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Effect of anthocyanin absence on white berry grape (*Vitis vinifera* L.)

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Summary

In grapevines, white berried cultivars are characterized by the absence of anthocyanins, which are the main pigments in *V. vinifera* fruits. These varieties produce berries with a yellowish color. The pigments responsible of this hue are still not well defined. In this paper, spectrophotometric analyzes were carried out using non-invasive methods (reflectance spectra) and destructive quantifications (chlorophyll and carotenoid quantifications) to describe the variation in color of three white grape varieties during ripening. A decrease in chlorophylls and carotenoids was found. Changes in the proportion of blue (450-500 nm) and red (630-700 nm) absorption bands were underlined. The contribution of melanin-like pigments (oxidation products) is also discussed. In general, our results indicate that the yellow color of white cultivars is not related to the activation of specific biosynthetic pathways. It is most likely due to a series of catabolic processes (and to their relative intensity), which become visible and that are possibly stimulated by the anthocyanin absence.

K e y w o r d s : *Vitis vinifera;* grapevine; reflectance spectroscopy; pigments; melanin-like pigments.

Introduction

The fleshy fruit skin color, thus its pigment composition, is an important trait for angiosperm eco-physiology and evolutionary development. It has a central role in dissemination because it attracts fruit-feeding vertebrates, generating strict co-evolutionary patterns between fleshy-fruited plants and frugivorous animals (Willson and Whelan 1990). Considering grapes, as for many other berries, the seed dispersion is carried out by birds, and in particular by common starling (*Sturnus vulgaris* L.) (Hardie 2000). Seed-dispersing birds are able to distinguish colors and prefer red and black fruits. For these reasons, the natural evolution of the wild grapevines (*Vitis vinifera* L. subsp. *sylvestris* Gmel.) produced fruits strongly pigmented thanks to high anthocyanin accumulation. However anthocyanins in fruit skins may play other roles, like antioxidant as well as UV and visible light protectant, in addition or in alternative to attraction of fruit eating birds (Close and Beadle 2003).

It should be noted that white berried wild grapevines have not been yet described in the wilderness (Arroyo García and Revilla 2013). For this reason, white mutation should be considered a non-adaptive trait for seed dispersal and/or other eco-physiological aspects. Contrarily, white grape cultivars have arisen by mutations of red cultivars (Kobayashi *et al.* 2004, Fournier-Level *et al.* 2010). Kobayashi *et al.* (2004) and Walker *et al.* (2007) found that the loss of pigmentation in white cultivars of *V. vinifera* is associated with a mutation in *VvMYBA1* and *VvMYBA2*, which regulate anthocyanin biosynthesis.

During millennia of grape cultivation, humans selected a range of berry colors, including white grape varieties, to differentiate the production. In fact, fruit color has a central role for the direct consumer attraction as well as for wine-making. For this reason, color is one of the most important ampelographic traits and it is one of the main features to be considered to assess by phenotyping the genotype quality potential (Rustioni *et al.* 2013a).

Tissue spectral signature of grapevine varieties in the visible and NIR ranges is ruled by content, composition and localization of pigments, epicuticular waxes and water content (Butler and Norris 1960, Rustioni *et al.* 2012). All these factors influence fruit spectral characteristics (McClure 1975). In relation to pigment, chlorophyll, carotenoid and anthocyanin contents and proportions determine fruit color and external appearance (Saure 1990, Abbott 1999) and represent markers of their commercial value. Focusing on the optical properties of pigments, only recently the contribution of different compounds to the tissue color in grapes has been approached at the molecular scale (Rustioni *et al.* 2013b). On the other side, no information about the compositional basis and the physiological implication of white grape color are available in literature. During maturation, green berries gradually vary their color towards yellow up to amber. The intensity depends on grape variety and growing conditions. The biochemical explanation of this modification surely involves pigments concentration and their balance in the skin tissue. Nevertheless, specific experimental evidences are not available to describe this phenomenon.

