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THE ROLE OF DIFFERENT CULTURAL PRACTICES ON POLYPHENOLS EVOLUTION DURING RIPENING AND ON WINE TASTE IN BLACK AND WHITE *Vitis vinifera* VARIETIES UNDER GLOBAL WARMING SCENARIO

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1. INTRODUCTION

1.1. CLIMATE CHANGE AND VITICULTURE

Human activities have been increasing greenhouse gas concentration in the last 60 years, causing the rise of air and ocean temperatures (Oreskes, 2004), and the increase of precipitation intensity and heat wave frequency (IPCC, 2014). Published studies predicted that the global warming will accelerate during the 21st century (Cox et al., 2000), with severe implications for agriculture (Olesen and Bindi, 2002).

The cultivation of *Vitis vinifera* L. grapevine for the production of wine takes place in the temperate climate zone of both hemispheres (Keller, 2010), and climate plays an important role on vine performances and wine quality (van Leeuwen et al., 2004). Therefore, the ongoing climate change represents new challenges for viticulture: on one side new winemaking areas may arise in regions that until now were considered too cold for the cultivation of grapevine, but in zones currently characterized by warm and dry climate, detrimental effects on yield and grape composition are expected to grow (Shultz, 2000; Jones, 2007; Jones et Alves, 2012; Fraga et al., 2013). Studies conducted in cool climate regions such as Bordeaux area and Alsace, have already described that global warming caused earlier onset of all the phenological phases and increased the sugar concentration at harvest (Jones and Davis, 2000; Duchêne and Schneider, 2005). These modifications could also be considered positively in zones where grapes struggle to reach optimal maturity, but in areas characterized by warm climate, the rise of temperature and the occurrence of drought has been causing negative effects on sensory attributes of berries (Sadras and Moran 2013a) and on the evolution of flavonoids (Sadras and Moran, 2012). Considering in particular the cultivation of black berry variety, when the growing season presents high temperatures and absence of rainfall, the strong acceleration of sugar accumulation often causes the decoupling between technological and phenolic maturity, and resulting wines can be characterized by high alcohol level, low acidity, poor color and unpleasant astringency and bitterness.

In the last few years, the issue of counteracting the negative effects of climate change on the black berry variety cultivated in warm regions was largely studied, and different approaches were chosen to slow down sugar accumulation with the aim to match technological and phenolic maturity. The limitation of carbohydrate sources was the first method adopted to reduce the rate of soluble solids accumulation and their concentration at harvest and, given the knowledge on grapevine physiology, it was decided to apply different techniques to decrease the photosynthetic activity of the youngest part of the canopy, which is the most active in the ripening period. For this reason, all the technical solutions were carried out

around veraison and are represented by innovative application of common cultural techniques. Among these solutions, leaf removal of the apical part of the shoots (Pallotti et al., 2013a; Poni et al., 2013), spraying of the upper part of the canopy with the antitranspirant di-1-*p*-menthene (Palliotti et al., 2013b, Gatti et al., 2016) and the elimination of the youngest part of the canopy by trimming (Filippetti et al., 2015; Herrera et al.,2015; Bondada et al.,2016) resulted effective in limiting sugar accumulation, and only minor interferences were found on the synthesis of berry flavonoids.

Another technique which was largely studied in the past, and that in the new scenario of global warming can be adopted to counteract the relative negative effects, is minimal pruning (Zheng et al., 2017). The higher bud load determined the increase of yield and the reduction of berry size that, respectively, caused the slowing down of the sugar accumulation and the increase of anthocyanin concentration.

Finally, in a recent paper was described a new approach for coupling technological and phenolic maturity which is based on the delay of winter spur pruning (Frioni et al., 2016). Authors reported that this technique, if performed in a particular phenological phase, was able both to reduce sugar level and to increase anthocyanin concentration, showing new perspectives in the study of the technical solutions for the viticulture of warm regions.

All the above mentioned techniques showed appreciable results in the contrast of the decoupling between technological and phenolic maturity, and their application appears feasible in commercial vineyards since no particular machinery is needed. Nevertheless, in most of the previous studies, the evaluation of anthocyanins and tannins was conducted analyzing the total amount of these compounds accumulated in the berry, which not necessarily represents the part that can be found in the resulting wines.

As well known, the phenolic maturity has been studied since the beginning of the eighties (Glories, 1984) and the knowledge on these complex topic has been improved in recent years (de Freitas et al., 2000; Kennedy et al., 2000; Kennedy et al., 2001; Cheynier et al., 2006; Rolle at al., 2011). Many factors affect the concentration and composition of the phenolic compounds extracted during vinification and the extractability of anthocyanin, skin tannins and seed tannins showed different behavior during ripening (Allegro et al., 2016).

Considering the strong impact on grape maturation of the global warming and of the cultural techniques adopted to mitigate its negative effects, a deep description of their consequences on berry flavonoids, on the basis of new findings and trough original approaches, has become essential to understand the changes that berry flavonoids undergo during ripening.

It appears clear that the topic of the phenolic maturity assumes a particular importance in the study of the effects that climate change and cultural techniques have on black berry varieties and, as a consequence, on their polyphenols and on the wine sensory attributes elicited by these compounds. Commonly is assumed that phenolic maturity is optimal when the color intensity is high, due to the high concentration of anthocyanins in grape and to the high extraction into wine, and the perception of rough sensations such as astringency and bitterness is low. Although a number of papers described the behavior of anthocyanins and tannins during ripening and their role on wine tastes, it has not been clarified yet which are the mechanisms that determines the enhancement of the phenolic maturity and which are, in detail, the characteristics of the different flavonoids that causes undesired sensations. Lately, some interesting results are coming from the study of the interactions between berry flavonoids and non-phenolic compounds of skin and flesh and, in particular, the findings which explains the ability of cell wall material to adsorb tannins (Bindon et al., 2010; Bautista-Ortín., 2014) represented the starting point of innovative researches on the extraction of phenolic compounds. These studies have already revealed important implications from the practical point of view (Bautista-Ortín et al., 2016; Bindon et al., 2016) and hopefully further investigations could be able to explain in more detail the matter of the phenolic maturity.

The consequences of climate change on the white berry varieties were studied to a lesser extent, probably because the problem of fastening the technological ripening can be apparently solved by the advance of harvest, with less implications than those found for the black berry cultivars. Nevertheless, Sadras et al. (2013b) reported that elevated temperatures increased pH of Chardonnay and Semillon and reduced both green and citrus aromas, confirming the results of early works, which indicated that high temperatures enhanced the degradation of malic acids (Lakso and Kliever, 1975) and the reduction of aromatic intensity (Reinolds and Wradle, 1993; Belancic et al., 1997). Therefore appears that climate change could determine negative effects on white wines but little is known about the impact of global warming on the phenolic compounds and on the tastes and textures they elicit, since, differently than what happens in the red wine vinification, white grapes doesn’t undergo to the maceration of skin and seeds and the release of flavonoids is weak. Despite these considerations, studies on this matter could be beneficial not only to overcome the lack of knowledge on the effects of climate change on white berry varieties but also to understand the role of tannins and flavonols in the perception of rough sensation such as astringency and bitterness which may be present also in white wines.

