Effects of altered microclimatic conditions on ripening and anthocyanin profile of "Nebbiolo" grapes.

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Artificial shading and cluster exposure were applied in order to alter the microclimatic conditions of the fruit zone in Nebbiolo grapes through two years of field trials. Particular importance was given on evaluating the influence of solar radiation and berry temperature on ripening and anthocyanin profile of the berries. Three fruit zone light exposure regimes were set up using plastic netting or leaf removal. Shading was applied from fruit set to harvest and leaf removal took place at fruit set. Both net shading and leaf removal decreased berry temperature compared to the non-treated vines. In net-shaded vines, photosynthesis of basal leaves decreased, but it did not change significantly in the upper ones. The fruit zone shading had no impact on vine yield and cluster weight. Fruit zone shading had a negative effect on total soluble solids (TSS) and anthocyanin accumulation. Generally, fruit shading induced a decrease in dihydroxylated anthocyanin concentration and an increase in trihydroxylated anthocyanin concentration. Fruit zone leaf removal did not affect either berry size or cluster weight or yield at harvest. It did not increase TSS and did not alter either the total anthocyanin content or the anthocyanin profile, although direct sun light exposure caused excessive sunburn damage to the clusters.

Introduction

The canopy microclimatic conditions influence berry development and ripening because berry and canopy sunlight exposure and temperature, in particular, have important influence on vine metabolism.

The photosynthetic activity of the leaves is maximum between 26 to 30 °C, when the net solar radiation is at saturating level (1000 to 1200 µmol m^{-2} sec^{-1}); under these levels photosynthetic activity is significantly reduced (Iacono 2004). Canopy shading can reduce total soluble solid (TTS) in the berries and may also cause a decrease of berry size (Morrison and Noble 1990) and a slower and more prolonged growth period (Rojas-Lara and Morrison 1989). Moreover, canopy shading delays the berry anthocyanin accumulation (Rojas-Lara and Morrison 1989) but has no significant effect on their final content (Rojas-Lara and Morrison 1989, Morrison and Noble 1990). The effects of light on fruit composition seems to depend upon the extent to which berry temperature augment as a result of increased sunlight exposure; in fact berry high temperature can inhibit color development (Bergqvist et al. 2001, Yamane et al. 2006, Mori *et al.,* 2007; Tarara et al. 2008; Chorti *et al.,* 2010, Azuma *et al.,* 2012).

Nevertheless, a different impact of fruit shading on berry development and weight at harvest was observed in relation to the timing of the shading application (Morrison and Noble 1990; Spayd et al. 2002; Downey et al. 2004; Chorti et al 2010). The effect of canopy shading on TSS accumulation is not clear (Rojas-Lara and Morrison 1989; Morrison and Noble 1990; Dokoozlian and Kliewer 1996; Haselgrove et al. 2000; Bergqvist et al. 2001; Spayd et al. 2002; Downey et al. 2004) but it is generally accepted that an excessive cluster shading reduces berry anthocyanin accumulation (Rojas-Lara and Morrison 1989; Morrison and Noble 1990; Gao and Cahoon 1994; Dokoozlian and Kliewer 1996; Haselgrove et al. 2000; Bergqvist et al. 2001; Spayd et al. 2002) because light is an important limiting factor, especially during the early stages of ripening (Haselgrove et al. 2000). Shading not only decreases the content of total anthocyanins, but also affects the ratio between free and acylated forms of anthocyanins and/or between 3'- and 3'-5' hydroxylated anthocyanins (Downey et al. 2004; Tarara et al., 2008; Chorti et al., 2010; Azuma et al., 2012).

More than to light conditions, anthocyanin accumulation responds to the temperature level; in particular, many studies agree that excessively high temperatures are detrimental to skin anthocyanin accumulation (Bergqvist et al. 2001; Spayd et al. 2002; Yamane et al., 2006), influencing both synthesis and potential degradation of the molecules (Mori et al., 2007). However, the effect of the temperature level can vary greatly among the stages of development (Yamane et al. 2006). The critical temperature for net anthocyanin accumulation lies between 30 and 35°C, but this could vary depending on the variety (Spayd et al. 2002).