Material and Methods

P l a n t m a t e r i a l a n d g r o w i n g c o n d i t i o n s : The *Vitis vinifera* L. accessions used in this study were all cultivated in the same germplasm collection vineyard, located in the Regional Research Station of Riccagioia (Lombardy region, Northern Italy) already described in a previous article (Rustioni *et al.* 2013a). The site is located in the Oltrepò pavese viticultural area (lon-

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gitude 9°05', latitude 44°58', elevation 144 m.a.s.l.) on a hilly terrace with a slight east exposition with a typical clay soil (Udic Paleustalfs fine silty, mixed, superactive, mesic following the USDA soil taxonomy; Soil Survey Staff 1999). Adopting Koeppen's classification (Strahler and Strahler 2003), the experimental site has a mesothermal climate with transitional characteristics between Oceanic (Cfb) and Mediterranean (Csa) types. The annual average temperature is 11.7 °C and the average temperature of January and July is 0.7 and 22.4 °C, respectively. The pluviometric regime presents an annual rainfall varying between 600 and 800 mm with two maxima (in spring and autumn). The winter and the summer minima correspond to the Oceanic and Mediterranean weather types, respectively. Plants were spaced by 2.5 m (interrow) , 1 m (intrarow) for about 4000 plants/ha, and trained to the classic Guyot system, leaving after winter pruning a two-bud spur and a cane of 10-12 buds. The interrow soil was kept weed-free by two glyphosate herbicide treatments yearly.

Experimental layout: three white berried grapes were selected: 'Ribolla Gialla', 'Pedro Ximenez', 'Moscato di Alessandria' (syn. Muscat of Alexandria). A bunch for each accession was harvested at different phenological stages, following the BBCH scale (Lorenz *et al.* 1995): pre-veraison (77 BBCH), veraison (81 BBCH) and harvest time (89 BBCH). For each accession and sampling time, four berries were studied in 2013 ripening season. On each one, weight, axial and abaxial diameters, reflectance spectra in four different positions, chlorophyll *a*, chlorophyll *b* and carotenoids content by destructive quantifications, were determined.

Berry reflectance detection: Overall 36 reflectance spectra were obtained using a spectrometer Jaz System (Ocean Optics, B.V.), completed with a Channel with a DPU Module and ILX511b Detector, OFLV-3 Filter, L2 Lens and 50 µm Slit as installed options. A reflection probe QR600-7-VIS125 was coupled to the spectrophotometer. The instrument was set up with a NIR/Vis light source 4095 power setting, and the integration time was corrected in order to give the best percentage of reflection during the calibration. Collected spectra ranged between 340-1025 nm with steps of about 0.3 nm. In this work, the visible spectral changes (450-750 nm), will be presented and discussed, with a particular focus on the variations in absorbance in the blue (450-490 nm) and the green (490-560 nm) regions. Experimental spectra were the average of 20 single instrumental spectra. The spectra were calculated as percentage of reflectance (% R) in comparison with a reference blank spectrum obtained by WS-1 Diffuse Reflectance Standard made of PTFE (Ocean Optics, B.V.). Also a dark spectrum was taken with the light path blocked.

Spectra elaboration: The relationships between reflectance signal and chromophore content is non-linear. Thus, the reflectance spectra were converted to approximate to the (quasi) linear relationship between pigment content and optical reflectance-based indexes using the following formula:

$$A = log\ (1 \cdot R^{-1})$$

where A is absorbance and R reflectance. Average absorbance spectra of each berry, were then rescaled according the detected concentration chlorophyll *a* as follows:

$$Ac = (Ca)^{*}(A_x) \cdot A_{678}^{-1}$$

where Ac is the absorbance corrected in relation to the measured chlorophyll *a*; Ca is the concentration of chlorophyll *a*; A_x is absorbance at each wavelength; A_{678} is the absorbance at 678 nm.

In order to evaluate pigments content in terms of contribution to the variation in color of the berries during the different phenological stages, we studied the modifications in the proportion of blue (450-500 nm) and red (630-700 nm) component in the absorbance spectrum. Therefore, we normalized the overall absorbance spectra to the chlorophyll *a* maximum peak of absorbance at 678 nm as follows:

$$Ar = Ac_x \cdot Ac_{678}^{-1}$$

where Ar is the relative absorbance; Ac_x is the Ac value at each wavelength; Ac_{678} is the *Ac* value at 678 nm.