1.2. SCOPE OF THE PhD

Considering the matters discussed in the introduction, PhD activities were focused on the evaluation of the effects that different cultural techniques may have on phenolic compounds of grape and wine in the current global warming scenario, with particular focus on the role that skin cell wall material played on phenolic maturity.

In detail, three studies were carried out on the following issues:

a) effects of delayed winter spur pruning on vine performances and grape composition in black berry cv. Merlot;
b) role of cell wall material on phenolic maturity of cv. Merlot berries;
c) effects of cluster sunlight exposure on grape composition and wine sensory traits in white berry cv. Grechetto gentile.

a) The study reported by Frioni et al. (2016) demonstrated the aptitude of the delayed pruning to reduce sugar and to ameliorate anthocyanin concentration at harvest. These results are very promising to counteract the acceleration of sugar ripening and the decupling between technological and phenolic maturity, and for this reason, this technique was tested on the black berry cv. Merlot. Phenological phases, vegetative parameters and yield components were recorded, but a particular point of interest was the study of the technique implications on phenolic compounds. For this reason, the analysis of berry flavonoids at harvest evaluated both total amount and extractable portion of each compound, the latter obtained using a model hydroalcoholic solution that simulated the winemaking conditions. The set of results gave the possibility to evaluate into deep the influence of delayed pruning on the concentration, composition and extractability of anthocyanins, skin tannins and seed tannins at harvest.

b) Since phenolic maturity was a key-point matter for evaluating the delay of winter spur pruning, it was decided to conduct a study on cv. Merlot berries, to better understand the changes on berry flavonoids during the last phases of ripening and the relations between cell wall material and the main polyphenolic compounds. Part of this work was carried out in the laboratories of the University of Bologna and part in collaboration with the Enology team of the University of Murcia (Spain), headed by professor Encarna Goméz-Plaza. Berries were sampled in the last 20 days before harvest, and the analysis of total and extractable portion of anthocyanins, skin tannins and seed tannins were performed to report the influence of ripening on their concentration, composition and extractability. Moreover, considering that skin cell wall material is able to bind berry tannins, preferentially those galloylated and of high molecular weight (Bindon et al., 2010; Bautista-Ortín., 2014), it was decided to study

the properties of skin cell wall material in the last part of ripening. In particular, interactions between the skin cell wall material of berries at different stage of ripening and a commercial seed tannin were carried out in a wine-like medium, and were determined the concentrations and compositions of the tannins remained in solution. Sharing the knowledge and specializations of the Viticulture team of the University of Bologna and the Enology team of the University of Murcia, it was possible to adopt innovative solution in the study of the phenolic maturity.

c) Considering that climate change is leading to seasons characterized by higher solar irradiance and higher temperatures, which influence the composition and the sensory attributes of both red and white wines (Sadras and Moran 2013b), it was decided to set up the third experiment of the PhD on Grechetto gentile cultivar, in order to investigate the role of sunlight exposure on grape composition and wine traits of a white berry variety. As for the previous experiments, the main point of interest was the effect on phenolic compounds, in fact was determined the concentration and composition of flavonols, skin tannins and seed tannins of the berries sampled at harvest. The vinifications took place in the experimental winery of ASTRA (Tebano, Faenza, Italy), following a particular protocol designed to emphasize differences of phenolic tastes, such as astringency and bitterness.

In the setting up of each experiment were adopted original approaches that leaded to better understand the influence of climate change and the results of the technical solutions on vine performances, grape composition and wine quality, focusing the investigations on phenolic compounds and their tastes.

2. EFFECT OF DELAYED WINTER SPUR PRUNING ON VINE PERFORMANCE AND GRAPE COMPOSITION IN *Vitis vinifera* L. CV. MERLOT

2.1. INTRODUCTION

In the last few decades, an accelerated sugar accumulation on *Vitis vinifera* L. berries was observed in many cultivation areas and different factors contributed to this phenomenon. For instance, yield limits adopted in the production of origin appellation wines to preserve the quality of the products, obliged grapegrowers to reduce crop load that, together with the enhancement of canopy management, resulted in a higher accumulation of sugar. Another factor that contributed strongly on the acceleration of sugar accumulation and that will not change in the short term, is global warming. The significant temperature increase of the last decades caused earlier onset of all the phenological phases, shorter phenological intervals and increased soluble solids concentrations at harvest in Bordeaux area (Jones and Davis, 2000). Duchêne and Schneider (2005) described similar results in Alsace, where from 1972 to 2002 was observed a 2% vol rise in the potential alcohol of Riesling grapes. It was also demonstrated that earlier ripening observed in Australia vineyards was mainly due to the increase of temperature (Petrie and Sadras, 2008). Elevated temperatures disassembled sugar and anthocyanin ripening, in fact while sugar accumulation is accelerated, the onset of anthocyanin biosynthesis was delayed and lower concentrations were reported at harvest (Sadras and Moran, 2012). Furthermore the accumulation of these compounds can even be inhibited if temperature rise over 30-35 °C (Mori at al. 2007, Movahed et al., 2016). High temperature also changed sensory attributes of berries and wines, and the responses are strongly linked to the variety (Sadras et al., 2013a,b). Therefore hot climate often seems to cause a decoupling between technological and phenolic maturity which brings grapegrowers to choose one of the two following options: 1) harvest at technological maturity to reach the right balance between alcohol content and acidity, with the risk of poor color and unpleasant astringency; 2) harvest when phenolic compounds has reached the desired characteristics to obtain a good extraction of anthocyanins and to avoid the risk of astringent tannins, but reaching too high alcohol and too low acidity levels.

Among several approaches adopted by researchers and viticulturists to counteract the negative effect of climate change, the first one was to control sugar accumulation by limiting the source of photosynthates at the beginning of ripening. The removal of apical leaves around veraison, which at that time strongly contributes to berries carbohydrate supply (Poni et al., 1994), was successfully used to slow down sugar accumulation. In fact, the mechanical

defoliation apical to the cluster zone lowered the sugar concentration at harvest without any detrimental effect on anthocyanins and total phenolics on Sangiovese berry (Palliotti et al., 2013a). Similar results were found in a study conducted on Sangiovese potted vines by Poni et al. (2013), in which postveraison hand defoliation caused a temporary reduction of sugar accumulation after the treatment, and lead to lower soluble solids concentration at harvest. Photosynthesis limitation of the upper part of the canopy with the antitranspirant di-1-*p*-menthene, showed appreciable results in decreasing sugar accumulation, but variable effects on anthocyanin concentration were reported (Palliotti et al., 2013b; Gatti et al., 2016). Referring to similar physiological assumption, severe shoot trimming performed around or after veraison in order to eliminate the youngest part of the canopy, reduced sugar concentration at harvest without changes on the concentration of anthocyanins (Herrera et al., 2015; Filippetti et al., 2015; Bondada et al., 2016).

A different approach to reduce the decoupling between phenolic and technological maturity was based on the bud load increase, as reported by Zheng et al. (2017). Through the application of minimal pruning on Tempranillo vines, the authors showed a delay in sugar of more than two weeks and an increase of anthocyanin concentration via the reduction of berry size.

