BUNCH EXPOSURE TO DIRECT SOLAR RADIATION INCREASES ORTHO-DIPHENOL ANTHOCYANINS IN NORTHERN ITALY CLIMATIC CONDITION

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Abstract

Aims: Berry anthocyanin contents were studied in eight different field experiments involving various grapevine (*Vitis vinifera* L.) cultivars (Syrah, Croatina, Sangiovese, Nebbiolo, Nebbiolo Rosé, Carmènere and Cannonau) in a variety of sites located in Northern Italy.

Methods and results: In each experiment, global irradiance was reduced by approximately 95% by applying specially designed boxes to selected bunches before the onset of ripening. Variations in anthocyanin accumulation and profile were studied on sun exposed and shaded bunches. Exposed bunches were subjected to less favorable temperatures for biosynthetic metabolism than shaded bunches, due to frequent excessive temperatures around noon.

Conclusions: Exposure to direct solar radiation induced an earlier and faster anthocyanin accumulation and a higher anthocyanin concentration across all the experiments, at least from veraison to mid ripening. In fact, the light induced elevated rate of anthocyanin accumulation did not always result in a higher anthocyanin content at grape maturity. In the cultivars with a relatively high proportion of acylated forms, sun exposure resulted in a relative increase in the quantities of non-acylated glucoside forms, while the acylation profile of cultivars that generally had a lower proportion of acylated forms was only minimally influenced by sun exposure.

Significance and impact of the study: A key observation resulting from anthocyanin profiling is the strong positive correlation between light exposure and increases in the percentage of delphinidin-, cyanidin- and petunidin-3-monoglucoside, that is, anthocyanins containing an ortho-dihydroxyl group.

Key words: anthocyanins, bunch exposure, microclimate, grape ripening, *Vitis vinifera*

Résumé

Objectif: Cette étude se fixe principalement pour but d'augmenter nos connaissances concernant l'influence de l'exposition directe aux radiations solaires sur le profil des anthocyanes, dans différentes régions de l'Italie du Nord, pour différents cépages de raisin (*Vitis vinifera* L.): Syrah, Croatina, Sangiovese, Nebbiolo, Nebbiolo Rosé, Carmènere et Cannonau.

Méthodes et résultats: Pour chaque expérience, l'irradiation globale a été réduite approximativement de 95 % au moyen de boîtes spécialement conçues pour les grappes sélectionnées avant le début de la maturation. Les variations d'accumulation d'anthocyanes et leurs profils ont été étudiés sur des grappes exposées au soleil et à l'ombre. Les grappes au soleil ont dû supporter des températures moins favorables pour le métabolisme des anthocyanes que celles restées à l'ombre, du fait de fréquentes températures excessives autour de midi.

Conclusion: L'exposition au rayonnement solaire direct entraîne une meilleure accumulation et une plus forte concentration en anthocyanes pour toutes les expériences, tout au moins durant la première partie de la phase de maturation. Mais ce taux élevé d'accumulation d'anthocyanes n'a jamais été maintenu jusqu'à la fin de la maturation. Pour les cépages possédant une proportion relativement importante de formes acylées, l'exposition au soleil a entraîné une augmentation relative des quantités de formes de glucosides non acylés ; les cépages qui possèdent généralement une proportion moins importante de formes acylées n'ont été que peu influencés par l'exposition au soleil, en ce qui concerne le profil acylique.

Signification et impact de l'étude: Le profil des anthocyanes a fourni une observation clé : la forte corrélation positive entre l'exposition lumineuse et l'augmentation du pourcentage de delphinidine-, cyanidine- et petunidine-3-monoglucoside, à savoir les anthocyanes contenant un groupe ortho-dihydroxyle.

Mots-clés: anthocyanes, exposition des grappes, microclimat, maturation du raisin, *Vitis vinifera*

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INTRODUCTION

Several research works have studied the effects of bunch exposure on the accumulation of grape anthocyanins (DOWNEY *et al.*, 2006). According to the conditions affecting the surface energy balance, exposure to solar radiation has a direct positive correlation with berry temperature, so it is difficult to distinguish between the physiological effect of solar irradiance and temperature (MILLAR, 1972). However, as SPAYD *et al.* (2002) demonstrated by exposing Merlot grapes to direct sunlight while maintaining temperature conditions comparable to shaded bunches, light may improve anthocyanin accumulation. More recently, with the same experimental equipment, a more complex interaction between solar radiation and berry temperature has been reported in Merlot grapes, showing that low incident solar radiation alone did not compromise total anthocyanin accumulation, whereas a combination of low light and high berry temperatures did (TARARA *et al.*, 2008). These results underline the importance of evaluating solar irradiance and berry temperature together for the interpretation of the effect of bunch microclimate on anthocyanin accumulation.

In vineyard conditions, bunches may be differently exposed to solar irradiance. When a bunch is not shaded by the canopy, both direct and diffuse irradiance components affect its surface, while on canopy-shaded bunches, irradiance is predominantly diffusive. As a consequence of the surface energy balance, the temperature of exposed berries during the daytime is generally higher than the air temperature, whereas the temperature of shaded berries is close to the air temperature (MILLAR, 1972). Thus, it is important to consider the combined effect of light and heat exposure to define when high temperature becomes a limiting factor that may overcome the possible positive effect of direct sunlight. Based on several research works (DELOIRE and HUNTER, 2005; DOWNEY *et al.*, 2004; TOMASI *et al.*, 2003; SPAYD *et al.*, 2002; BERGQVIST *et al.*, 2001; DOKOOZLIAN and KLIEWER, 1996; JACKSON and LOMBARD, 1993; KELLER and HRAZDINA, 1998; TARARA *et al.*, 2008; CRIPPEN and MORRISON, 1986), it is possible to conclude that generally in cool regions, where temperature is a limiting factor, the increase in bunch exposure results in an increase in berry anthocyanin content, while in warmer regions the opposite result is usually observed.

Anthocyanin content not only in terms of total amount per unit weight of grapes but also in terms of composition is an important contributor to wine style and quality (ALLEN, 1998). Thus, grapes with the same total anthocyanin content may produce wines with different hue and intensity of color. In grape skin, five different anthocyanidins are present in the form of 3-monoglucoside: delphinidin, cyanidin, petunidin, peonidin and malvidin (KENNEDY *et al.*, 2005). They can be esterified by p-coumaric, acetic and, to a lesser extent, by caffeic acid (KENNEDY *et al.*, 2005). In relation to the number of B-ring substitution, it is possible to separate anthocyanidins with two B-ring substitutions (cyanidin and peonidin) from those with three B-ring substitutions (delphinidin, petunidin and malvidin). This classification can be correlated to the activity of flavonoid-3'-hydroxylase (F3'H) and flavonoid-3',5'-hydroxylase (F3'5'H), which convert dihydrokaempferol to dihydroquercetin and both to dihydromyricetin (HOLTON and CORNISH, 1995). These dihydroflavonols have one, two and three -OH substituents on a B-ring, respectively. Dihydroflavonol-4-reductase (DFR) converts dihydroflavonols to leucoanthocyanidins, which are subsequently converted to anthocyanidins by leucoanthocyanidin oxygenase (ADAMS, 2006). The Petunia DFR enzyme preferentially converts dihydromyricetin to leucodelphinidin; dihydroquercetin is a low affinity substrate, and dihydrokaempferol is not converted to leucopelargonidin. This distinct specificity explains the preferential accumulation of delphinidin derivatives and the lack of pelargonidin pigments in Petunia (HOLTON and CORNISH, 1995). Considering that pelargonidin is not found in grape berry skin, it is possible to suggest a similar mechanism in the grapevine as well.

