7.82 ^a	T1 108.99 ± 25.86 ^a	T2 90.56 ± 16.91 ^a	S 107.61 ± 22.60 ^a
able 1d - ENZYMATIC ACTIVI Guaiacol peroxidases (GPX) Ascorbate peroxidases (APX)	TIES T0 100,000 ± 7.82 ^a 100.000 ± 9.97 ^b	T1 108.99 ± 25.86 ^a 115.01 ± 26.94 ^b	T2 90.56 ± 16.91 ^a 134.67 ± 11.77 ^b	S 107.61 ± 22.60 ^a 941.27 ± 75.59 ^a

2.2.1. Reflectance spectroscopy

The browning intensity index showed a slight, yet significant, darkening of the grape skins during ripening (Table 1a). Similar results were already obtained in skins⁵⁷ and seeds^{27,29,30}. Obviously, the photooxidative process caused by sunburn produced stronger symptoms and higher values of this index.

According with the available literature, all the chlorophyll-related indexes confirmed the photosynthetic pigment degradation produced by both ripening^{45,57,61} and photooxidative sunburn processes^{19,35,36}.

2.2.2. Spectrophotometric analyses

The spectrophotometric analyses of skin extracts (Table 1b) confirmed the data obtained by reflectance spectroscopy concerning the degradation of chlorophylls and, in general, photosynthetic pigments: also carotenoids showed similar trends. In general, it is possible to note a similar decreasing trend during ripening, which is emphasized in consequence of photooxidative sunburn. In fact, photosynthetic pigment management is considered among the most important protective strategies against photo-oxidation in plants^{19,35,62}. Furthermore, no significant differences were obtained concerning the ratio chlorophylls/carotenoids, indicating a strong relation among the degradation of both groups of pigments.

Considering the chlorophyll a/chlorophyll b ratio, during ripening, chlorophyll b underwent a stronger decrease with respect to chlorophyll a, while photooxidative sunburn produced the opposite effect. Different results were obtained in previous studies, where photooxidative sunburn produced stronger degradation of chlorophyll b with respect to chlorophyll a^{19} ; however, in this paper, the authors found an important contribution to the variability due to the genotype*treatment interaction, also suggesting a cultivar classification, based on their susceptible or tolerant responses to radiation excess.

Concerning the total phenolic content, it was possible to note a decrease in the molecular concentration during ripening, accentuated by photooxidative sunburn. This decrease could also be ascribed to either a lower extractability or a lower reactivity to the Folin Ciocalteu reagent of the oxidized molecules.

As expected by the total phenolic and carotenoid quantifications, the antioxidant activity of the extracts decreased, especially in consequence of photooxidative sunburn, according to an oxidative stress caused by light. Although degradation events cannot be excluded, it is also possible that the decrease in the antioxidant activity has been due to the consumption of antioxidants by active metabolic responses to the excess of light in berry tissues.

2.2.3. Organic acids

The organic acids of grape are represented by tartaric acid, malic acid, citric acid and succinic acid. Table 1c summarizes the changes in concentrations of these compounds in berry skins during ripening and after photooxidative sunburn.

Tartaric acid concentrations (average at ripening stage: 1.30 ± 0.59 mg/100 mg grape skins) remained stable up to the harvest, in agreement with Cholet et al.⁶³. This was expected because tartaric acid is synthetized in the first stages of grape berry development and it is accumulated up to veraison. The photooxidative sunburn had a negligible effect on tartaric acid.

Malic acid represented, together with tartaric acid, the major organic acid of grape (average at ripening stage: 1.48±0.64 mg/100 mg grape skins). It also kept similar concentrations during ripening, showing a little and not significant decrease in concentration. This is because malic acid is mainly synthetized before veraison⁶⁴. The photooxidative sunburn led to a significant increase in the content of malic acid. On the contrary, Moreno and Peinado⁶⁵ reported that malic acid decreased, probably as a consequence of an

increase in energy demand by berry metabolism. The availability of malic acid for these responses is dependent on the ripening stage, since malic acid is transported into the vacuole during maturation⁶⁶.

As expected, citric acid was detected in amounts lower than tartaric and malic acids (average at ripening stage: 0.27±0.04 mg/100 mg grape skins), in accordance to literature⁶⁵. Citric acid is one of the substrates for berry respiration and it is converted to malic acid in the late stages of maturation⁶⁷. In fact, during ripening, a general decrease of citric acid in the berry pulp is expected⁶⁸. Nevertheless, in our samples, no significant differences were found related to the treatments (photooxidative sunburn or ripening), probably due to the different tissues analyzed (berry skins instead of pulps).

Succinic acid was found in low amounts in the grape skin for all the varieties analyzed and it was not affected by ripening (average at ripening stage: 0.50±0.20 mg/100 mg grape skins). Succinic acid is a low concentrated organic acid in the berries of *Vitis vinifera*⁶⁵. Similarly, negligible differences were detected due to the photooxidative sunburn.