The Nebbiolo variety is considered one of the greatest wine varieties long-lived and prized by collectors. It is, however, very sensitive to terroir and it is characterized by elevated vigor and reduced berry skin color. Its berry skin anthocyanin profile is distinguished by the presence of peonidin-3-glucoside as the most abundant anthocyanin, followed by malvidin-3-glucoside (Guidoni et al. 2008). Its low total anthocyanin content could result in wines with insufficient color. This study aimed to investigate the impact of altered cluster temperature and sunlight microclimatic conditions on berry development and composition of Nebbiolo grapes, focusing on anthocyanin accumulation and composition.

Materials and Methods

In 2006, the study was conducted in a commercial vineyard of Vitis vinifera cv. Nebbiolo located in Vignane (Northwest Italy) which has been planted in 1996 on a 16 ° slope with a north-western exposure. Due to a severe cutworm (*Noctua spp*.) infestation in the spring of 2007, the study had to be moved to a nearby, similar commercial vineyard. In both vineyards vine rows were planted across the maximum slope and vines were pruned to a single 10-bud cane and shoots were vertically trained and managed according to the standard practices for this cultivar and the region.

In both years, three fruit zone light exposure levels were set up in the vines by artificial shading or leaf removal: naturally occurring shade (experimental control, C), shaded fruit-zone from fruit set to harvest (FH), fruit- zone leaf removal at fruit set (LR).

The shading was obtained using 2-m wide 2220 WO Iride Due anti-hail, run resistant, double thread nets (Arrigoni Spa, Como, Italy). The nets, made of UV stabilized HDPE, had a 16 % light screening factor. The nets were placed vertically along the fruit zone, folded in two, in order to form a one-meter wide double layer. The LR treatment consisted in removing 5 to 6 basal leaves from the main shoots and 2 to 3 basal lateral shoots, in order to obtain maximum cluster light exposure. All three treatments were arranged within three complete randomized blocks with 15-vine plots. Sampling was performed on the 10 central vines.

In order to monitor and evaluate light conditions, photosynthetically active radiation (PAR) sensors (model S-LIA-M003, Onset Computer Corporation , Pocasset, MA, U.S.A.) were positioned at fruit zone level and connected to data loggers (Hobo H 21-002 Micro station logger, Onset). Berry temperature -was monitored by thermocouples (model TC6-T, Onset Computer Corporation) inserted just beneath the berry skin and connected to data loggers (Hobo H12 Type T thermocouple logger, Onset Computer Corporation). In order to monitor the air temperature at cluster level, sensors were placed (Hobo H08-032-08) at the fruit zone. PAR and berry temperature sensors were applied in two replicates of each treatment. Data were recorded once every 20 minutes throughout fruit growth from veraison (8 August) to harvest (20 September). Mean diurnal PAR (0500 to 2100 hr) and temperature (000 to 24 hr) patterns were generated from hourly means calculated from the raw data.

In several stages of the growing season, the leaf photosynthetic rate was measured by means of a ADC-LCA3 gas-analyzer equipped with a Parkinson leaf chamber.

Starting from the beginning of veraison, every 2 weeks samples were taken in order to determine TSS concentration by refractometer, and anthocyanin concentration and profile by HPLC (Perkin Elmer Series 200 Diode Array Detector, Perkin Elmer Ltd., Buckinghamshire, U.K., Di Stefano et al. 1991). For anthocyanin extraction, berry skins were removed from the pulp, and placed in 40 mL of acidified methanol (1 % HCl, v/v) (Revilla et al. 1998). The samples were placed in an oven at 30 °C for 72 hours. Individual anthocyanins were identified by comparing the retention time of each chromatographic peak with available data in literature (Di Stefano et al. 1995). The concentration of individual anthocyanins was expressed as mg kg^{-1} of berry fresh weight using malvidin-3-O-glucoside chloride (Extrasynthèse, Genay, France) as external standard. Total anthocyanin amount was calculated as the sum of the concentrations of the free and derivative anthocyanin forms.

Cluster and berry weight, yield per vine, bunch rot incidence and cluster sunburn damage were evaluated at harvest.

Data were subjected to one-way analysis of variance (ANOVA) using the SAS procedure (SAS Institute, Cary, NC).