In reference to methylation, anthocyanins may be grouped into three classes: non methylated forms (cyanidin and delphinidin); one methylation (peonidin and petunidin); and methylation in 3' and 5' positions (malvidin). The enzymes responsible for these differences are O-methyltransferases (HOLTON and CORNISH, 1995; DOWNEY *et al.*, 2004). It is also useful to classify anthocyanins in relation to the presence/absence of an ortho-dihydroxyl group as it accounts for the different susceptibility to oxidation, which is also an important aspect of the anthocyanin profiling analysis for the evaluation of the grape enological properties. Thus, it is possible to separate ortho-diphenol pigments (delphinidin, cyanidin and petunidin) from non ortho-dihydroxylated molecules (peonidin and malvidin).

Some recent studies (SPAYD *et al.*, 2002; DOWNEY *et al.*, 2004; RISTIC *et al.*, 2007) have indicated that bunch shading increases the trihydroxylated and the acylated anthocyanin forms.

The main aim of this study was to increase our knowledge of the influence of bunch exposure to direct solar radiation on the anthocyanin profile by exploring a range of different cultivars, selected for their diverse anthocyanin profiles, in different sites of Northern Italy. Important cultivars in the Italian viticulture were

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preferentially chosen to acquire data for the local winegrowers .

MATERIALS AND METHODS

1. Experimental sites and plant material

The *Vitis vinifera* L. cultivars used in this study were selected for their different levels of expected anthocyanin accumulation in berry skin in terms of both quantity and profile (composition in each anthocyanin, in acylated and non-acylated form). The experimental design involved eight trials carried out over two years (four experiments per year) in four sites with a variety of climatic conditions (Table 1). In the first season (2005), Syrah, Croatina, Sangiovese and Nebbiolo were tested in a site located in Oltrepò Pavese (Pavia, Lombardy). In the second season (2006), the studies were carried out on Nebbiolo Rosé in Albese (Piedmont), Nebbiolo in Valtellina (Lombardy), Carmènere and Cannonau (syn. Grenache) in Tavazzano (Lombardy). The geographical and topographical data of the sites are presented in Table 2, and a description of the vineyards is presented in Table 3.

Based on Koeppen's climate classification (STRAHLER and STRAHLER, 2003), all experimental sites except Valtellina have a mesothermal climate with transitional characters between oceanic and Mediterranean climates. The rainfall pattern presents two maxima (in spring and fall). The Valtellina area has a mountain climate that can be classified as endo-alpine (FAILLA *et al.*, 2004).

Table 1 – Years, sites and cultivars involved in the experiments.

Experiment	Year	Site	Cultivar	Code
1	2005	Oltrepò Pavese	Croatina	CR OP 05
2	2005	Oltrepò Pavese	Sangiovese	SG OP 05
3	2005	Oltrepò Pavese	Syrah	SY OP 05
4	2005	Oltrepò Pavese	Nebbiolo	NE OP 05
5	2006	Albese	Nebbiolo Rosé	NR AL 06
6	2006	Valtellina	Nebbiolo	NE VT 06
7	2006	Tavazzano	Carmènere	CM TA 06
8	2006	Tavazzano	Cannonau	CA TA 06

Table 2 – Geographical location and topographical description of the experimental sites.

Site	Township	Region	Soil and landscape	Geographical coordinates		Elevation	Slope direction	Slope inclination
				Long.	Lat.	m asl	angular degree (°)	
Oltrepò Pavese	Torrazza Coste	Lombardy	Clay soil on a hilly terrace	9°05’	44°58’	144	-	-
Albese	Serralunga d’Alba	Piedmont	Clay soil on a hilly slope	7°59'	44°38'	293	200	20
Valtellina	Sondrio	Lombardy	Glacial tills on mountainside	9°51’	46°10	316	152	25
Tavazzano	Tavazzano con Villavesco	Lombardy	Coarse textured sandy soil on alluvial plains	9°25’	45°19’	82	-	-

Table 3 – Main characteristics of the vineyards involved in the experiments.

Site	Row direction	Bunch azimuth	Vine age	Training system	Row x Vine spacing	Canopy height	Location of the fruiting zone
		angular degree from North (°)	years		m	m	m
Oltrepò pavese	SE-NW	43	15	Single Guyot	2.5 x 1.0	1.8	0.5-0.8
Tavazzano	N-S	17	10	Single Guyot	4.0 x 1.0	2.5	0.5-0.8
Albese	E-W	290	15	Single Guyot	2.5 x 1.0	1.8	0.5-0.8
Valtellina	N-S	152	30	Single Guyot	1.7 x 1.0	1.8	0.5-0.8

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2. Experimental treatments, temperature monitoring and grape sampling

The experiments started in the last week of July, except in Valtellina where the research started at the end of August due to the lateness of the site. The three experimental treatments (boxed, sun exposed and leaf shaded bunches) were performed as follows. In each experiment, between the lag phase corresponding to the pheno-phases 32-33 of berry growth (Eichorn and Lorenz scale; LORENZ *et al.*, 1995) and veraison, boxes capable of reducing global irradiance by roughly 95% were applied to selected bunches (boxed bunches). At the same time, canopy manipulations (leaf removal or shoot positioning) were performed to keep some clusters completely exposed to direct sunlight (sun exposed bunches). Other bunches were chosen inside the canopy and, where necessary, their position was slightly modified in order for them to be naturally shaded by a single leaf layer on average, thus exposing them only to diffuse radiation and to occasional sun flecks (leaf shaded bunches). In the 2006 experiments, the latter treatment was not performed due to the generally thinner foliage bunch coverage.

Each experiment was conducted on at least 10 plants, which were selected according to correct vine balance (crop load vs. leaf surface ratio), evaluated by visual inspection, and to proper grape ripening potential, based on local experience (generally more than one square meter of exposed leaves per expected kilogram of grapes). In order to take into proper consideration the differences due to the individual physiological status of each vine, as well as to minimize the possible influence of the slight leaf removal around the sun exposed bunches, each treatment (i.e., sun exposed, leaf shaded and boxed bunches) was replicated on each vine. Three (Exp. 7 and 8) or four (Exp. 1-6) bunches per treatment were collected at each sampling time (biological replication).



Photo 1 – The shading boxes derived from the standards defined by the World Meteorological Organization for screen boxes of meteorological stations made of white reflective laminated paper.

The design of the shading boxes was derived from the standards defined by the World Meteorological Organization (W.M.O.) for screen boxes of meteorological stations (W.M.O., 1996). The boxes were made of white reflective laminated paper measuring 200 x 200 x 250 mm^3 (L x W x H), and a set of double parallel angled slats were positioned on all sides of the box, except on the top surface. The boxes had vents in order to maximize airflow and, therefore, to minimize the differences between temperature and relative humidity in the boxes and the outside air conditions measured in a meteorological station. Boxes were applied to bunches below the canopy to avoid any possible disturbance on the rest of the canopy (Photo 1).

During ripening, berry temperature was measured using American National Standards Institute (ANSI) standard thermocouples with a copper-constantan sensor by insertion of the probe into the berry center. Data were collected by a specific Data Acquisition System (DAS). The thermocouple-DAS system had a temperature range of -65 - 130 °C, an accuracy of ± 1.5 °C and a resolution of ± 0.1 °C. The thermocouple tip was inserted into a berry that had been previously pierced with a spike. The measurement period ranged from pre-veraison to harvest with a time step of one minute. Once a week, each sensor was moved into a new berry. Moreover, air temperature in the boxes and in a standard radiation shield located just above the canopy were monitored with the same sensors. According to these observations, the temperatures in the boxes were similar to those measured by a standard meteorological station.