Chlorophyll, carotenoid and xanthophyll extraction: Just after the detection of the optical properties, pigments concentrations were determined on the same berry sample. All the four berries for each accession were separately squeezed to isolate the exocarp from the pulp. The exocarps were ground in liquid nitrogen, and 1.5 mg of $CaCO_3$ salt (Sigma-Aldrich Co) were added to prevent the formation of pheophytins, and to neutralize the tissue samples avoiding cis/trans isomerisation when extracting carotenoids (Krasnow *et al.* 2010). All procedures were carried out in green subdued light to minimize light-associated degradation of chlorophylls and carotenoids (Kamffer *et al.* 2010). Ethanol (Sigma-Aldrich Co) 95 % was used for the extraction.

A Jasco model 7800 recording spectrophotometer was used to quantify chlorophyll *a*, chlorophyll *b* and carotenoids plus xanthophylls concentrations following Lichtenthaler (Lichtenthaler 1987).

The berry surface was calculated using the measured diameters. Berries were compared to prolate or oblate spheroids (a = b > c in oblate ellipsoid of revolution and a = b < c in prolate ellipsoid of revolution, where a, b and c are the semi-principal axes length). The surfaces (S) were calculated as follows:

The concentrations measured by destructive quantifications were then converted in µg·cm^{-2} to be compared with the surface optical properties.

$$S(oblate) \approx 2\pi \left(\alpha^2 + c^2 \frac{\operatorname{arctanh}(\sin(\text{œ}))}{\sin(\text{œ})}\right)$$

$$S(prolate) \approx 2\pi \left(c^2 + ac \frac{(\text{œ})}{\sin(\text{œ})}\right)$$

$$\text{œ} = \arccos\left(\frac{c}{a}\right)$$

Results and Discussion

In Fig. 1 pigments content variation during the three different phenological stages are reported. The bars represent respectively chlorophyll *a* (µg·cm^{-2}), chlorophyll *b* (µg·cm^{-2}), total chlorophylls (µg·cm^{-2}) and carotenoids plus xanthophylls (µg·cm^{-2}). From pre-veraison to harvest

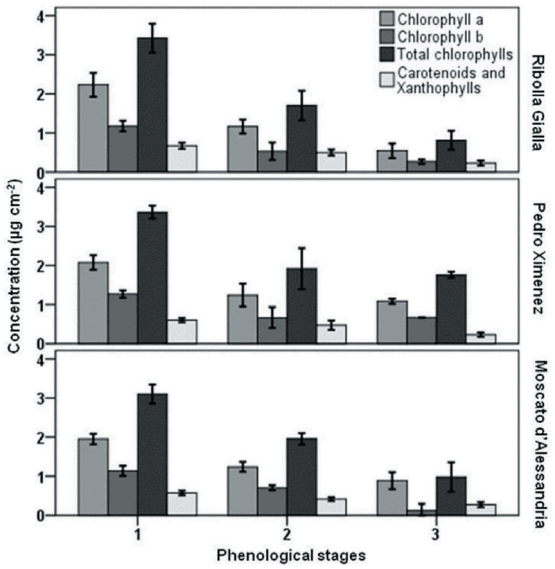

Fig. 1: Pigments content variation during the three different phenological stages, pre-veraison (**1**), veraison (**2**), harvest (**3**). Error intervals indicate standard errors. The bars represent chlorophyll a (µg·cm^{-2}), chlorophyll b (µg·cm^{-2}), total chlorophyll (µg·cm^{-2}) and carotenoids plus xanthophylls respectively (µg·cm^{-2}).