In the last years, innovative studies on the delay of winter spur pruning showed good perspectives in mitigating the negative effects of the global warming. Early work proved that this technique was able to reduce damages of spring frost, since in unpruned shoots, apical buds development inhibits the burst of basal bud, whose shoots grew after spur-pruning (Howell and Wolpert, 1978). Recent studies reported that late winter pruning on Cabernet Sauvignon vines determined a delay of 4-5 days in the main phenological events and lowered soluble solids concentration of about 1 °Brix at harvest (Martin and Dunn, 2000). Moreover Friend and Trought (2007) described that later was performed the winter pruning, greater was the delay of sugar ripening of Merlot berries. Post-budburst spur pruning on Sangiovese vines caused the reduction of sugar concentration and the increase of phenolic compounds, but different responses were reported in relation to the phenological stage in which vines were pruned (Frioni et al., 2016).

Forcing vine re-growth in late-spring or early-summer by leaving 6 nodes to the developing shoots and removing from them each leaf, lateral and developing cluster, all the phenological phases of the new vegetative and productive cycle was dramatically delayed and grape of the re-growth vines ripened in cooler condition, showing at harvest lower sugar levels and higher concentrations of anthocyanins and tannins (Gu et al., 2012).

In the present experiment, winter spur pruning was delayed after budburst to retard the phenological phases and to reduce sugar concentration at harvest with the aim to achieve good balance between technological and phenolic ripening in Merlot grapes. In order to describe the effects of this technique on the phenolic maturity, this work provided detailed information on the characteristics of anthocyanins, skin and seed tannins, extracted both with strong solvents and hydroalcoholic solutions.

2.2. MATERIALS AND METHODS

2.2.1. Plant material and experimental layout

The study was conducted in the 2014 and 2015 season in a 12-year-old irrigated vineyard of *Vitis vinifera* L. cv. Merlot (clone R3 grafted onto SO4 rootstock), located in Valsamoggia, Bologna, Italy (latitude 44°28'N; longitude 11°07'E). Vines were spaced 1 m within the row and 3 m between the rows and were trained to a vertically shoot positioned (VSP) spur pruned cordon. Each vine was winter-pruned leaving 5 nodes of two buds (10 buds per vine). During the growing season were left 10 shoots per vine by shoot thinning. Shoots were trimmed twice, in June and July, and pest management was carried out according to Emilia-Romagna region standard practices.

Four randomized blocks were created in two adjacent rows and five vines per treatment were assigned in each block (total 60 vines). Spur pruning treatments were based on the phenological phase: pruning carried out during dormancy (CK); pruning carried out when both control vines and shoots developed on the apical part of unpruned canes were at the stage BBCH53 – inflorescence clearly visible (DA); pruning carried out when control vines and shoots developed on the apical part of unpruned canes were at the stage BBCH57 – inflorescences fully developed, flowers separating (DB). In 2014 and 2015 winter pruning was carried out respectively on 28 and 26 January for CK, 17 and 29 April for DA, 30 April and 11 May for DB.

2.2.2. Leaf area measurement and yield components

After harvest 20 representative shoots per treatment were removed from plants within the blocks and the area of main and lateral leaves were measured with a LI-3100 A (Li-cor, Lincoln, Nebraska USA). The leaf area of each vine was calculated multiplying the average leaf area of the 20 shoots by the number of shoots per vine.

At harvest (7 October 2014 and 16 September 2015) the yield of the tagged plants was weighted and the number of cluster counted. The incidence of cluster rot was assessed by

estimating the surface with symptoms and the cluster compactness was estimated using International Organization of Vine and Wine (OIV) code 204 (OIV, 1983).

2.2.3. Berry sampling

In both years, total soluble solids, pH and titratable acidity were analyzed every ten days from veraison, by sampling 50 berries from the five vines of each block (200 berries per treatment), while at harvest, samples of 25 berries were sampled from each tagged plant (500 berries per treatment). In the same date, samples of 80 berries were taken from the five vines of each block (320 berries per treatment) and then divided into three subsamples which were used to determine: a) total anthocyanins (20 berries); b) total tannins (20 berries); c) extractable anthocyanins and tannins (40 berries). The berries for the determination of must biochemical parameters were immediately processed, while the other samples were frozen and stored at –20°C.

2.2.4. Biochemical analysis of musts

Must samples were analyzed to determine the soluble solids concentration using a temperature-compensating Maselli R50 refractometer (Maselli Misure, Parma, Italy). Must pH and titratable acidity were measured using a Crison Titrator (Crison Instruments, Barcelona, Spain).

2.2.5. Extraction of anthocyanins and tannins using a model hydroalcoholic solution

Whole (not ground) skins and seeds from 40 berries were soaked separately and shaken daily, in different tubes containing 80 mL of a hydroalcoholic solution for 15 days at 28°C. The duration and the temperature imposed to the extractions were chosen to simulate the winemaking conditions and so to determine the concentration of extractable anthocyanins and tannins. The hydroalcoholic solution comprised 6 g/L tartaric acid, 40 mL/L 1 N NaOH, 100 mg/L potassium metabisulphite and a proportion of ethanol that raised from 0 to 13% in the first 12 days of extraction. This concentration was reached by adding every two days 2 mL of ethanol absolute (12 mL total) to simulate alcoholic fermentation (Allegro et al., 2016). The extracts were centrifuged (15 minutes, 13000 rpm) and an aliquots of the supernatant (400 µL) were dried under vacuum at 20°C. Pellets were stored at -20°C.

2.2.6. Exhaustive extraction of anthocyanins and tannins

Total anthocyanins were extracted from the skins of 20 berries by soaking the peeled skins in 100 mL methanol for 24 h, then storing the extracts at –20°C (Mattivi et al., 2006). Total tannins were extracted from the skins and seeds of 20 berries grounded separately to a fine powder, before extracting 1 mg of the sample in 1 mL 70% (v/v) acetone in water, for 24 h in dark room (Downey, et al., 2003). Skin and seed extracts were then centrifuged (15 minutes,

13000 rpm) and two 400 µL aliquots of the supernatant were dried under vacuum at 20°C. Pellets were stored at -20°C.

2.2.7. Anthocyanin determinations

Total and extractable anthocyanins were separated by HPLC as described by Mattivi et al. (2006), using a Waters 1525 instrument equipped with a diode array detector (DAD) and a reversed-phase column (RP18 250 x 4 mm, 5 µm) with a pre-column (Phenomenex, Castel Maggiore, BO, Italy). The concentration was determined by measuring absorbance at 520 nm. A calibration curve was established using a malvidin-3-glucoside standard (Sigma-Aldrich, ST. Louis, MO, USA).

2.2.8. Tannin determinations

Total and extractable skin and seed tannins were measured by HPLC with the equipment described above. The tannin content was determined by acid-catalyzed cleavage in the presence of excess phloroglucinol (Kennedy and Jones, 2001). The separation of monomer subunits and cleaved proanthocyanidins was carried out following the two different HPLC methods proposed by Downey et al. (2003). The concentrations of free monomers and hydrolyzed terminal subunits were determined from standard curves prepared with commercial standards of catechin, epicatechin, epicatechin-gallate and epigallocatechin (Extrasynthese, Genay, France) by measuring absorbance at 280 nm (Downey et al., 2003). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy and Jones, 2001).