Berries were sampled three (Exp. 3, 5-8) or four times (Exp. 1-2, 4) during the ripening period, from when color change of sun exposed grapes was almost complete until the fruit achieved full maturity, according to sugar (°Brix) accumulation.

3. Total sugar and anthocyanin analysis

On each sampling date, bunches were collected into plastic bags, stored in a thermal box, and shortly after they were taken to the laboratory to be frozen. Before analyses, a few hundred berries per biological replication were classified into three classes based on the visual assessment of their color: fully colored, just pink or green. Then, two sub-samples of berries, equally composed of the different color classes according to the relative bulk sample, were selected from each sample. The first sub-sample (composed of at least 20 berries) was set aside for anthocyanin determination (as of mg per kg of berries). The second sub-sample was softly pressed by hand within a plastic bag, and the juice was used for total sugar

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determination (°Brix) by a temperature-compensated digital refractometer (DBR 32 Sinergica®).

For anthocyanin determination, the skins of at least 20 frozen berries were separated from the pulp and extracted for 24 hours in 100 ml methanol at room temperature. The total extract was filtered to remove plant debris, dried out in a rotary evaporator at 37 °C and dissolved in an aqueous solution of 0.3% perchloric acid - methanol (73:27 v/v).

Total anthocyanin concentration was evaluated by measuring the absorbance of this solution at a wavelength of 520 nm and referring the values to a malvidin-3-glucoside calibration curve (Jasco 7800 spectrophotometer).

Anthocyanin profile was determined at 520 nm using a Shimadzu HPLC LC-10 AD (Shimadzu Co. Tokyo, Japan) connected to a Shimadzu UV-VIS detector SPD-10 A. HPLC analysis of the extract was conducted with the following parameters: flow rate 0.45 ml/min; temperature 40 °C; column Purospher RP18, 5 µm (250 x 4 mm) (Merck, Darmstadt, Germany); solvent A: methanol; solvent B: aqueous 0.3% perchloric acid; elution: linear gradient from 27% to 43% A in 32 minutes, from 43% to 68.5% in 13 minutes, from 68.5% to 100% in 2 minutes, then isocratically with 100% A for 3 minutes; re-equilibration time: 5 minutes; and loop: 10 µl. A calibration curve was established using malvidin-3-monoglucoside and results for all anthocyanins, identified according their retention times, were expressed as « malvidin-3-monoglucoside equivalent » (mg per kg of berries).

The anthocyanin profiles were outlined as the relative levels of delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside and their acetic and p-coumaric esters.

4. Microclimate analysis

To quantify the temperature status of the grape berries in physiological terms, the organ hourly mean temperatures were converted into « Normal Heating Hours » (NHH), an analogous of the « Growing Degree Hours » (GDH) proposed by ANDERSON *et al.* (1986), based on a non linear response of plant metabolism to temperature. NHH summation should be considered as a more useful agro-meteorological index for growing condition monitoring compared to the classical linear growing degree summation approach. It takes into account the effect of temperature regimes on anthocyanin biosynthesis without any consideration of the possible effects of temperature on anthocyanin losses by oxidation or any other catabolic mechanisms. The NHH model is based on the temperature response curve of biosynthetic metabolism. By defining one NHH as one hour at the optimal temperature for anthocyanin biosynthetic metabolism, one hour at a temperature divergent from the optimal one will be a fraction of NHH according to the response curve reported in Fig. 1. The procedure for computing NHH was based on a ≤ function that varies from 0 to 1 (WANG and ENGEL, 1998). The function gives 0 for temperatures outside minimum and maximum cardinals (T_{min} = 10 °C and T_{max} = 35 °C, respectively) and 1 for temperatures at optimum (T_{opt} = 25 °C):

$$NHH(T) = [2(T-T_{min})^{\alpha} (Topt-T_{min})^{\alpha} (T-T_{min})^{2\alpha}]/(T_{opt}-T_{min})^{2\alpha}, \text{ for } T_{min} \leq T \leq T_{max}$$

$$\text{and } NHH(T) = 0 \text{ for } T<T_{min} \text{ or } T>T_{max}$$

$$\text{where } \alpha = \ln[2/\ln((T_{max}-T_{min})/(T_{opt}-T_{min}))]$$

The choice of 10, 25 and 35 °C as minimum, optimum and maximum temperature, respectively, was made according to the expected effect of temperature on biosynthetic metabolism, with special attention to the recent results obtained by MORI *et al.* (2007) as to anthocyanin accumulation in grapes.

The actual ability of the boxes to reduce global solar irradiance was checked in some preliminary tests lasting several days with different sky conditions. On average, the global solar irradiance inside the box was approximately 5% that of the external one. The global solar radiation inside the canopy behind a single leaf layer and in close proximity to the bunches selected for sampling was measured repeatedly during some selected days in different sky conditions. The global solar radiation behind a leaf layer ranged between 40 and 60% of the external one.

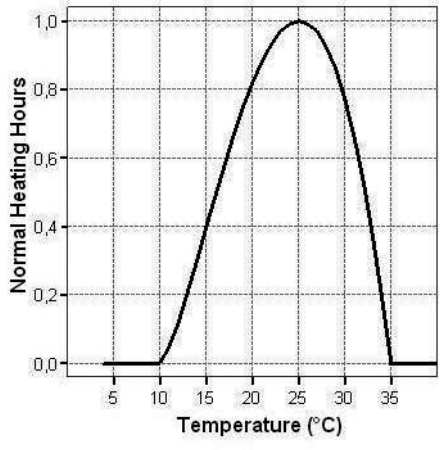


Figure 1 – Shape of the β function proposed to estimate the Normal Heating Hours in relation to berry temperature.

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5. Statistical data processing

For each experiment, the effect of light availability on metabolic profile was statistically tested by mixed factor analysis of variance (ANOVA) with treatment as between subject factors and sampling times as within subject factors. This was followed by Tukey's HSD test (P=0.05) to compare treatment means within each sampling time. Data were analyzed with SPSS statistical software (version 16.0, SPSS Inc. Chicago, Illinois).

RESULTS

1. Light and temperature conditions

During the ripening period, the temperature patterns (mean air temperature and heat summation) differed among the experimental sites (Table 4). As expected, the most divergent pattern was recorded in Valtellina, where the onset of ripening was delayed compared to the other sites, causing grape maturation to occur at a cooler temperature. The highest temperatures were recorded in Oltrepò Pavese, where the peak occurred during the 31st, 33rd and 35th week of the year (the 1st, 3rd and 5th week of the ripening cycle, respectively). On average, Tavazzano, Albese and Valtellina sites had similar and lower temperatures for the entire duration of the ripening period.

2. Grape temperature status

The diurnal temperature patterns of sun exposed and shaded berries displayed considerable diversity (Fig. 2). During daytime, berry temperature was generally higher in sun exposed than in shaded berries because of the direct exposure to solar radiation. The extent of the temperature differences ranged from less than 1 °C on the cool and cloudy days of September and October to more than 10 °C on the hot and clear days of August. In general, the greatest difference was recorded in experiments 1-5, while smaller divergences were recorded in experiments 6-8 (data not shown).