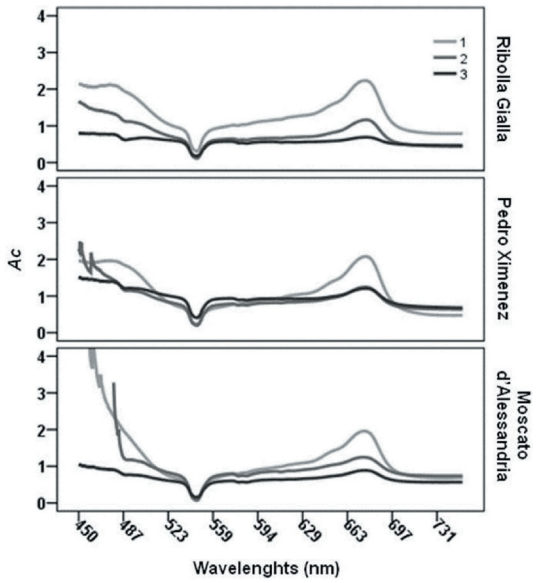

Fig. 2: Absorbance corrected in relation to the measured chlorophyll a (Ac) during the three different phenological stages for each accession. Pre-veraison (**1**), veraison (**2**), harvest (**3**).

a decrease in all pigments concentration is visible for each accession. These results are in agreement with others already published (Baumes *et al.* 2002, Downey *et al.* 2004). Both chlorophylls (*a* and *b*) are components of the photosynthetic membranes; chlorophyll *a* is the major pigment, while chlorophyll *b* is an accessory pigment (Lichtenthaler 1987). In our case in general chlorophyll *a* in respect to total chlorophylls varies from 63 to 67 % during ripening.

To limit the transmittance impact, Fig. 2 represents the absorbance spectra proportional to the measured chlorophyll *a*, during the three different phenological stages in each accession. The spectral range is 450-750 nm. A general diminution in the absorption spectra is visible from pre-veraison to harvest. Spectral absorption variation in the blue (450-490 nm) and in the red (630-700 nm) during the maturation process for all the accessions, clearly shows the degradation of carotenoids and chlorophylls, respectively. In these ranges the absorbance values differentiated significantly the first versus the third stages, for all the accessions. Basically both chlorophylls and carotenoids plus xanthophylls degraded.

In order to understand the causes of the green-yellowish colour changing during ripening, we decided to compare the relative contribution of blue and red absorption bands. With this purpose we normalized the absorbance spectra by the chlorophyll *a* peak at 678 nm for all the sampling times and accessions (Fig. 3). Part of the blue green range (450-550 nm) was analysed. In this case the normalization for the red part of the spectrum allowed to highlight the proportional variation in the blue green region through a relative absorbance spectrum. Pigments degradation during ripening was confirmed by destructive quantifications. However, chlorophylls and carotenoids had a different degradation intensity. The rate of carotenoids in relation to the total studied pigments content was 15.8 % at pre-veraison, 19.9 % at veraison and 31.4 % at harvest. Similar

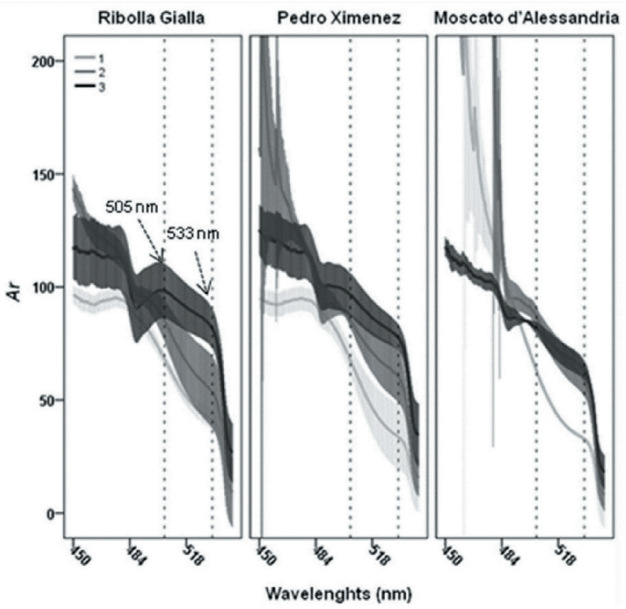

Fig. 3: Detail of the relative absorbance (Ar) spectra (means ± standard error), during the three different phenological stages for each accession. Pre-veraison (**1**), veraison (**2**), harvest (**3**). The dashed lines represent the maximum in absorbance at 504.89 and 533.28 nm during the second and the third sampling times.

results were obtained by Merzlyak (1998): in their case a decrease in absorbance around 678 nm was accompanied by a slight decrease in the blue range, characteristic of chlorophyll breakdown and carotenoids retention during leaf senescence and fruit ripening. In agreement with the destructive quantifications and the bibliographic data, at 450-500 nm (blue) in two out of the three studied cases, the relative absorbance increased during ripening (Fig. 3).