The seed tannin content was assigned to free monomers, terminal subunits and extension subunits, whereas the skin tannin content was assigned to terminal subunits and extension subunits. The mean degree of polymerization (mDP) was calculated by summing terminal and extension subunits and dividing by terminal subunits (Downey et al., 2003).

The anthocyanin and tannin concentrations were expressed as mg per kg of berries (mg kg^{-1}), in order to take into account variations in berry weight that modify the concentration of these compounds during ripening.

2.2.9. Statistical analysis

A combined analysis of variance over years was performed using the mixed procedure available in SAS v9.0 (SAS Institute, Inc., Cary, NC, USA). Treatment comparisons were analyzed using the Tukey test with a cut off at $P \leq 0.05$.

2.3 RESULTS AND DISCUSSION

The performances of the vines spur pruned after budburst were compared with those of the vines pruned during dormancy, in order to verify the effectiveness of this technique in delaying phenological phases and in coupling technological and phenolic maturity. Phenolic maturity was deeply investigated by the analysis of total and extractable anthocyanins and tannins.

2.3.1. Enviromental conditions and phenology

Climatic conditions of the two growing seasons appeared similar from April to June, while afterwards they strongly differed: from July to September, the average air temperature was 2.5 °C lower in 2014 than in 2015 and precipitations were 262.9 mm in 2014 and only 71.2 mm in 2015 (Figure 1).

In Table 1 are reported the dates of budburst, bloom and veraison of all the treatments. The delay of DA and DB phenological phases was large at budburst but decreased at bloom, caused by the reduction of the interval budburst-bloom in the delayed pruned vines. On the contrary, the number of days between bloom and veraison was almost constant in all the treatments and as a consequence the delay of DA and DB veraison was still noticeable, as also found by Martin and Dunn (2000). Bloom-veraison interval appears more stable than budburst-bloom and our results are in concordance with those of a long-term study conducted on the phenology of many *Vitis vinifera* L. varieties (Tomasi et al., 2011), in which is reported that over a period of 46 years, this interval showed the lowest variation among all the phenological intervals.

Figure 1. Average air temperature and precipitations from April to September in the years 2014 (A) and 2015 (B). Bars indicate the mm of rainfall; lines indicate temperatures.

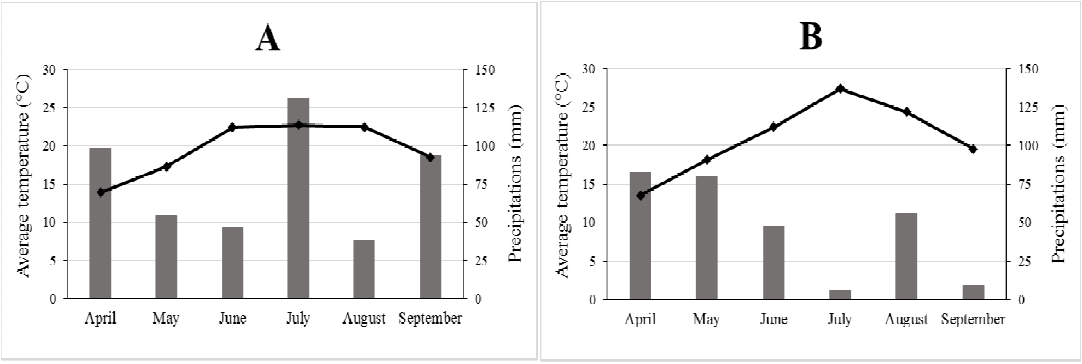


Table 1. Phenological phases and intervals of the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB), in the years 2014 and 2015.

	2014			2015		
	CK	**DA**	**DB**	**CK**	**DA**	**DB**
Budburst	29 Mar	27 Apr	10 May	14 Apr	8 May	21 May
Bloom	30 May	12 Jun	22 Jun	1 Jun	10 Jun	23 Jun
Veraison	2 Aug	13 Aug	20 Aug	1 Aug	6 Aug	13 Aug
Budburst – bloom (n° of days)	62	46	43	48	34	33
Bloom – veraison (n° of days)	64	62	59	61	56	51

2.3.2. Vegetative behavior, yield components and grape composition

Both main leaf area and lateral leaf area decreased delaying the pruning, in fact vines pruned later in the season (DB) showed the lowest values (Table 2). The reduction of main leaf area is due to the smaller surface of the single leaf, while lateral leaf area was negatively affected by the smaller number of leaves in 2014 and by the smaller surface of the single lateral leaf in 2015 (data not reported).

Table 2. Vegetative parameters, rot incidence and cluster compactness of the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB). Average data of the years 2014 and 2015.

Parameter	**CK**	**DA**	**DB**	**Year effect**	**Year x treatment interaction**
Main LA (m^2 / vine)	3.57 a	3.06 ab	2.45 b	***	ns
Lateral LA (m^2 / vine)	2.30 a	2.01 ab	1.14 b	ns	ns
Total LA (m^2 / vine)	5.87 a	5.07 ab	3.59 b	*	ns
Rot incidence (%)	0.13	0.08	1.03	***	ns
Cluster compactness (1-9)	4.85	4.58	5.38	ns	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

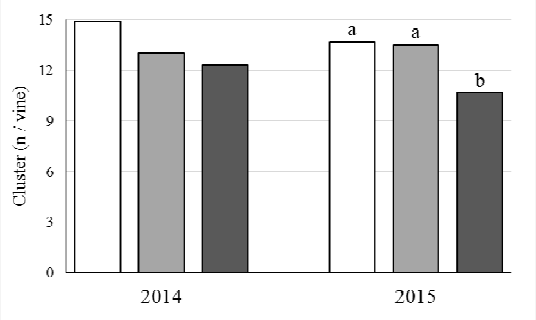
No difference in cluster rot was reported but the effect of the climatic conditions of the two years influenced this parameter: the heavy rain occurred in September 2014 favored rot incidence, while in 2015 no infection was detected. Cluster compactness was not affected by delayed spur pruning.

Yield was reduced of about 45% and 70% in DA and DB vines respectively, mainly due to the reduction of cluster weight and in less extent to their number (Table 3). Also the weights of DA and DB berries was lower than that of CK, as reported in a similar study conducted on cv. Sangiovese (Frioni et al., 2016). Cluster and berry weights resulted higher in 2014 than in 2015, probably because of the greater water availability of the first season.

In the second year of the trial, it was noted the decrease of cluster number in DB treatment (Figure 2), which suggested a carry-over effect on bud fertility linked to the loss of storage reserves in the previous year (Candolfi-Vasconcelos and Koblet, 1990). We may assume that in our study, the removal of the developing shoots with late pruning, could have depauperated the carbohydrate and nitrogen reserves, being less available for flower induction in shoots growing from the basal buds.

Similarly, Frioni et al. (2016) found a drop of bud fertility in the second year of trial, on vines submitted to the delay pruning treatment at the beginning of May (BBCH55).

Figure 2. Number of clusters per vine in 2014 and 2015, of CK ( ), DA ( ) and DB ( ).