Two examples of the hourly mean temperature in exposed, leaf shaded and boxed bunches observed at the Oltrepò Pavese site are reported in Fig. 2. The first example (Fig. 2A) corresponds to the second week of the experiment during which anthocyanin accumulation was in progress in all the tested cultivars. The second example (Fig. 2C) corresponds to the last three days of the

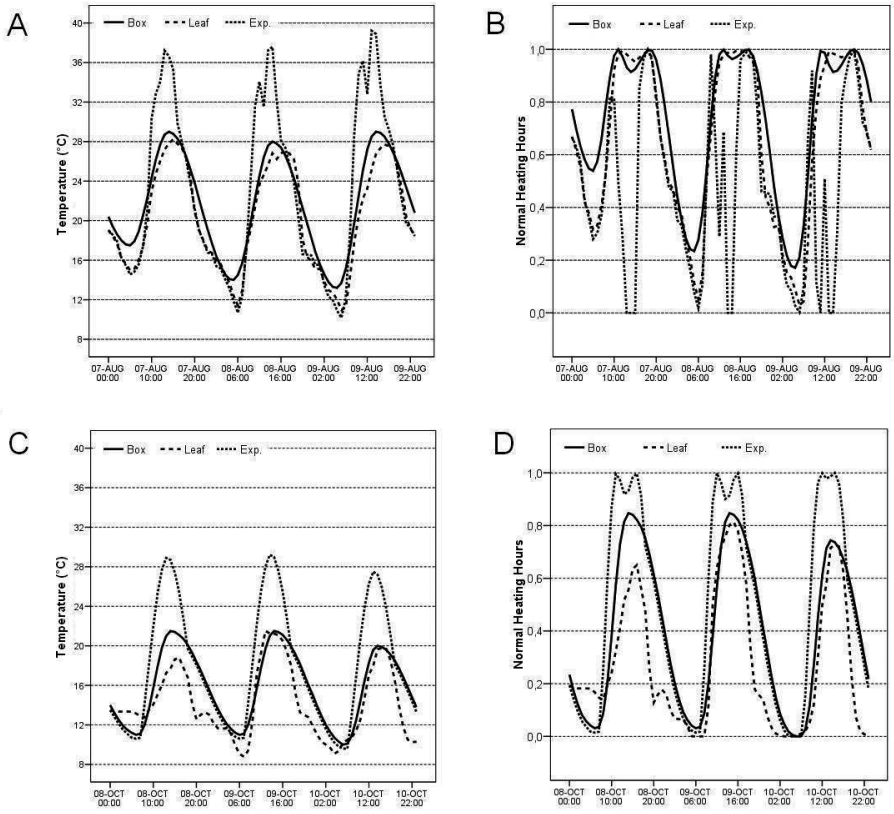


Figure 2 – Hourly courses of berry temperatures (A and C) and relative Normal Heating Hours (NHH) conversion (B and D) in exposed, leaf shaded and boxed bunches.
Data are from the experiment conducted in Oltrepò Pavese (A-B) on the 32nd week of the year (August 7-9, 2005) and (C-D) on the last period of the experiment (41st week; October 8-10, 2005).

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experiment when only the grapes from the two late ripening cultivars (Nebbiolo and Croatina) were still on the vines. The most evident differences between exposed and shaded bunches were recorded during midday. During that time, due to row orientation and bunch azimuth, exposed bunches were reached by direct solar radiation and their temperature exceeded that of shaded bunches by 4-12 °C, while the temperature regime during the morning and the late afternoon was essentially equivalent. Leaf shaded bunches occasionally had a maximum temperature slightly lower than boxed bunches. The minimum temperatures of boxed bunches were generally 2-3 °C higher than those of exposed and leaf shaded ones. The latter two experimental treatments generally did not show differences in minimum temperature.

Comparable findings were recorded in Albese and Tavazzano, though in the latter case the differences were smaller, which coincided with the similar results obtained in Valtellina (data not shown).

When the hourly berry temperatures were converted into NHH to take into consideration their expected physiological effectiveness, it was observed a marked variability. In this Oltrepò Pavese experiment, the exposed bunches during the selected three days of the first phase of the ripening period showed a dramatic reduction of NHH accumulation around noon, due to excessive temperature, while at the same time of day, both leaf shaded and boxed bunches maintained a high accumulation rate (Fig. 2B). In the last three days of the experiment, the situation was reversed with exposed bunches accumulating an overall higher amount of NHH, particularly during the period around noon (Fig. 2D). In the Tavazzano and Valtellina experiments, where the differences in bunch temperature were not so marked, differences in NHH accumulation were also not very noticeable (data not shown).

In general, a negative relation between light availability and NHH accumulation was observed over most of the ripening period, with the exception of the last weeks (Fig. 3). Direct sun exposure of bunches reduced the NHH accumulation, particularly during the starting phase of the ripening period, when higher temperature regimes led to an increase in the temperatures recorded in exposed bunches. Around noon the exposed bunches were above the optimal ranges for biosynthetic metabolism, with a different frequency in relation to the year, the site and the specific weather conditions. The effect of direct solar radiation in reducing NHH accumulation was particularly evident in experiments 1-5, while in experiments 6 and 7 the differences were minimal or even negligible as in the case of experiment 8. These differences might have been caused by the different patterns of cloud cover and atmospheric clearness conditions that occurred in the different experimental sites and years. As a result, boxed bunches had the highest NHH accumulation rate throughout the ripening season, with the exception of the last two weeks of experiments 1-5. The temperature response of leaf shaded bunches followed the trends of boxed bunches, with lower NHH accumulation rates. In the final weeks, in both Oltrepò Pavese and Albese sites, the exposed bunches accumulated more NHH than the shaded and boxed ones. In Tavazzano, exposed and boxed bunches did not show any difference, while in Valtellina, boxed bunches had the highest NHH accumulation.

Table 4 – Weekly air mean temperature and summation of active temperature on base 10 °C recorded during the ripening period in the different experimental conditions.

Week of the year	Oltrepò Pavese 2005		Albese 2006		Valtellina 2006		Tavazzano 2006	
	Temperature	Heat sum	Temperature	Heat sum	Temperature	Heat sum	Temperature	Heat sum
	°C	GDDs	°C	GDDs	°C	GDDs	°C	GDDs
31	25.6	108.9	20.7	74.9	-	-	23.8	96.4
32	22.5	87.7	22.1	84.7	-	-	21.5	80.8
33	23.5	94.6	19.3	65.1	-	-	20,0	70.3
34	21.7	81.7	21.7	81.9	-	-	21.7	81.9
35	24.3	99.9	19.9	69.3	19.8	68.6	19.2	64.6
36	22.6	87.9	23.3	93.1	22.7	88.7	23.1	91.9
37	21,0	76.9	18.7	60.9	18.8	61.9	19.6	67.5
38	18.3	57.9	19.6	67.2	19,0	63.3	19.6	67.4
39	19.9	69.3	17.3	51.1	17.8	54.3	17.5	52.3
40	15.4	37.8	18.7	60.8	15.6	39.2	-	-
41	15.4	38.1	-	-	13.3	22.9	-	-
42	-	-	-	-	13.7	26.1	-	-

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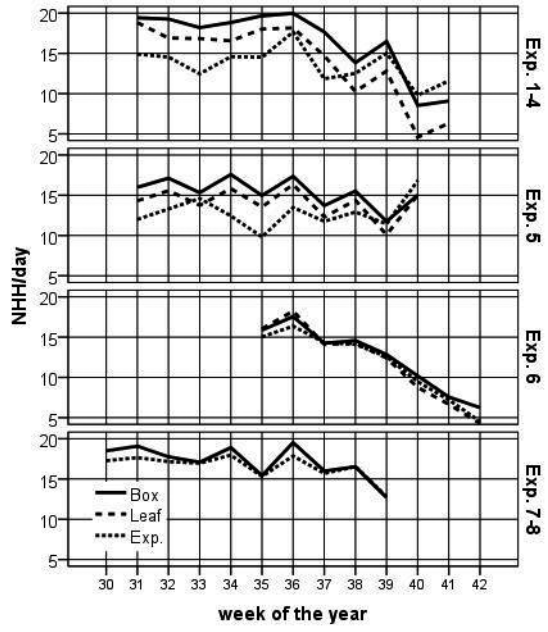


Figure 3 - Courses of the average daily Normal Heating Hours accumulation during the ripening period in the different experimental sites (see table 1) in exposed, leaf shaded and boxed bunches.