2.2.4. Ascorbic acid and reduced glutathione

Ascorbic acid increased during ripening (average at ripening stage: $24.81\pm23.70 \ \mu g/100 \ mg grape skins$). An accumulation of ascorbic acid can be related to an increase in the antioxidant responses⁶⁸ as well as to the exposure of berries to sunlight during ripening⁶⁹. Ascorbic acid dropped to undetectable levels in the samples exposed to the photooxidative sunburn treatment (Table 1c), probably because of its complete oxidation by the ROS induced by the intense exposure to light³⁴. Although the content of ascorbic acid was very different among the varieties investigated (i.e. Cortese grapes had the highest content, with values of 52 μ g/100 g skin, which was 50-folds higher in comparison to Moscato variety), no differences were found in the samples exposed to photooxidative sunburn (data not shown).

Reduced glutathione (average at ripening stage: $8.20\pm2.21 \ \mu g/100 \ mg$ grape skins) showed a trend similar to that of ascorbic acid: it increased during the ripening and it was not detectable after the photooxidative sunburn treatment (Tab. 1). The complete oxidation of reduced glutathione during photooxidative sunburn related well with its antioxidant properties, in particular ROS scavenging. In general, the ascorbate-glutathione cycle can play an important role to counteract the damaging effects of photooxidative sunburn for the maintenance of the redox state of the tissues, by scavenging the hydrogen peroxide and other ROS⁷⁰.

Taken together, these data highlighted that, regardless of the initial content of antioxidant compounds, the intensity/duration of the photooxidative sunburn treatment used in this work completely depleted an important part of the antioxidant system.

2.2.5. Enzymatic activities

Guaiacol peroxidases (GPX), ascorbate peroxidases (APX) and catalase (CAT) are enzymes involved in the enzymatic antioxidant system operating in plants^{9,10}. The trend of these enzymes was very similar among the varieties investigated (data shown in supplementary materials). The activity of APX and CAT dramatically increased after the photooxidative sunburn treatment, whilst no significant changes were found among the different times of ripening (Table 1d). Differently, GPX activity did not change in any experimental condition (Table 1d). APX and CAT are two of the main heme enzymes involved in the H₂O₂ homeostasis and the increase in both their expressions and activities in responses to increasing levels of H₂O2 in the cell are widely known⁷¹. Starting from this knowledge, our results seem to describe a progressive worsening of oxidative stress conditions induced by the photooxidative sunburn treatment. Considering the decline in the contents of ascorbic acid and reduced glutathione, it is very interesting to

observe that the greater increase was that related to CAT, an enzyme that is able to detoxify the H_2O_2 without the request of reducing power. Considering that these enzymes are localized in different cell compartments, the results suggest that the oxidative stress induced by photooxidative sunburn involved the whole cellular environment⁹.

2.3. MULTIVARIATE ANALYSIS

The main aim of this study was to investigate chemical and enzymatic changes in grape berry during ripening and after photooxidative sunburn treatment, in order to gain new information about the antioxidant responses involved in these two processes. In particular, as far as it concerns the photooxidative sunburn effects, the experimental conditions allowed to simulate a very hard stress, useful to unravel the possible roles of the different components of the antioxidant system (*i.e.* enzymatic and non-enzymatic activities). Moreover, the impairment of the protective activities was related to damage effects, evaluated by non-disruptive (*i.e.* reflectance spectroscopy analysis) and disruptive (*i.e.* spectrophotometric and chromatographic analyses) approaches.



Figure 3: Distribution of the samples based on the first two functions obtained by the PCA analysis. Samples are distributed by function 1 in relation to their oxidation. The function 2 separate the samples based on their sampling time. Connecting lines indicate the centroids of each treatment.

The Analysis of Variance (ANOVA) was used to identify explained and unexplained variance, highlighting the dominant effect of the treatment with respect to the one ascribable to cultivar (Fig. 2). Among the 22 parameters analyzed, only in 3 of them the variance was not mainly explained by the treatment. Moreover,

data obtained by chemical and enzymatic analyses were summarized by PCA (Fig. 3). It produced five functions with eigenvalues higher than 1, representing 87.03 % of the total variability (43.98%; 15.19%; 14.64%; 8.64% and 4.58% for PC1, PC2, PC3, PC4 and PC5, respectively). In supplementary materials, it is possible to find the average values, including the error bars, of the functions that describe the combination of treatment and the cultivar effects. The components do not discriminate the cultivars, confirming the effectiveness of the normalization adopted in this study (Supplementary materials). Fig. 3 shows the distribution of the samples based on the first two functions obtained by the PCA analysis. Samples are distributed by function 1 in relation to their oxidation state. TO samples are located to the right of the graph, and, moving to the left, we find, in order, T1; T2 and S samples. The function 2 separates the samples based on their sampling time. Centroids of T0 and S samples are in the upper side of the graph, while T1 and T2 are in the lower part in progressive order. Thus, function 1 summarizes processes that are coherent and common in both ripening and photooxidative sunburn processes, while function 2 highlights the modifications that are characteristic of ripening but not involved in photo-oxidation. To point out the principal components involved in these processes, Tab. 2 reports their matrix.