Technological ripening was retarded by delayed pruning, as found also in previous studies (Martin and Dunn, 2000; Friend and Trought, 2007; Frioni et al., 2016). The concentrations of soluble solids of DA and DB grapes at the same data were respectively 0.8 and 1.5 °Brix lower than CK, and pH resembled the same trend of sugars. On the contrary, titratable acidity of DA and DB grapes was respectively 1.1 and 2.3 g/L higher than that of CK. In the two

Table 3. Yield components, grape composition and leaf-to-fruit ratio of the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB).

Parameter	2014			2015			Average 2014-2005			Year effect	Year x treatment interaction
	CK	DA	DB	CK	DA	DB	CK	DA	DB		
Yield/vine (kg)	3.22 a	1.95 b	1.11 c	2.19 a	1.04 b	0.49 c	2.70 a	1.49 b	0.80 c	***	ns
Cluster (n / vine)	14.9	13.0	12.3	13.7 a	13.5 a	10.7 b	14.3 a	13.2 ab	11.5 b	ns	*
Cluster weight (g)	197.1 a	135.7 b	86.4 c	160.4 a	75.0 b	46.6 c	178.7 a	105.3 ab	66.5 b	***	ns
Berry weight (g)	2.47 a	2.22 b	1.83 c	1.96 a	1.54 b	1.26 c	2.21 a	1.88 b	1.54 c	***	ns
Soluble solids (°Brix)	22.2 a	21.6 b	21.3 b	25.6 a	24.5 b	23.3 c	23.8 a	23.0 b	22.3 c	***	ns
pH	3.51 a	3.42 b	3.36 b	3.77 a	3.70 ab	3.60 b	3.64 a	3.56 b	3.48 c	**	ns
Titratable acidity (g/L)	5.92 b	6.74 ab	7.70 a	4.99 c	6.32 b	8.04 a	5.45 c	6.53 b	7.87 a	***	ns
Leaf-to-fruit ratio (m^2 / kg)	1.93 b	2.68 ab	3.94 a	3.14 c	6.06 b	9.30 a	2.53 c	4.37 b	6.62 a	***	ns

Different letters within a row indicate significant differences in each of the two years and in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

years of study, ripening was affected by the different climatic conditions, since an overall increase of sugar concentration and pH, associated with lower levels of titratable acidity, were observed in the hot and dry 2015.

Leaf-to-fruit ratio increased in the delay pruned vines but, since also the value of CK was above the optimal for an adequate ripening (~ 1.5 m^2 /kg of grape), no incremental effect on sugar concentration of DA and DB berries should have been induced compared to CK (Kliewer and Dokoozlian, 2005).

It could be hypothesized that three main factors may have contributed to the lower sugar concentration of delay pruned grapes: 1) the delay in the onset of veraison shortened the interval veraison-harvest reducing the time for sugar ripening; 2) the prolonged vegetative competition due to the late development of shoot could have interfered with the accumulation of soluble solids (Frioni et al., 2016); 3) the lost of storage reserves, due to the removal of the developing shoots with late pruning, could have limited the contribution to the berry ripening of the carbohydrates from the permanent organs of the plants.

2.3.3. Phenolic maturity

No difference between the concentrations of total anthocyanins, expressed as mg/kg of berries, was observed in the average values of the two years of study (Table 4), but this parameter resulted higher in DA and DB berries in 2014, while in the following year was higher in CK (Figure 3). Total glicosilate and acetate anthocyanins behaved similarly to total anthocyanins, but total cumarate were lowered by the delay of pruning. Despite the retard in the onset of anthocyanin accumulation was reported to be a cause of the reduction of their content at harvest (Sadras and Moran, 2012), in our Merlot berries of 2014 the delay of veraison was associated to higher concentration at harvest. The increase of total anthocyanin concentration after the delay of ripening was due not only to the growth of the skin-to-pulp ratio, but also to the rise of anthocyanins synthesis that comes to light looking at the results expressed in mg per gram of skin (Figure 4). Higher leaf-to-fruit ratio is reported to stimulate the accumulation of both sugars and anthocyanins (Pastore et al., 2011), but in our Merlot berries of 2014, the rise of anthocyanins was associated to lower level of sugars. These particular results, also found by Frioni et al. (2016) in a similar study, showed the ability of the delayed pruning to dissect the accumulation of primary (sugars) and secondary (anthocyanins) metabolites favoring unexpectedly the latter. Further investigations that takes into account the factors involved in the accumulation of sugars and in the balance between the synthesis and degradation of anthocyanins during ripening, also from the molecular point of view, are needed to clarify the mechanism that underlie these particular behavior.

The opposite results registered in 2015 when DA and DB berries showed lower concentration of anthocyanins at harvest could be due to the diverse climatic condition that were recorded around the veraison of each treatments. In fact, full veraison of CK berries correspond to a temporary decrease of the maximum temperatures (29.9°C), which determined optimal conditions for the biosynthesis of anthocyanins (Mori at al. 2007, Movahed et al., 2016). On the contrary, the return of high temperatures in the days in which took place the veraison of DA berries (36.1°C) and DB berries, (34.0 °C), could have depressed the biosynthesis of anthocyanins, as reported in a recent study on Cabernet Sauvignon (Lecourieux et al., 2017).

Table 4. Total and extractable anthocyanins, extractability of anthocyanins and skin-to-pulp ratio of the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB). Average data of the years 2014 and 2015.

Parameter	**CK**	**DA**	**DB**	**Year effect**	**Year x treatment interaction**
Total anthocyanins (mg/kg of berries)	1459	1459	1535	***	**
Glicolsilate	1058	1100	1182	***	**
Acetate	227	211	224	***	**
Cumarate	174 a	148 b	129 b	***	***
Total anthocyanins (mg / g of skin)	10.9 a	9.9 b	10.6 a	***	***
Extractable anthocyanins (mg/kg of berries)	512	509	543	***	**
Glicolsilate	388	403	438	***	*
Acetate	81	73	77	**	ns
Cumarate	42 a	33 b	28 c	ns	***
Extractability (%)	35.8	35.1	35.5	*	ns
Skin / pulp ratio (g / g)	0.172 b	0.200 a	0.199 a	**	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

Figure 3. Total anthocyanins (mg / kg of berries) in 2014 and 2015, of CK ( ), DA ( ) and DB ( ).