3. Grape ripening timing and extent

In all experiments, the severe shade condition induced by the boxes caused a delay in berry color change. At the first sampling time (5 to 20 days after the onset of visible symptoms of veraison in exposed bunches), most berries from exposed bunches were partially or totally colored, while leaf shaded and boxed bunches showed a delay in color development with a higher proportion of green and/or just pink berries (Table 5). The degree of delay in berry color change varied between experiments; however, it was completely recovered at the final sampling time as no berries were classified as green or just pink (data not shown).

At the beginning of the ripening period, in all the experiments, boxed and leaf shaded bunches had a lower °Brix level than the exposed bunches, with some minor differences in extent and statistical significance. In some experiments the differences tended to disappear by the end of ripening (Fig. 4).

Anthocyanin accumulation at ripening time showed a wide range of variability across the different cultivars and sites (Fig. 5). Croatina and Sangiovese had the highest levels (800-1300 mg/kg grapes); Syrah, Carmènere and Nebbiolo in Oltrepò Pavese had intermediate levels (500-800 mg/kg grapes); Nebbiolo Rosé and Nebbiolo in Valtellina had low levels (300-400 mg/kg grapes); and Cannonau had very low levels (50-150 mg/kg grapes).

Total anthocyanin levels in the different grape cultivars displayed a different accumulation behavior in response to the bunch microclimatic conditions (Fig. 5). In Croatina, Sangiovese, Carmènere and Cannonau (Exp. 1, 2, 7 and 8, respectively), the exposed bunches had higher anthocyanin concentrations compared to the other two experimental treatments throughout the whole ripening period (not significant for the 3rd Croatina sampling time). Syrah (Exp. 3) displayed marked differences between treatments, with higher levels in exposed bunches at the beginning of the ripening period; however, these differences were reduced and were not statistically significant over the succeeding ripening phase. Anthocyanin accumulation in Nebbiolo exposed bunches (Exp. 4) began rapidly but slowed down towards the end of the ripening period, therefore, by the end of ripening, bunches that had ripened in boxes had the same quantity of pigments than the exposed ones. A similar pattern was detected in Nebbiolo Rosé (Exp. 5) and Nebbiolo in Valtellina (Exp. 6).

4. Cultivar anthocyanin profiles

The choice of the experimental cultivars was based on their different anthocyanin profile, and, as expected, they exhibited considerable variation at ripening time (Table 6) without any particular relation with the accumulation levels. Based on the average data among all the treatments, these differences can be summarized as follows:

- Acylation: Carmènere and Syrah had the greatest proportion of acylated anthocyanins (~35-40%). Cannonau and Croatina had similar percentage of this form of pigments (~20%) but different proportion of coumaroyl and acetyl glucosides: Cannonau had ~14% of coumaroylated anthocyanins, whereas Croatina had the same percentage of the two forms of acylated pigments. In Nebbiolo, Nebbiolo Rosé and Sangiovese, non-acylated glucosides represented more than 89% of the anthocyanin profile.

- B-ring: The profiles of Cannonau, Carmènere, Syrah and Croatina were dominated by malvidin (64-82%). In Sangiovese, Nebbiolo Rosé and Nebbiolo the percentage of malvidin was approximately 30-40%, but these cultivars differed for the remaining part of the profile: in Nebbiolo and Nebbiolo Rosé there was a high percentage of peonidin (41-47%) that was generally representative of their anthocyanin profile, while in Sangiovese the remaining part of the profile was divided in equal proportions between the other pigments.

5. Exposure and anthocyanin profile

Based on the statistical analysis of the quantity of anthocyanins, considering both the single molecules and their summation according to the different criteria, the

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most consistent differences between treatments as for anthocyanin concentration and profile were found in acylation rate (Fig. 6) and B-ring ortho-diphenol groups (Fig. 7).

a) Acylation

In the cultivars with a relatively high proportion of acylated forms (i.e., Croatina, Syrah, Carmènere and Cannonau), sun exposure treatments induced a decrease in the proportion of acylated forms. Conversely, the percentage of acylated forms was greater in bunches that had ripened in boxes (Fig. 6). These results were mainly due to changes in p-coumaric esters (data not shown). Leaf shaded bunches did not show significant differences from exposed bunches. Sangiovese had higher proportions of acylated forms in boxed bunches compared to exposed bunches at the first and particularly at the third sampling time with no differences at the second sampling time, while at ripening time, boxed and sun exposed grapes had similar and low proportions of acylated forms in comparison to the higher level in leaf shaded bunches. Quite consistently, the ratio of acylated forms in Sangiovese decreased when sun exposure increased. The

Table 5 – Effect of bunch exposure on berry coloring at the onset of grape ripening.

Site	Cultivar	Year	DAV 1	Treatment	%		
					Green	Just pink	Fully colored
Oltrepò Pavese	Croatina	2005	20	Exposed	6	29	66
				Leaf shaded	12	48	40
				Boxed	19	69	12
Oltrepò Pavese	Sangiovese	2005	14	Exposed	2	24	74
				Leaf shaded	28	43	29
				Boxed	53	34	14
Oltrepò Pavese	Syrah	2005	15	Exposed	11	21	68
				Leaf shaded	20	40	40
				Boxed	38	55	7
Oltrepò Pavese	Nebbiolo	2005	17	Exposed	6	57	36
				Leaf shaded	12	58	30
				Boxed	25	61	14
Albese	Nebbiolo Rosé	2006	10	Exposed	3	8	90
				Boxed	16	53	31
Valtellina	Nebbiolo	2006	19	Exposed	2	18	80
				Boxed	16	67	17
Tavazzano	Carmènere	2006	18	Exposed	3	2	95
				Boxed	13	1	86
Tavazzano	Cannonau	2006	5	Exposed	45	33	22
				Boxed	94	6	0

[1] DAV = days after onset of veraison in exposed bunches

Table 6 – Anthocyanin profiles of the grape cultivars analyzed: average data of the different experimental conditions at ripening time.