Results and Discussion

PAR and temperature. 2007 season was warmer than 2006, especially in the spring (Figure 1). The high spring temperature, in 2007 accelerated all phenological phases in comparison to 2006.

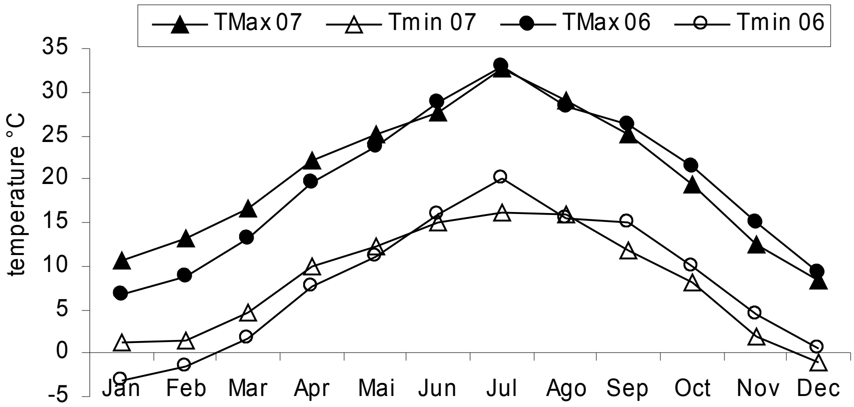
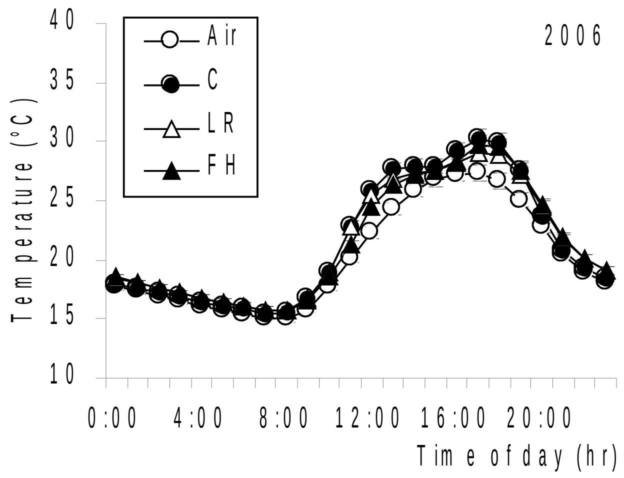


Figure 1. Monthly average of daily maximum and minimum temperatures over the two growing seasons (data come from *Regione Piemonte* meteorological stations located few kilometers from the vineyards).

In both years cluster temperature always resulted higher than ambient air temperature, measured at cluster height level, during daytime. During nighttime, on the other hand, cluster and ambient temperature were similar (Figure 2). Air daily temperature at the canopy level was higher in 2007 than in 2006, confirming data from meteorological station.

In 2006, control cluster temperature was slightly higher than both LR and FH. In 2007, the average cluster temperature of all treatments was higher than the year before. The warmer temperature conditions of 2007 increased the cluster temperature differences between the control and the other treatments, which, during the afternoon varied up between 2.5 °C and 3°C depending on the treatment (Figure 2).



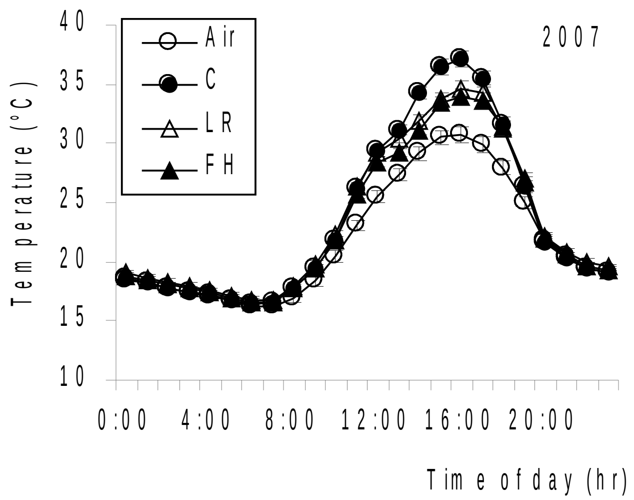


Figure 2. Mean diurnal berry temperature from veraison to harvest in 2006 and 2007. **C:** control vines; **LR:** leaf-removed vines; **FH:** vines shaded from fruit set to harvest; **Air:** air temperature at fruit zone level (means of 40 days ± standard error).