From 500 to 550 nm a general increase in relative absorbance was visible during maturation. Two maximum relative absorption peaks, appeared for the three accession

spectra at 505 and 533 nm, respectively. A spectral contribution at these wavelengths could be ascribed to oxidation products and, in particular, to melanin-like pigments. In fact, similar spectral modifications were observed during sunburn symptoms appearance (Rustioni *et al.* 2014). This result suggest a significant role of the oxidation products in berry colour modification during ripening. Melanin-like pigments accumulation might be the natural consequence of the H_2O_2 rapid increase, which starts during the so-called oxidative burst phenomenon and characterizes the beginning of ripening (Pilati *et al.* 2007). In red varieties it is not possible to distinguish the appearance of yellow hue, because of the overlapping absorption of anthocyanins at similar wavelengths. Moreover, anthocyanins may play an antioxidant role against ROS (reactive oxygen species) which lead to the formation of melanin-like pigments in white grapes during ripening. Anthocyanins antioxidant activity is well known: these pigments are able to capture free radicals by donation of phenolic hydrogens (Castañeda-Ovando *et al.* 2009). It has been described that red/black berries extracts possess a high scavenging activity towards ROS. A linear correlation between antioxidant capacity and anthocyanins content in ripe fruits of blackberries, raspberries and strawberries has also been reported (Wang and Lin 2000).

White berried *V. vinifera* cultivars are the consequence of the inactivation of anthocyanin biosynthesis (Kobayashi *et al.* 2004), thus it is consistent to propose that grape green-yellowish colour is principally related to catabolic pathways instead of specific pigment accumulation. In this case, the modification in colour seems not to be related to the activation of specific pigments accumulation in response to evolutionary adaptations. However, the anthocyanins absence, the chlorophylls/carotenoids degradation ratio, and the ripening characteristic oxidative burst, promote the appearance of the well known yellowish colour of these fruits. This experiment is a starting point to better understand the physiological implications on the yellowish colour variations in the white skinned varieties.

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References

Abbott, J. A.; 1999: Quality measurement of fruit and vegetables. Postharvest Biol. Technol. **15**, 207-225.

Arroyo García, R. A.; Revilla, E.; 2013: The current status of wild grapevine populations (*Vitis vinifera* ssp *sylvestris*) in the mediterranean basin, chapter 3, 51-71. In: D. Poljuha, B. Sladonja (Eds): The Mediterranean Genetic Code - Grapevine and Olive, Agricultural and Biological Sciences.

Baumes, R.; Wirth, J.; Bureau, S.; Gunata, Y.; Razungles, A.; 2002: Bio-generation of C13-norisoprenoid compounds: experiments supportive for an apo-carotenoid pathway in grapevines. Analyt. Chem. Acta **458**, 3-14.

Butler, W. L.; Norris, K. H.; 1960: The spectrophotometry of dense light-scattering material, Arch. Biochem. Biophys. **87**, 31-40.

Castañeda-Ovando, A.; Pacheco-Hernández, M. D. L.; Páez-Hernández, M. E.; Rodríguez, J. A.; Galán-Vidal, C. A.; 2009: Chemical studies of anthocyanins: A review. Food Chem. **113**, 859-871.

Close, D. C.; Beadle, C. L.; 2003: The ecophysiology of foliar anthocyanin. Bot. Rev. **69**, 149-161.

Downey, M. O.; Harvey, J. S.; Robinson, S. P.; 2004: The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. Aust. J. Grape Wine Res. **10**, 55-73.