Site	Cultivar	Year	Anthocyanins %							
			non-acylated	*p*-coumaric esters	acetic esters	delphinidin	cyanidin	petunidin	peonidin	malvidin
			Sum of acylated and non-acylated forms							
Oltrepò Pavese	Croatina	2005	80.5	10.6	9,0	11.5	2.0	11.2	11.9	63.5
Oltrepò Pavese	Sangiovese	2005	97.6	1.6	0.8	13.9	19.2	15.1	14.2	37.6
Oltrepò Pavese	Syrah	2005	60.1	24.9	15,0	6.4	1.3	9.4	13.1	69.7
Oltrepò Pavese	Nebbiolo	2005	91.4	5.1	3.5	6.5	10.7	5.5	41.1	36.2
Piedmont	Nebbiolo Rosè	2006	89.8	6.2	4.1	5.7	10,2	5.0	46.6	32.5
Valtellina	Nebbiolo	2006	91.2	4.5	4.3	2.8	5.2	3.2	46.8	42.0
Tavazzano	Carmènere	2006	63.6	19.8	16.6	7.8	1.4	9.4	7.1	74.3
Tavazzano	Cannonau	2006	82.4	13.7	3.9	1.1	1.4	1.9	13.5	82.0

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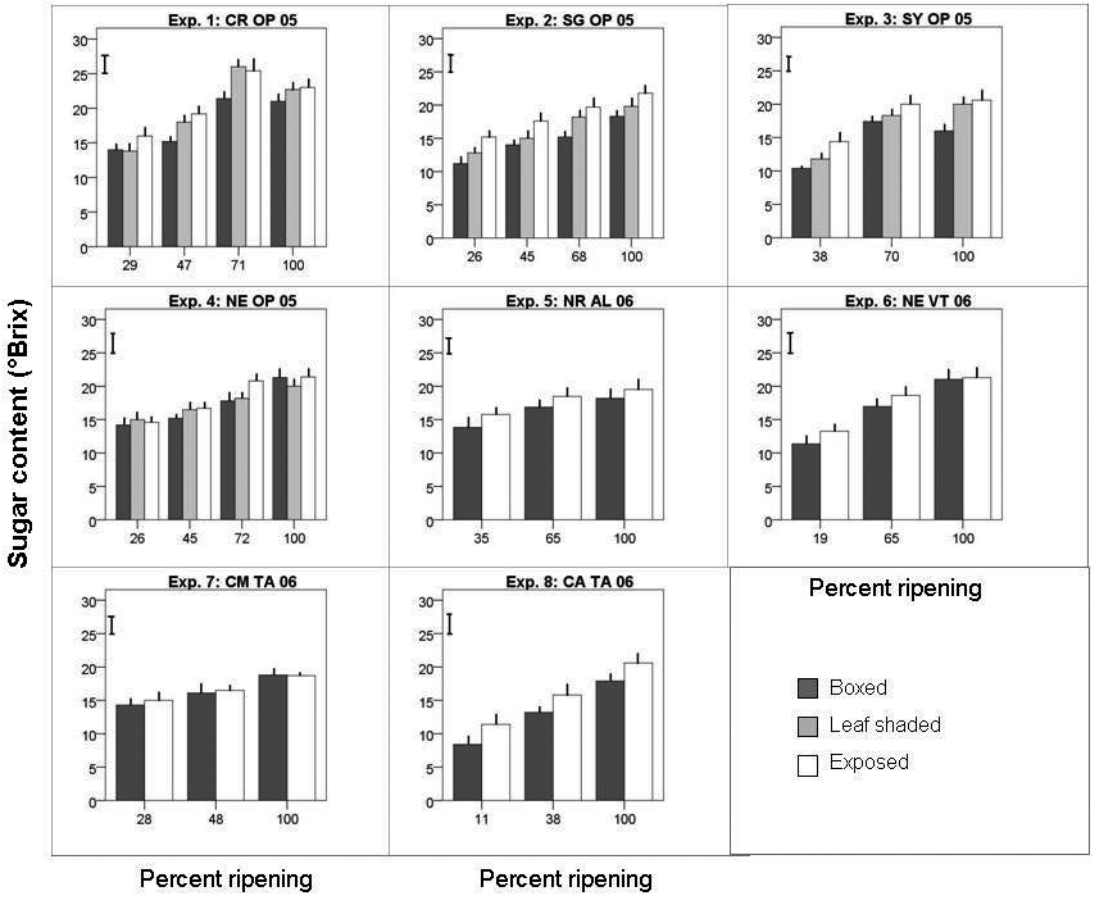


Figure 4 – Sugar content (mean + 0.5 S.D.) during grape ripening in relation to the bunch microclimate conditions in the eight experiments (see table 1).

The X-axes representing the time courses have been rescaled in percent of the final ripening time.
In each graph bars represent the least significant difference between means according to Tukey's test (P=0.05).

acylation profiles of Nebbiolo and Nebbiolo Rosé, which generally had low percentages of acylated forms, were only minimally influenced by sun exposure.

b) B-ring

Focusing on the B-ring, it can be observed that sun exposure usually caused an increase in the percentage of ortho-diphenols compared to boxed bunches (Fig. 7), with only two exceptions where the percentage of these pigments was higher in shaded bunches (2nd sample of Syrah experiment and 3rd sample of Nebbiolo Rosé experiment). Moreover, the proportion of ortho-dihydroxylated pigments often increased proportionally to increased radiation availability, with the lowest values in boxed bunches, intermediate values in leaf shaded bunches, and the highest values in exposed bunches.

DISCUSSION

Eight experiments were performed with seven different cultivars in four different sites to compare sun exposed to shaded bunches in terms of anthocyanin accumulation and profile. Direct light availability resulted in an earlier and stronger anthocyanin accumulation in all the experiments, at least at the beginning of the ripening period, from veraison to mid ripening. This relative increase in anthocyanin accumulation was not always maintained throughout ripening. As a result, at the final stage of ripening, the different grape cultivars showed a wide variety of responses to light conditions: Croatina and Sangiovese had the highest levels of anthocyanin and showed the ability to respond to light availability up to the last stage of grape ripening, unlike the other tested cultivars. In Syrah, Nebbiolo and Nebbiolo Rosé, which in general had lower levels of anthocyanin, shaded and/or boxed bunches reached the same levels of anthocyanin accumulation as sun exposed bunches over the final stages of the ripening period. This behavior could be related to their intrinsic limitation in anthocyanin accumulation and, therefore, a lower sensitivity to light and temperature conditions. A third physiological pattern was observed in Carmènere and Cannonau grapes where the differences

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detected at the onset of maturation were maintained until grape ripening.

These results were obtained in temperate (Exp. 1-5, 7-8) and cool (Exp. 6) conditions, where under midday sun the exposed bunches often reached excessive temperatures for biosynthetic metabolism, while box shaded bunches generally remained within a favorable temperature range for biosynthetic metabolism. By assuming the biosynthetic metabolism versus temperature curve depicted in Fig. 1, it was possible to quantify the gain in thermal units per bunch in different microclimate conditions in terms of NHH. Following this model, the highest thermal differences between sun exposed and shaded bunches were recorded in experiments 1-4, where sun exposed bunches gained 13-15 NHH per day during the first part of the ripening season in comparison to 18-20 NHH per day for boxed bunches. In the other experiments (Exp. 5-8), the differences were lower; however, light availability overcame the possible inhibitory effect of unfavorable temperature, always inducing an earlier and stronger anthocyanin accumulation. In the second stage of the ripening period, when there were only small variations in daily NHH accumulation in the different microclimatic conditions, cultivar dependent responses were evident, leading to different effects on the final accumulation (Fig. 5).

In summary, in the environmental conditions tested in this study, which were essentially representative of a mesothermal climate, direct sunlight seemed to play a major role in inducing the onset of anthocyanin accumulation; however, in our experimental conditions, a possible role of high berry temperature in inducing anthocyanin synthesis cannot be excluded.