Global irradiance (PAR) from veraison to harvest, resulted higher in 2007 when compared to 2006 (Figure 3), both in maximum and average values. In both years, leaf removal contributed to significantly increase cluster light exposure and,. As expected and wished, shading nets caused a significant reduction of PAR incidence on fruit zone.

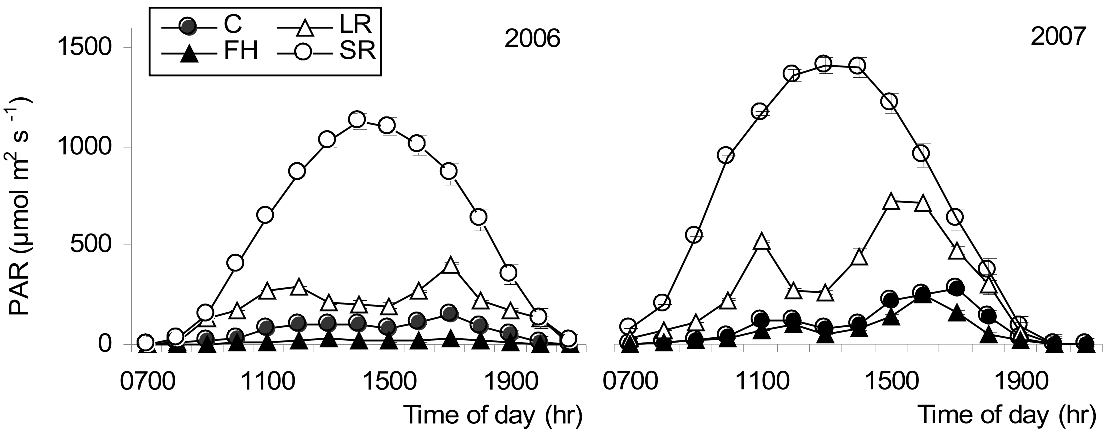
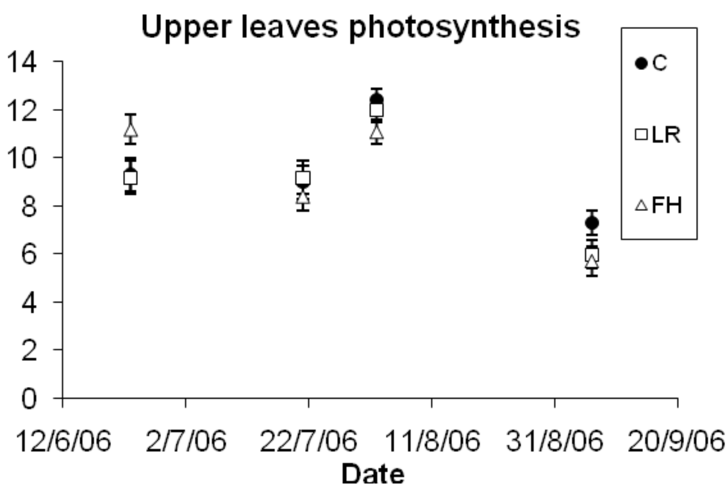


Figure 3. Mean diurnal PAR incidence, from veraison to harvest in 2006 and 2007. C: control vines; LR: leaf-removed vines; FH: vines shaded from fruit set to harvest; SR: above canopy solar radiation.

Although in FH vines PAR was limited by the net while in LR vines clusters were direct exposed to the incident solar radiation, LR and FH vine berry temperature was similar in both years probably due to the different air circulation conditions. In both years, the presence of net or leaf removal reduced berry temperature compared to natural shading (Figure 2).

In net shaded vines (FH), photosynthesis of basal leaves decreased, but the upper leaves didn't show a compensative activity . Leaf removal didn't affect photosynthetic activity (Figure 4).