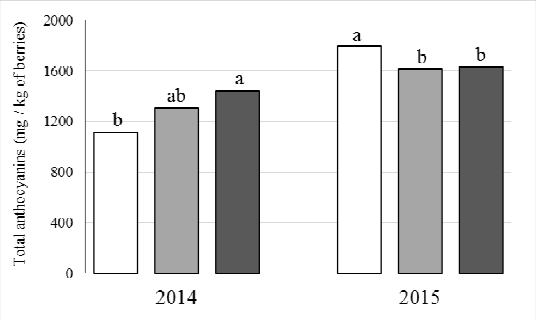
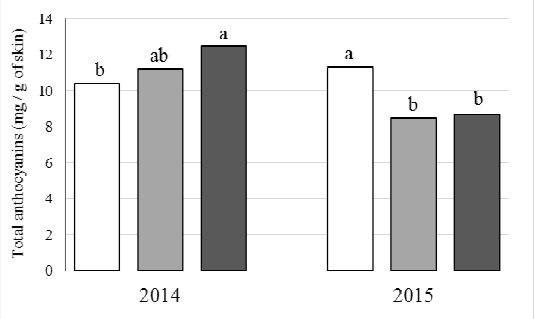
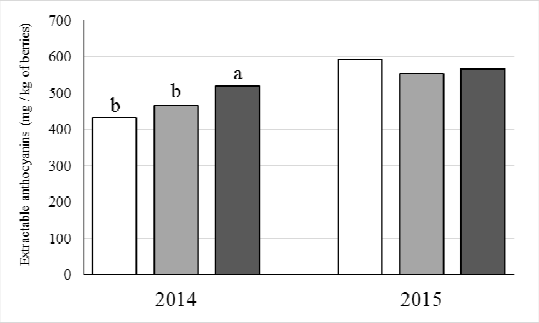


Figure 4. Total anthocyanins (mg / g of skin) in 2014 and 2015, of CK ( ), DA ( ) and DB ( ).



In 2014, extractable anthocyanins of DB grapes resulted higher than those of CK and DA, while in 2015 no significant difference was detected (Figure 5). The extractability of anthocyanins, calculated as the percentage of the extractable portion relative to the total amount, did not show any difference in the average of the two years of trial, indicating that the degradation of skin cell structure which takes place during ripening and leads to higher extraction (Río Segade et al., 2008) was not affected by the delay of pruning. This result is important from a practical point of view, since the extractable anthocyanins represent the portion that can be obtained in wine. Given that and above all the considerations about the synthesis and the extractability of these phenolic compound, appears that delayed pruning did not determine detrimental effects on the color extractability obtained from the maceration of these grapes.

Figure 5. Extractable anthocyanins (mg / kg of berries) in 2014 and 2015, of CK (▭), DA (▭) and DB (▭).



The percentage of each anthocyanin was calculated to study the composition of the total amount and the extractable portion (Table 5). After both extractions, the percentages of di-substituted anthocyanins, cyanidin and peonidin, rose with the delay of pruning, while the percentage of malvidin, a tri-substituted form, decreased. Similar results were found when yield constrain was achieved by cluster thinning or pre-bloom defoliation (Guidoni et al, 2008; Pastore et al., 2013), supporting the hypothesis that the reduction of productivity caused by the delay of ripening could influences anthocyanins with mechanism similar to those induced by cluster thinning, which increased source / sink ratio.

Considering the results of total skin tannins (Table 6), it appears that the highest concentration was found when pruning was delayed at BBCH-57 (DB). The higher skin-to-pulp ratio certainly contributed to this increase, but also the different state of ripening could have interfered in the fate of total skin tannins. Downey et al. (2003) described the decrease of these compounds from veraison to harvest, indicating that unripen grapes showed higher level of skin tannins. Since the interval between veraison and harvest was 15 days shorter for DB grapes than for CK, and the sugar level was 1.5 °Brix lower in DB than CK, it is possible that the concentration of skin tannins resulted higher in DB berries, also for the incomplete ripening of these grapes. Both terminal and extension sub-units resulted higher in DB berries than CK and DA and, since extension sub-units increased proportionally more than the terminal, also the mDP of DB skin tannins resulted higher than those of the other treatments. The overall increase of total skin tannins observed in 2015 is linked with the higher skin-to-pulp ratios found in this year respect those of the previous (Figure 6).

Table 5. Composition (%) of total and extractable anthocyanins in the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB). Average data of the years 2014 and 2015.

Parameter	CK	DA	DB	Year effect	Year x treatment interaction
Total anthocyanins					
Delphinidin	21.3	23.5	23.5	ns	ns
Cyanidin	7.3 c	11.6 b	15.0 a	ns	ns
Petunidin	15.1	14.8	13.9	ns	ns
Peonidin	13.6 b	17.4 a	19.3 a	ns	ns
Malvidin	42.7 a	32.8 b	28.3 c	ns	ns
Extractable anthocyanins					ns
Delphinidin	14.4	16.5	16.9	**	ns
Cyanidin	5.4 c	9.1 b	12.7 a	ns	ns
Petunidin	14.1	14.4	13.6	ns	ns
Peonidin	13.1 c	17.7 b	20.3 a	ns	ns
Malvidin	53.0 a	42.3 b	36.5 c	**	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

The concentrations of extractable skin tannins resembled those of total, with the highest concentration found in DB treatment (Figure 7). As a consequence, no change of skin tannin extractability was found after delayed pruning. Since DB terminal and extension subunits increased proportionally, no difference in the extractable skin tannin mDP was observed. As found by Allegro et al. (2016), the mDPs of the extractable portion were lower than those of the total, due to the weaker extraction efficiency of the hydroalcoholic solutions compared to that of acetone (Downey and Hanlin, 2010).

Table 6. Concentration and mean degree of polymerization (mDP) of total and extractable skin tannins (mg / kg of berries), and extractability of skin tannins in the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB): Average data of the years 2014 and 2015.

Parameter	CK	DA	DB	Year effect	Year x treatment interaction
Total skin tannins	953 b	986 b	1244 a	**	ns
Terminal sub-units	114	111	130	**	ns
Extension sub-units	839 b	875 b	1114 a	**	ns
mDP	8.6	8.9	9.6	ns	ns
Extractable skin tannins	459 b	448 b	573 a	ns	ns
Terminal sub-units	78 b	73 b	92 a	ns	ns
Extension sub-units	381 b	375 b	481 a	ns	ns
mDP	5.9	6.2	6.3	ns	ns
Extractability (%)	49.8	46.3	48.0	**	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

Figure 6. Total skin tannins (mg / kg of berries) in 2014 and 2015, of CK ( ), DA( ) and DB ( ).

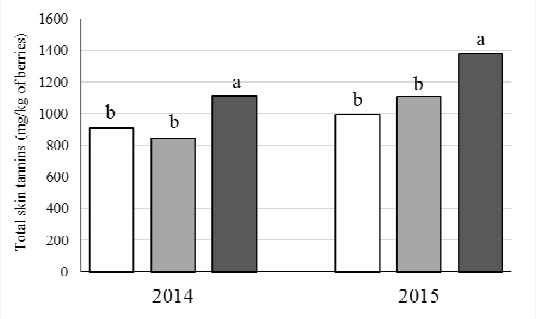
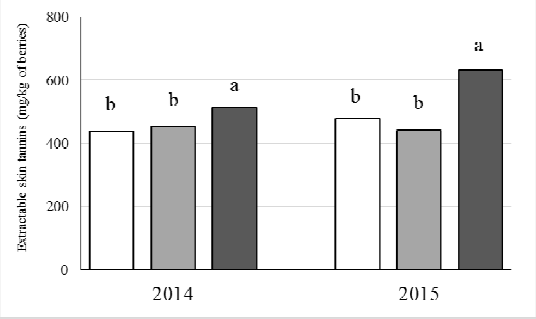


Figure 7. Extractable skin tannins (mg / kg of berries) in 2014 and 2015, of CK ( ), DA ( ) and DB ( ).