When considering the influence of light availability on anthocyanin profile, a number of studies (DOWNEY *et al.*, 2004; HASELGROVE *et al.*, 2000; TARARA *et al.*, 2008) indicated that bunch shading generally causes an increase in the proportions of esterified anthocyanins. These authors also noted that not all cultivars have a similar response. Accordingly, in the current study, the anthocyanin profiles of Nebbiolo and Nebbiolo Rosé, which are two closely related cultivars with very low

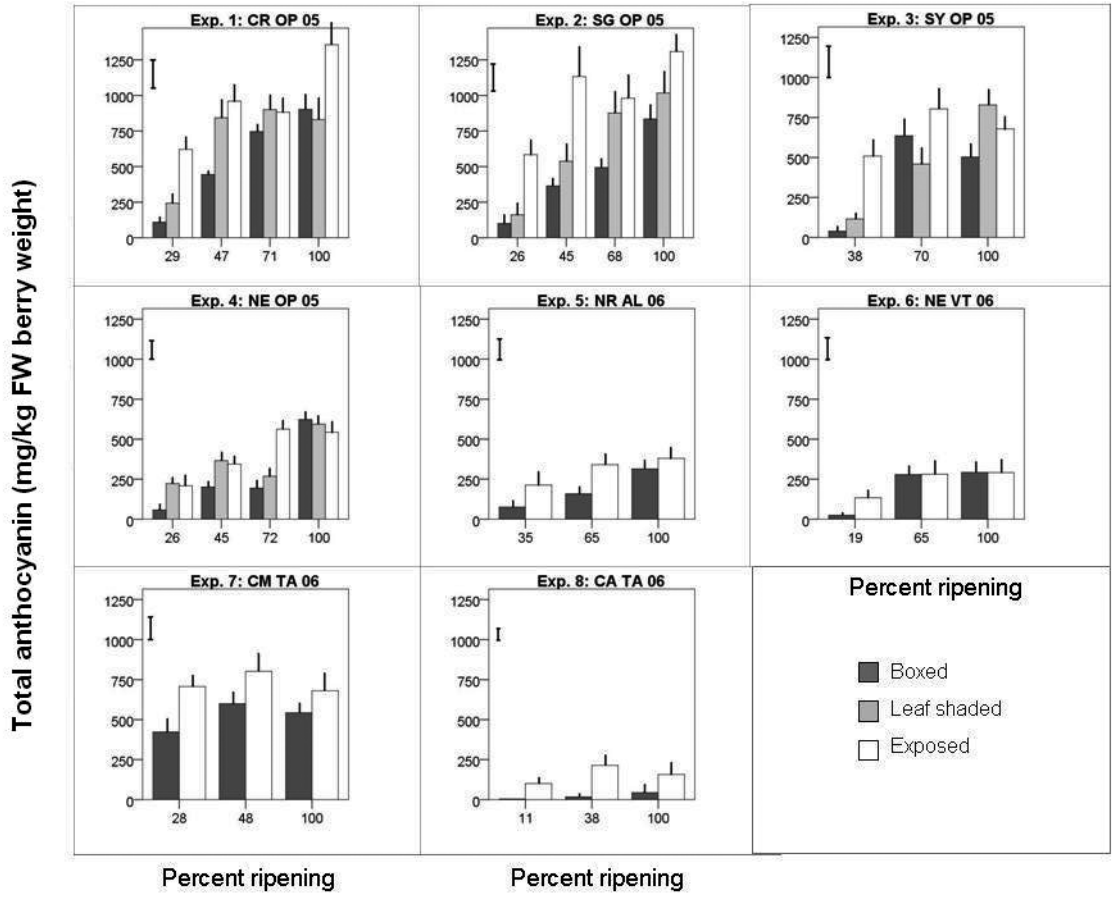


Figure 5 – Accumulation pattern (mean + 0.5 S.D.) of total anthocyanins in relation to the bunch microclimate conditions in the eight experiments (see table 1).

The X-axes representing the time courses have been rescaled in percent of the final ripening time.
In each graph bars represent the least significant difference between means according to Tukey's test (P=0.05).

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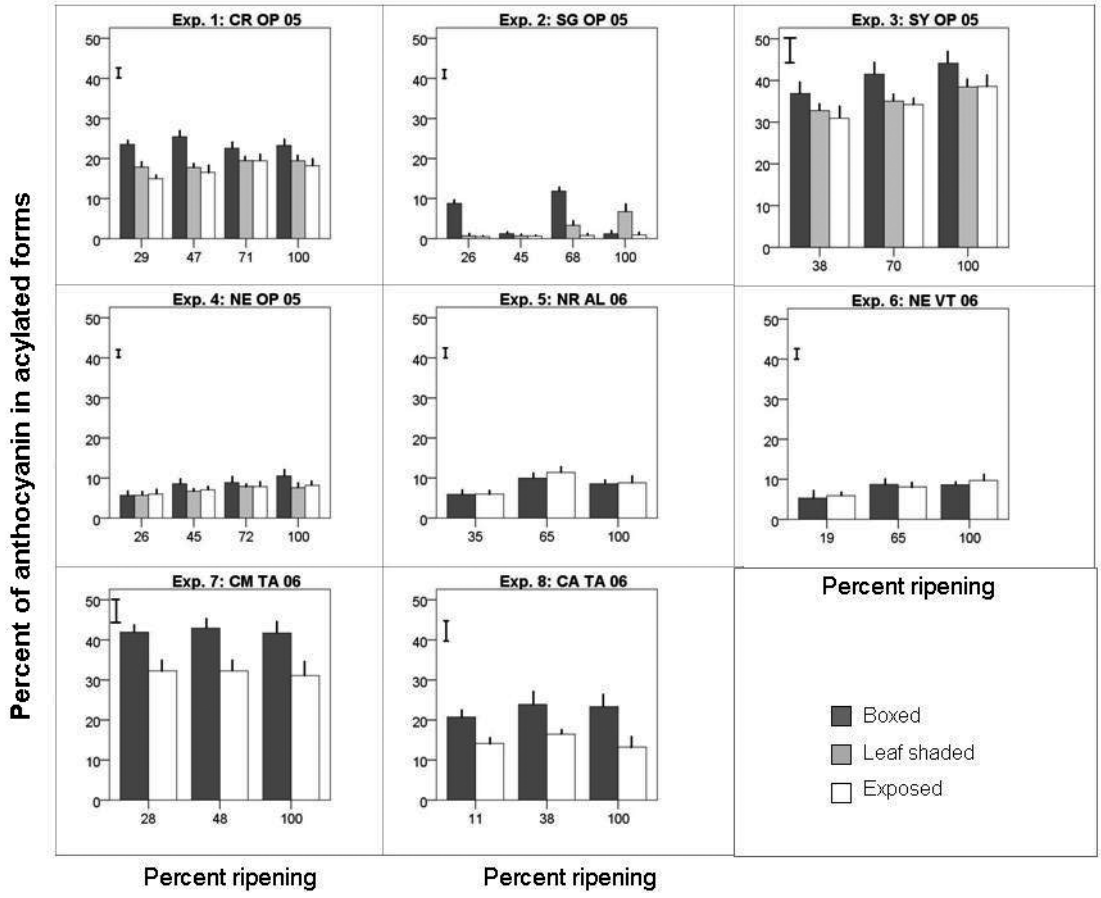


Figure 6 – Proportion (in percent) of anthocyanin in acylated forms (mean + 0.5 S.D.) during grape ripening in relation to the bunch microclimate conditions in the eight experiments (see table 1).
The X-axes representing the time courses have been rescaled in percent of the final ripening time.
In each graph bars represent the least significant difference between means according to Tukey's test (P=0.05).

levels of acylated forms, did not show any significant changes in response to the treatments.