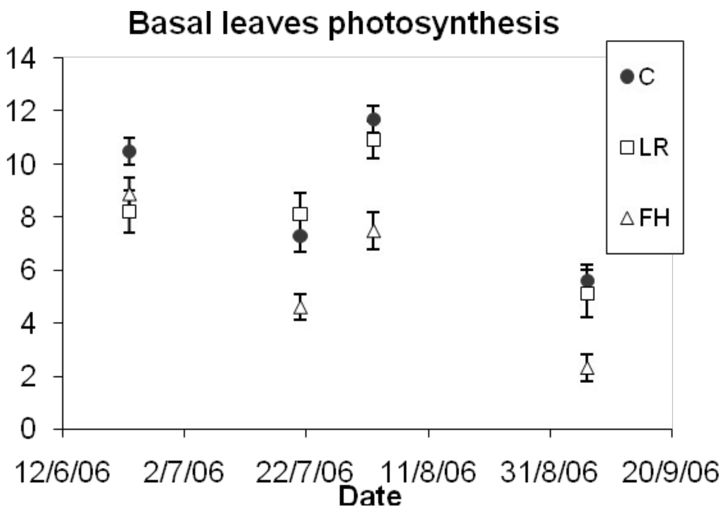


Figure 4. Net photosynthesis of upper and basal leaves measured in 2006. C: control vines; LR: leaf-removed vines; FH: vines shaded from fruit set to harvest.

Berry development and composition. Shading had no impact on vine yield and cluster weight. Neither did leaf removal at fruit set in 2006, in agreement with Main and Morris (2004), although results in other studies (Petrie et al. 2003; Poni et al. 2006) suggested that leaf removal had a negative effect on yield components, especially when performed at a very early stage. In our case the leaf removal preserved around 80 % of the vine leaves that was, probably, more than enough to guarantee all photosynthetic needs of the vinesIn 2007, though, due to serious sun burn damages in leaf removed treatment, cluster weight wasreduced (Table 1). There were no differences among treatments in bunch rot incidence. No differences were observed in relation to the berry weight, among the treatments but for this assesment we sampled avoiding the shriveled berries (Table2).

Artificial fruit zone shading, in 2007, had a negative effect on TSS accumulation (Table 2) in agreement with previous studies (Rojas-Lara and Morrison 1989, Dokoozlian and Kliewer 1996, Bergqvist et al. 2001). Leaf removal had no influence on TSS accumulation and content at harvest, in agreement with Percival et al. (1994), although other studies suggested that leaf removal around fruit set reduced TSS concentration (Poni et al. 2006; Joscelyne et al. 2007), probably as a result of a reduced whole-vine photosynthesis (Petrie et al. 2003). In our study, despite the impact of leaf removal on vine leaf area, especially in 2007, no differences were found between LR and C-vine TSS concentration in both years.

Table 1. Influence of cluster exposure level on grapevine yield, cluster weight, incidence of bunch rot, and cluster burning damages of Nebbiolo grapes at harvest, in 2006 and 2007. **C:** control vines; **LR:** leaf removed vines; **FH:** vines shaded from fruit set to harvest.

	Yield per vine (kg)	**Cluster weight (g)**	**Bunch rot (%)**	**Burning damage (clusters/vine)**
2006				
C	2.6 a[a]	330 a	25 a	0
LR	2.4 a	315 a	14 a	0
FH	2.2 a	278 a	47 a	0
2007				
C	1.7 a	266 a	0	0.70 b
LR	1.0 a	164 b	0	3.00 a
FH	2.2 a	308 a	0	0.08 b

[a]Means followed by different letters in columns, within the same year, indicate significant differences among treatments at $p \leq 0.05$.

Table 2. Influence of cluster sunlight exposure on berry weight and total soluble solid (TSS) accumulation during ripening of Nebbiolo grapes. C: control vines; LR: leaf-removed vines; FH: vines shaded from fruit set to harvest. [b]Means followed by different letters in rows, within the same year, indicate significant differences among treatments at $P \leq 0.05$.