Both total and extractable skin tannins were predominantly represented by epicatechin (Table 7), as found in other studies conducted on Merlot berries (Cohen et al., 2012; Yu et al., 2016). The percentage of epicatechin rose in the delayed pruning treatments, and it was counterbalanced by the decrease of epigallocatechin. No change between treatments were found in the composition of the extractable portion. Despite the composition of total skin tannins remained stable over the two years of study, that of the extractable portion varied, since in 2015 the percentages of catechin and epicatechin resulted higher than the previous year, counterbalanced by the lower percentages of epigallocatechin and epicatechin-gallate.

Table 7. Composition (%) of total and extractable skin tannins in the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB). Average data of the years 2014 and 2015.

Parameter	**CK**	**DA**	**DB**	**Year effect**	**Year x treatment interaction**
Total skin tannins					
Catechin	12.3	11.8	11.5	ns	ns
Epicatechin	69.3 b	75.6 a	75.4 a	ns	ns
Epigallocatechin	15.5 a	9.4 b	9.6 b	ns	ns
Epicatechin-gallate	2.9	3.2	3.5	ns	ns
Extractable skin tannins					
Catechin	16.3	15.7	15.9	**	ns
Epicatechin	64.9	61.2	64.9	**	ns
Epigallocatechin	16.5	20.8	16.3	**	ns
Epicatechin-gallate	2.3	2.3	2.8	**	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

Different behaviors of total seed tannins concentration were observed between the two years of study: in 2014 both delayed pruning treatments showed higher values than CK, mainly due to the increase of the monomeric sub-units (Table 8), while in 2015 no increase was detected for DA berries and a rise of about 50% was reported for DB (Figure 8). In the latter season, all the tannin fractions (monomer, terminal and extension sub-units) of each treatment varied in the same manner. The fate of total seed tannins appeared well correlated with the

modifications of the seed-to-pulp ratio, indicating that the reported increases were due to reduced berry size rather than variations occurred in the synthesis of tannins. The changes of the extractable seed tannin concentrations resembled those of total, but appeared of larger magnitude, in particular for DB treatment that showed increases of 28% and 63%, in 2014 and 2015 respectively (Figure 9).

Table 8. Concentration and mean degree of polymerization (mDP) of total and extractable seed tannins (mg / kg of berries), extractability of seed tannin and see-to-pulp ratio in the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB). Average data of the years 2014 and 2015.

Parameter	**CK**	**DA**	**DB**	**Year effect**	**Year x treatment interaction**
Total seed tannins	1769 b	1835 b	2274 a	***	**
Monomer sub-units	233 b	268 b	378 a	***	*
Terminal sub-units	252 b	267 b	333 a	***	*
Extension sub-units	1284 b	1300 b	1562 a	*	***
mDP	6.2	6.0	5.9	**	ns
Extractable seed tannins	946 b	1018 b	1401 a	***	***
Monomer sub-units	212 c	255 c	349 a	***	***
Terminal sub-units	167 b	169 b	222 a	**	***
Extension sub-units	568 b	594 b	829 a	***	***
mDP	4.4	4.5	4.7	*	ns
Extractability (%)	53.7 b	55.8 ab	61.5 a	*	ns
Seed / pulp ratio (g / g)	0.056 b	0.063 ab	0.072 a	**	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

Figure 8. Total seed tannins (mg / kg of berries) in 2014 and 2015, of CK ( ), DA( ) and DB ( ).

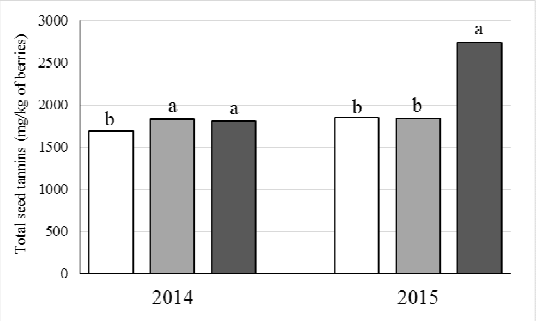
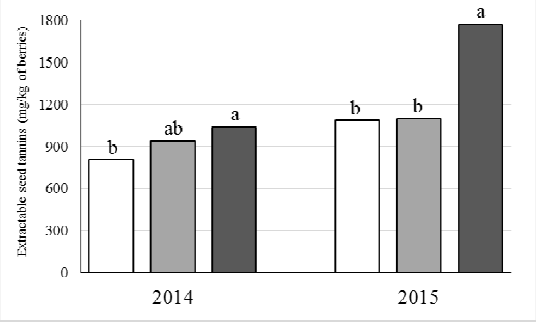


Figure 9. Extractable seed tannins (mg / kg of berries) in 2014 and 2015, of CK ( ), DA ( ) and DB ( ).



These results were due to the step increase of seed tannin extractability that was found delaying the pruning. The mDPs of total seed tannins resulted higher than those of the extractable portion, as also reported by Bautista-Ortín et al. (2012), but no difference was found between treatments.

Considering the composition of total seed tannins, epicatechin resulted the most representative flavan-3-ol, as reported also by Cohen et al. (2012) in a study on Merlot berries, and in our trial was not affected by treatments, while the percentage of catechin rose with the delay of pruning, counterbalanced by the decrease of epicatechin-gallate (Table 9). Conversely, no difference was found between the percentages of the tannins extracted with the wine-like solution, but, since higher was the level of the extractable seed tannins in DB treatment, higher is the concentration of each flavan-3-ol. Between them, epicatechin-gallate was considered responsible of rougher sensation in wine (Vidal et al., 2003) and these findings allow to postulate the risk of increasing undesired sensations when the pruning was delayed at the phenological phase BBCH57 (DB).

Table 9. Composition (%) of total and extractable seed tannins in the following treatments: control (CK), delayed pruning at BBCH-53 (DA) and delayed pruning at BBCH-57 (DB). Average data of the years 2014 and 2015.

Parameter	CK	DA	DB	Year effect	Year x treatment interaction
Total seed tannins					
Catechin	25.2 b	26.8 ab	27.9 a	***	***
Epicatechin	54.2	54.9	54.6	ns	ns
Epicatechin-gallate	20.6 a	18.3 ab	17.6 b	***	ns
Extractable seed tannins					
Catechin	32.1	34.0	33.3	***	***
Epicatechin	56.4	55.0	55.7	***	***
Epicatechin-gallate	11.5	11.0	11.0	***	ns

Different letters within a row indicate significant differences in the average values of the two years.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

2.4. CONCLUSIONS

The present work has provided detailed information about the effects of two consecutive years of delayed pruning after budburst, carried out at the stages BBCH53 and BBCH57, on the vegetative and productive behavior of vines and on the technological and phenolic maturity of the grapes. This new technique retarded all the phenological phases, reduced yield and lowered sugar concentration at harvest. The latter result was one of the main goal of our study. The effects were more evident when vines were pruned later (BBCH57), but in this case the over year recurrence of the same technique lead to reduce yield under acceptable level (0.49 kg/vine in 2015) due to the carry-over effect that influenced negatively bud fertility. The analysis of the extractable portion of the phenolic compounds have important implications from a practical point of view, and two main results can be identified: 1) extractable anthocyanins were not reduced by the delay of pruning compared to control; 2) skin and seed tannins resulted higher when pruning was delayed at BBCH57 (DB), while no effect was observed in DA treatments. Considering the results of a previous study conducted on Shiraz (Ristic et al., 2010), in which the quality of wine was positively correlated with higher concentration of anthocyanins and skin tannins but negatively with those of seed, we can not exclude that the increase of extractable seed tannins found in DB berries, could determines negative effects on the sensory attributes.