Fournier-Level, A.; Lacombe, T.; Le Cunff, L.; Boursiquot, J. M.; This, P.; 2010: Evolution of the VvMybA gene family, the major determinant of berry colour in cultivated grapevine (*Vitis vinifera* L.). Heredity **104**, 351-362.

Hardie, W. J.; 2000: Grapevine biology and adaptation to viticulture. Aust. J. Grape Wine Res. **6**, 74-81.

Kamffer, Z.; Bindon, K. A.; Oberholster, A.; 2010: Optimization of a method for the extraction and quantification of carotenoids and chlorophylls during ripening in grape berries (*Vitis vinifera* cv. Merlot). J. Agric. Food Chem. **58**, 6578-6586.

Kobayashi, S.; Goto-Yamamoto, N.; Hirochika H.; 2004: Retrotransposon-induced mutations in grape skin color. Science **304**, 982-982.

Krasnow, M. N.; Matthews, M. A.; Smith, R. J.; Benz, J.; Weber, E.; Shackel, K. A.; 2010: Distinctive symptoms differentiate four common types of berry shrivel disorder in grape. Calif. Agric. **64**, 155-159.

Lichtenthaler, H. K.; 1987: Chlorophylls and carotenoids: pigments of photosynthetic membranes. Meth. Enzymol. **148**, 350-382.

Lorenz, D. H.; Eichorn, K. W.; Blei-Holder, H.; Klose, R.; Meier, U.; Weber, E.; 1995: Growth Stages of the Grapevine: Phenological growth stages of the grapevine (*Vitis vinifera* L.: ssp. *vinifera*). Codes and descriptions according to the extended BBCH scale. Aust. J. Grape Wine Res. **1**, 100-103.

McClure, W. F.; 1975: A spectrophotometric technique for studying the browning reaction in 217 tobacco. Transaction of ASAE **18**, 380-383.

Merzlyak, M. G.; 1998: Light-induced pigment degradation in leaves and ripening fruits studied *in situ* with reflectance spectroscopy. Physiol. Plant. **104**, 661-667.

Pilati, S.; Perazzolli, M.; Malossini, A.; Cestaro, A.; Dematte, L.; Fontana, P.; Dal Ri, A.; Viola, R.; Velasco, R.; Moser, C.; 2007: Genome-wide transcriptional analysis of grapevine berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at veraison. BMC Genom. **8**, 428.

Rustioni, L.; Basilico, R.; Fiori, S.; Leoni, A.; Maghradze, D.; Failla O.; 2013a: Grape colour phenotyping: development of a method based on the reflectance spectrum. Phytochem. Anal. **24**, 453-459.

Rustioni, L.; Di Meo, F.; Guillaume, M.; Failla, O.; Trouillas, P.; 2013b: Tuning color variation in grape anthocyanins at the molecular scale. Food Chem. **141**, 4349-4357.

Rustioni, L.; Maghradze, D.; Failla, O.; 2012: Optical properties of berry epicuticular waxes in four Georgian grape cultivars (*Vitis vinifera* L.). S. Afr. J. Enol. Vitic. **33**, 138-143.

Rustioni, L.; Rocchi, L.; Guffanti, E.; Cola, G.; Failla, O.; 2014: Characterization of grape (*Vitis vinifera* L.) berry sunburn symptoms by reflectance. J. Agric. Food Chem. **62**, 3043-3046.

Saure, M. C.; 1990: External control of anthocyanin formation in apple. Sci. Hortic. **42,** 181-218.

Strahler, A. H.; Strahler, A.; 2003: Introducing Physical Geography, 3rd ed. J. Wiley & Sons, Inc., New York.

Walker, A. R.; Lee, E.; Bogs, J.; McDavid, D. A. J.; Thomas, M. R.; Robinson, S. P.; 2007: White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant J. **49**, 772-785.

Wang, S. Y.; Lin, H. S.; 2000: Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental Stage. J. Agric. Food Chem. **48**, 140-146.

Willson, M. F.; Whelan, C. J.; 1990: The evolution of fruit color in fleshy-fruited plants. Am. Nat. **136**, 790-809.