	Berry weight (g)			TSS (Brix)		
Year /dpv[a]	C	LR	FH	C	LR	FH
2006						
0	1.2 a	1.0 a	1.0 a	11.9 a	12.2 a	10.0 a
14	1.7 a	1.6 a	1.7 a	17.1 a	17.0 a	16.7 a
28	1.8 a	1.7 a	1.8 a	22.5 a	21.7 a	21.6 a
42	1.9 a	1.7 a	1.9 a	22.2 a	23.1 a	22.6 a
54	1.9 a	2.0 a	1.9 a	23.6 a	23.7 a	23.1 a
2007						
5	1.7 a	1.7 a	1.5 a	16.6 a	17.1 a	15.3 b
20	2.1 a	2.0 a	2.0 a	20.3 a	19.5 a	16.8 b
32	2.2 a	2.2 a	2.0 a	23.2 a	23.8 a	21.2 b
47	1.9 a	1.9 a	2.0a	26.4 a	25.9 a	24.2 b

[a]dpv: days post veraison

Anthocyanin accumulation. Anthocyanin concentration at harvest was higher in 2006 compared with 2007 (Table 3) for all treatments. This could have been the result of the higher berry temperature in 2007, exceeding 30 °C for several hours during the day. Many metabolic processes stop or are reduced at about 30 °C in the grapevine (Coombe, 1987), and with temperatures higher than 30 °C (Spayd et al. 2002, Yamane et al. 2006) skin anthocyanin accumulation can be inhibited. These limits are not yet well known and may depend on cultivar (Tarara et al. 2008).

Although several studies have found that light exposure has a positive effect on cluster anthocyanin concentration (Rojas-Lara and Morrison 1989, Morrison and Noble 1990, Gao and Cahoon 1994, Dokoozlian and Kliewer 1996, Haselgrove et al. 2000, Bergqvist et al. 2001, Spayd et al. 2002), other studies suggest that anthocyanin biosynthesis is not readily affected by sunlight (Downey et al. 2004). In the present study leaf removal increased cluster sunlight exposure but did not alter anthocyanin concentration compared to the control. This could be the result of the elevated berry skin temperature that may have overridden the positive effects of cluster light exposure, strongly suggesting that berry skin temperature has more influence on anthocyanin accumulation than light (Spayd et al. 2002; Tarara et al. 2008), and that the effect of temperature can vary greatly along development stages (Yamane et al. 2006). It has been underlined that at 100 µmol m^{-2} s^{-1} of incident solar irradiation, the effects of light on anthocyanin biosynthesis are indeed heavily dependent on the extent to which berry temperature is elevated as a result of increased sunlight exposure (Bergqvist et al. 2001).

In the present study, both C and LR PAR reached or exceeded 100 µmol m^{-2} s^{-1}, especially in 2007 (Figure 3), and berry temperature exceeded the threshold discussed above for several hours during the day (Figure 2).

Nonetheless, and in contrast to 2006, leaf removal caused an increase of the 3'-substituted / 3'-5'-substituted anthocyanin ratio compared to FH in 2007, mainly due to a decrease of 3'-substituted anthocyanin proportion.

Table 3. **Single and total anthocyanin concentration of Nebbiolo grape skins at harvest, under different sunlight exposure levels, in 2006 and 2007. C: control vines; LR: leaf-removed vines; FH: vines shaded from fruit set to harvest.** Means followed by different letters in columns, within the same number of days post veraison, indicate significant differences at $p \leq 0.05$.

	Anthocyanidin-3-glucoside (mg/kg berry)					TA (mg/kg berry)	di/tri ratio
2006	**Df**	**Cy**	**Pt**	**Pn**	**Mv**		
C	43.4 b	109.1 a	35.4 b	408.4 a	176.1 a	829 a	2.0 a
LR	53.2 a	96.4 a	42.9 a	340.7 a	204.8 a	796 a	1.5 b
FH	42.8 b	80.2 a	38.2 ab	369.7 a	211.8 a	805 a	1.6 b
2007							
C	33.2 a	83.4 ab	28.4 a	257.1 a	141.8 a	615 a	1.6 ab
LR	30.6 a	102.5 a	26.1 a	266.2 a	116.6 a	606 a	2.1 a
FH	25.6 a	36.0 c	25.0 a	177.5 b	158.5 a	482 b	1.1 b

Df: delphinidin-3-glucoside; **Cy:** cyanidin-3-glucoside; **Pt:** petunidin-3-glucoside; **Pn:** peonidin-3-glucoside; **Mv:** malvidin-3-glucoside.