The main finding of the study was that light exposure caused increases mainly in the percentage of pigments featuring an ortho-dihydroxyl group, that is, delphinidin, cyanidin and petunidin. Anthocyanins are not usually examined in relation to their oxidation reactivity, so it is hard to find other studies that discuss this aspect on pigment profiles. However, an ortho-dihydroxyl group is also present in other phenolics, such as flavonols, which can contain part of the molecule identical to the B-ring of anthocyanins. In this view, our results are in accordance with other studies that showed that UV light increases the accumulation of ortho-dihydroxylated flavonols, a behavior that has been interpreted as a biological response to protect plant tissue against such high energy radiation (COCKELL and KNOWLAND, 1999; KOLB *et al.*, 2003; MARKHAM *et al.*, 1998; OLSSON *et al.*, 1998). In *Vitis vinifera* cv. Merlot, TARARA *et al.* (2008) found that exposure to more intense solar radiation increases the concentration of quercetin-3-glucoside but has no apparent influence on the concentration of kaempferol-3-glucoside. In contrast, KOLB *et al.* (2003), in Bacchus grapes, did not find significant variation in the quercetin-3-glucoside:kaempferol-3-glucoside ratio between exposed and unexposed berries to UVB radiation. This behavior could be due to the fact that different flavonoid classes generally have different UV protection mechanisms. We can speculate that in grape berries flavonols may have an important role in UV screening, but the phenolic antioxidant protection is most related to anthocyanins (CLOSE and BEADLE, 2003). MONICI *et al.* (1994) evaluated the role of kaempferol and pelargonidin as photoprotectors. They concluded that both compounds contribute to plant protection: the action of pelargonidin seems more likely to derive from its strong radical scavenger action, whereas kaempferol can be considered a good screen against UV radiation (ROBARDS and ANTOLOVICH, 1997). This is probably because double bonds of A and C anthocyanin rings are conjugated with the B-ring and the 4'-hydroxyl group. This fact can be important in reaction with some oxidants, as shown by ANTOLOVICH *et al.* (2004) in the conjugation of the 4'-hydroxyl group with the alkene

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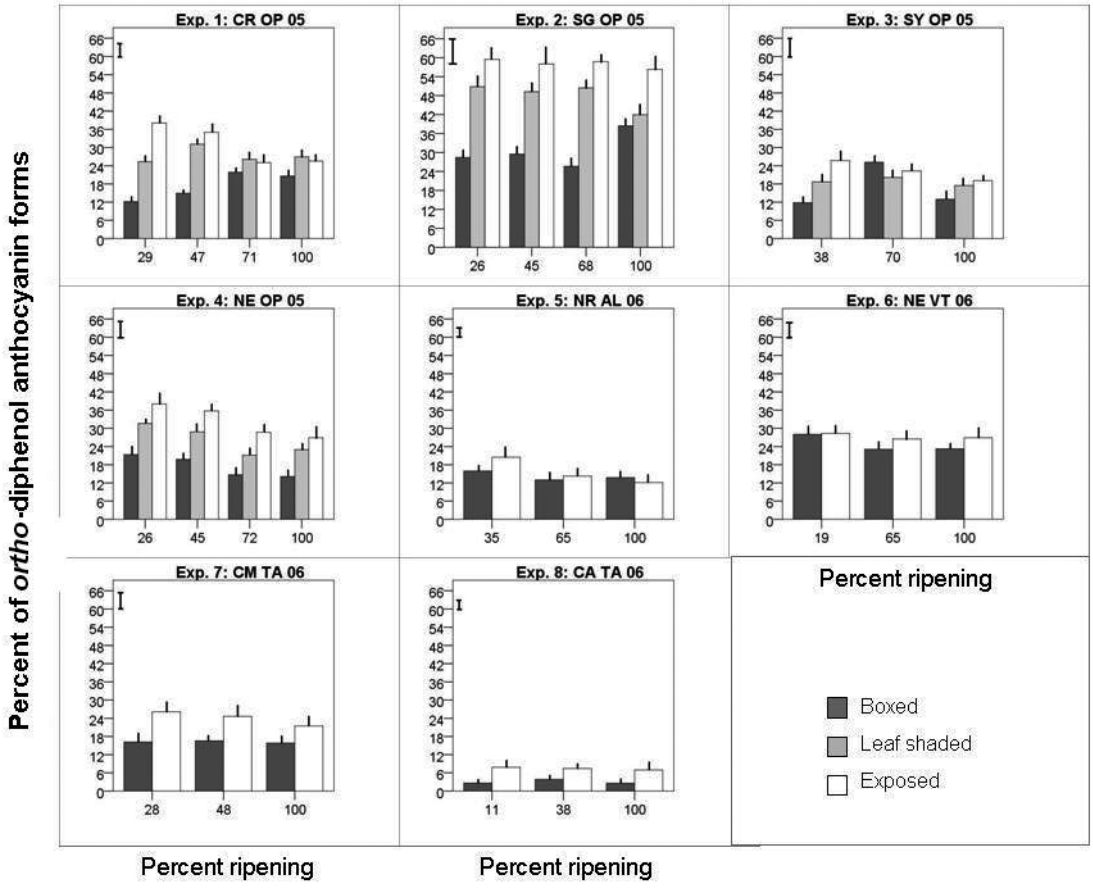


Figure 7 – Proportion (in percent) of ortho-diphenol (delphinidin, cyanidin and petunidin), acylated and non acylated anthocyanin forms (mean + 0.5 S.D.) during grape ripening in relation to the bunch microclimate conditions in the eight experiments (see table 1).

The X-axes representing the time courses have been rescaled in percent of the final ripening time. In each graph bars represent the least significant difference between means according to Tukey's test (P=0.05).

double bond in cinnamic acids. Therefore, also following the results of the present research work, it can be supposed that in grape berries, where both flavonols and anthocyanins are accumulated, the best protection strategy against excessive light condition could be an increase in ortho-diphenol anthocyanins.

CONCLUSION

The present research work indicated that in the climatic conditions of Northern Italy, which are representative mesothermal climates, direct bunch exposition to solar radiation stimulate anthocyanin accumulation in grapes in spite of the increase of berry temperature above the optimal range during midday. The different tested cultivars showed a different behavior in the second part of the ripening period, which seem partly related to the cultivar ability to accumulate anthocyanin, when the thermal differences between exposed and shaded bunches were very similar in term of favorable NHH accumulation for biosynthetic processes.

The common response of the tested cultivars to sun exposure in terms of grape anthocyanin profiles was the increase in the percentage of delphinidin, cyanidin and petunidin, that is, anthocyanins with an ortho-dihydroxyl group.

Summarizing our main results from the cultural and enological point of view, in the climatic condition of Northern Italy, bunch exposure would appear to have favored the production of musts and wines with more anthocyanins, due to a generally higher concentration of total anthocyanins and to the presence of a greater percentage of easily extractable pigments. However, as for color, the wine could have conceivably been less stable as a result of the higher proportion in non-acylated anthocyanins and ortho-dihydroxylated pigments, which are more susceptible to chemical oxidation (FULCRAND *et al.*, 2006; WATERHOUSE and LAURIE, 2006; RODRÌGUEZ-SAONA *et al.*, 1999).

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