		C	omponei	nt	
	1	2	3	4	5
BII	-,883	,152	,164	-,189	,048
IChl	,841	,348	,018	,055	-,131
IChI _{tot}	,783	-,241	,216	-,217	-,192
IChI _a	,845	-,153	,080	,004	-,203
IChI _b	-,329	,563	-,414	,511	-,029
Antioxidant activity	,866	,127	,152	-,147	-,135
Total Phenolic Content	,811	,395	,087	-,118	,176
Total Chlorophylls	,877	,426	,013	-,011	-,014
Chlorophyll a	,896	,375	,008	,022	,025
Chlorophyll b	,817	,515	,025	-,073	-,093
Carotenoids	,929	,319	,026	-,006	,042
Chlorophyll a / Chlorophyll b	,448	-,711	-,031	,309	,215
Clorophylls / Carotenoids	,443	-,361	,247	-,380	,562
Tartaric acid	-,064	,162	,934	,066	-,164
Malic acid	-,547	,214	,762	,005	-,057
Citric acid	-,262	,256	,808	,291	,135
Succinic acid	,269	-,059	,715	,519	,090
Ascorbic acid	,082	-,470	,097	,541	-,227
Glutathione	,561	-,483	-,097	,589	,080
Guaiaco lPeroxidase (GPX)	,101	,587	-,261	,405	,547
Ascorbate perxidase (APOX)	-,811	,290	,282	-,174	,160
Catalase (CAT)	-,643	,528	-,301	,285	-,216

Table 2: Matrix of the components of the first five functions,	having
eigenvalues higher than 1	

The first function, representing the common processes among ripening and photooxidative sunburn, is mainly related to brown and photosynthetic pigments (with few exceptions: only the *ICHIb* and the ratio chlorophyll a/chlorophyll b had higher impact in function 2 and the ratio chlorophyll/carotenoid in function

5); total phenolic content; antioxidant activity; and the enzymatic activities of APX and CAT. Considering the HPLC quantifications, malic acid and glutathione contents showed the highest values among the components of function 1.

The second function, discriminating ripening and photooxidative sunburn, is mainly related to the ratio chlorophyll a/chlorophyll b; chlorophyll b (both $ICHI_b$ and extract quantification); and the enzymatic activity of GPX. Again, considering HPLC quantification, the highest values were reached by glutathione and ascorbic acid.

The roles of oxidations in plants are gaining increasing physiological meaning. ROS, both by their oxidative or signaling activities, cause important changes in both berry composition and metabolism during ripening. In this study, photooxidative sunburn and natural ripening have been compared. In both cases, oxidations were confirmed to be involved in the berry responses; nevertheless, despite the similarities, the berry appeared to be able to modulate type and intensity of answer, by a different control of the redox activities, in relation to the two different oxidative stress conditions.

Considering that the levels of ascorbic acid and reduced glutathione were undetectable after the artificial sunburn treatment, this study supports the hypothesis that in these conditions, although the antioxidant enzymes preserved their functionality, they could no longer play a protective role, due to lack of substrate. Even if future evaluations, such as the NAD+/NADH and NADP+/NADPH ratios, could be useful to complete the description of the metabolic changes induced by photooxidative sunburn, these results allowed to highlight dramatic changes in the antioxidant system. Indeed, according to the decrease of both chlorophyll contents and the antioxidant activity evaluated by DPPH assay (Tab. 1), exocarp seemed to have quite entirely lost its ability to counteract the oxidative stresses. In these hard conditions, only some antioxidants, such as phenolic compounds and/or carotenoids, could still play a protective role.

CONCLUSIONS

In conclusion, this study allowed us to highlight the common responses as well as the differences among the physiological oxidation induced by natural ripening and the oxidative response to a strong abiotic stress such as photooxidative sunburn. It is worth to notice that the photooxidative sunburn symptoms were obtained under controlled conditions designed to simplify the natural overlapping of different stresses (e.g. temperature effect and, in general, meteorological and micro-meteorological variations). This approach, in one side is not completely representative of natural and physiological conditions. Future studies will have to take into account the complexity of the field conditions, designing experiments for the evaluation of possible vineyard treatment(s), in order to elucidate the effects of the photooxidative sunburn on both grape and wine characteristics.

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Laura Rustioni prepared the samples. She did the spectrophotometric analyses, including data elaboration and interpretation. She wrote mostly of the paper draft.

Daniela Fracassetti, and Antonio Tirelli, obtained the HPLC data, and they elaborated, discussed and interpreted them.

Bhakti Prinsi and Luca Espen studied the enzymatic activities, including data elaboration, interpretation and discussion.

Alessandro Ancelotti and Valerio Fauda contributed to the sample analyses during their master studies.

Filippo Geuna supported the spectrophotometric data elaboration.

Osvaldo Failla supervised the work and carefully revised the manuscript.







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