Net shading in 2007 depressed the anthocyanin biosynthesis beginning at the initial stages of observation. This result agrees with previous studies which showed that reduced sunlight could be a limiting factor for anthocyanin accumulation (Rojas-Lara and Morrison 1989, Morrison and Noble 1990, Gao and Cahoon 1994, Dokoozlian and Kliewer 1996, Haselgrove et al. 2000, Bergqvist et al. 2001, Spayd et al. 2002). However, in 2006, with lower fruit zone PAR values, higher anthocyanin concentrations were reached compared to 2007, confirming that high sunlight may not be an absolute necessity for anthocyanin biosynthesis in grape berries (Downey et al. 2004). Moreover, it could be assumed that the limiting effect of low sunlight exposure could become evident when berry temperature is also above the optimum level for anthocyanin biosynthesis. As a matter of fact, it has been observed that when shading does not alter berry temperature, it may have no impact on anthocyanin accumulation (Cortell and Kennedy 2006; Ristic et al. 2007), and an increase of anthocyanin biosynthesis can occur under low temperature conditions (20 °C rather than 30 °C) particularly following veraison, independently from light level (Yamane et al 2006). It has been suggested that low sunlight combined with high berry temperature may negatively affect anthocyanin biosynthesis (Tarara et al. 2008), but from the results of the present study it could be assumed that high sunlight combined with high temperature does not necessarily decrease anthocyanin biosynthesis.

Kliewer and Torres (1972) proposed that the effect of elevated temperature and cluster shading on anthocyanin accumulation depends, among other factors, on grape cultivar. The fact that cultivars are less or not at all affected has also been shown by others (Price et al. 1995, Spayd et al. 2002, Downey et al. 2004). The most sensitive cultivars may be those with high proportion of 3'- hydroxylated anthocyanins.

Net shading seems to have favored the synthesis of 3-coumaroyl-glucosides in this study (Table 4). It should be pointed out that in Nebbiolo the concentration in the skins of 3-coumaroyl-glucosides and of acylated anthocyanins was generally very low compared to non acylated ones.

Table 4. . Proportion of acylated and non acylated anthocyanins expressed as percentage of total anthocyanins concentration

		Acylation proportion (%)			
		nonacylated	acylated	3-acetyl-glucosides	3-coumaroyl-glucosides
2006	C	93.20 a	6.80 b	1.32 a	5.13 b
	LR	92.70 ab	7.30 ab	1.27 a	5.75 a
	FH	92.32 b	7.68 a	1.33 a	5.91 a
2007	C	88.40 ab	11.60 ab	2.70 a	8.40 b
	LR	89.50 a	10.50 b	2.86 a	7.19 c
	FH	87.75 b	12.25 a	2.09 ab	9.57 a

[a]Means followed by different letters in columns, within the same number of days post veraison, indicate significant differences at P ≤ 0.05.

Conclusions

In two years the attempt to modify the fruit zone microclimate by netting or leaf removal has had only limited success. The shading nets should have decreased sunlight and berry temperature but only the former was accomplished as the netting did not always reduce air temperature due to reduced air circulation in the fruit zone. This did not cause any particular problem in the cooler year (2006), but in the warmer one (2007) it resulted in elevated berry temperature underneath the shading nets causing an unfavourable microclimate for anthocyanin biosynthesis.

Fruit zone shading using nets caused a delay of berry maturation without affecting yield components at harvest. In 2006 all treatments reached the optimum anthocyanin concentration for Nebbiolo but in 2007 anthocyanins were much lower for all treatments even though sunlight conditions were potentially more favorable. In 2007 artificial shading not only reduced total anthocyanin accumulation, but also enhanced acylation with p-coumaroyl acid.

Fruit zone leaf removal increased sunlight exposure but did not increase anthocyanin accumulation. It caused excessive sunburn in exposed clusters and therefore qualitative and quantitative crop damage, rendering this practice not appropriate for areas of comparable climate conditions. Under these conditions, natural shading was more favorable for anthocyanin accumulation during both years of the study.

These results confirm the importance of temperature on anthocyanin concentration but it should be underlined that the interaction with sunlight can also influence the anthocyanin profile. Further studies are necessary in order to improve knowledge of the interactions between sunlight and berry temperature. From a practical point of view, fruit quality in Nebbiolo will benefit from conditions under which berry temperature does not rise over 30 °C, regardless of sunlight exposure.

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