Results of the presented study suggest that in our conditions it is not advisable to delay the pruning at BBCH57: the yield drop is too drastic and doubts arise after the increase of extractable seed tannins. On the other hand, delaying the pruning at BBCH53 determined a yield loss of about 50%, which can be acceptable for the production of high quality wines, associated with a decrease in the potential alcohol and no change in the phenolic maturity. These results support the applicability of this technique performed at BBCH53, in case viticulturists desire to slow down sugar accumulation without detrimental effects on phenolic maturity.

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3. PRACTICAL IMPLICATIONS OF FLAVONOID AND CELL WALL MATERIAL CHANGES ON PHENOLIC MATURITY IN *Vitis vinifera* L. CV. MERLOT

3.1 INTRODUCTION

The accumulation of berry flavonoids takes place in different phenological phases, as flavan-3-ol monomers and proanthocyanidins, commonly called tannins, are synthesized in the skins and seeds since the early stages of berry development, while anthocyanins appear on skin only at veraison. Genotype, temperature, sunlight exposure and water availability are only few of the different factors affecting the evolution of berry flavonoids during ripening (Kennedy et al., 2002; Pastore et al., 2013; Filippetti et al., 2015) and since many of them are season dependent, the concentration and composition of these compounds can vary strongly between years.

However, not only the concentration but also the extractability of anthocyanins and tannins from the tissues in which they are stored is a key determinant for the phenolic content of wine. Many researches were carried out to understand the changes of extractability during ripening and a number of contrasting results were found. The extractability of Tempranillo and Sangiovese anthocyanins increased with the progression of maturity (Hernández-Hierro et al., 2012; Allegro et al., 2016) while no difference was found for Shiraz grape (Fournand et al., 2006). Researchers also found opposite results concerning the extractability of seed tannins: Pinot Noir seed tannins become easier to extract approaching to the harvest (Pastor Del Rio and Kennedy, 2006), whereas Monastrell seed tannins, when extracted with a solution containing 12.5 % of ethanol, decreased during ripening (Bautista-Ortín et al., 2012).

In the last years, the interest of different researchers has been focused on the characteristics and properties of cell wall material (CWM) of berry skin and flesh, as the interactions between this material and berry flavonoids may have important implications on the concentration, composition and extractability of anthocyanins and tannins in the resulting wine (Bautista-Ortín, et al., 2016a).

The cell wall is mainly composed by lignin and structural polysaccharides (pectin and cellulose), and by lower amounts of proteins and phenols integrated in the structure (Bindon and Smith, 2013). Skin and flesh CWM composition differs among varieties but the quantity is much higher in the skin than in the flesh (Ortega-Regules et al., 2008a).

Anthocyanins and proanthocyanidins can be bound by CWM via hydrogen bonds and hydrophobic interaction (Le Bourvellec et al., 2004), and as these associations precipitate during vinification, they could reduce the phenolic content of wine (Bindon et al., 2010a;

Guerrero et al., 2013). Differences can be observed as regard variety. It was demonstrated that Monastrell pomace CWM had higher ability to remove proanthocyanidins compared to that of Cabernet Sauvignon and Syrah (Bautista-Ortín et al., 2015).

Moreover, interactions of flavonoids with cell walls may change the composition of the remaining flavonoids. Previous results have shown that Shiraz CWM presented a preference for the galloylated forms of seed proanthocyanidins (Bindon et al., 2010a), while Monastrell skin CWM didn't show this option (Bautista-Ortín et al., 2014). The same last authors reported that the interactions between grape CWM and proanthocyanidins are favored with higher molecular mass proanthocyanidins, which are considered responsible of wine astringency (Vidal et al., 2003). Furthermore CWM affinity for proanthocyanidins changes during ripening. Skin CWM of both Cabernet Sauvignon and Monastrell cvs. showed an increasing affinity for proanthocyanidins as ripening progressed, while flesh CWM of the first variety didn't display any changes in the interactions with the progression of maturity (Bindon et al., 2012; Castro-López et al., 2016).

Viticulturists and winemakers tend to associate the best grape phenolic maturity with high color and low astringency in must and wine, so the last part of ripening is a crucial period for them. Since there is no clear knowledge of the role and relationships between different berry compounds involved in the so-called phenolic maturity, the choice of the correct harvest date for obtaining the desired wine becomes difficult. The aim of this work is to enhance the understanding of the mechanisms driving phenolic maturity, studying the evolution of berry flavonoids and the properties of CWM in late ripening of Merlot grape.

3.2 MATERIALS AND METHODS

3.2.1. Plant material and yield components

The study was conducted in the 2014 and 2015 season in a 12-year-old irrigated vineyard of *Vitis vinifera* L. cv. Merlot (clone R3 grafted onto SO4 rootstock), located in Valsamoggia, Bologna, Italy (latitude 44°28'N; longitude 11°07'E). Vines were spaced 1 m within the row and 3 m between the rows and were trained to a vertically shoot positioned (VSP) spur pruned cordon. Each vine was winter-pruned leaving 5 nodes of two buds (10 buds per vine). During the growing season, the number of shoots was kept uniform by shoot thinning. Shoots were trimmed twice, in June and July, and pest management was carried out according to Emilia-Romagna region standard practices.

3.2.2. Berry sampling

Four replicates, each one consisting of five vines (20 vines total), were established in the vineyard. Three sampling per year were done: 18 September (47 days after veraison), 29 September (58 DAV), 7 October (66 DAV) for 2014 season and 27 August (26 DAV), 7 September (37 DAV), 16 September (46) for 2015 season. A random sample of 130 berries was collected from each replicate (520 berries for each sampling date) by cutting with scissors through the pedicel. Each sample was then divided into five subsamples which were used to determine: a) must biochemical parameters (25 berries); b) total anthocyanins (20 berries); c) extractable anthocyanins and tannins (40 berries); d) total tannins (20 berries); e) skin CWM composition and properties (25 berries). The berries for the determination of must biochemical parameters were immediately processed, while the remaining subsamples were frozen and stored at –20°C.

3.2.3. Biochemical analysis of musts

Must parameter subsamples (25 berries each) were analyzed to determine the soluble solids concentration using a temperature-compensating Maselli R50 refractometer (Maselli Misure, Parma, Italy). The must pH and titratable acidity were measured using a Crison Titrator (Crison Instruments, Barcelona, Spain).

3.2.4. Extraction of anthocyanins and tannins using a model hydroalcoholic solution

Whole (not ground) skins and seeds from 40 berries were soaked separately and shaken daily, in different tubes containing 80 mL of a hydroalcoholic solution for 15 days at 28°C. The duration and the temperature imposed to the extractions were chosen to simulate the winemaking conditions and so to determine the concentration of extractable anthocyanins and tannins. The hydroalcoholic solution comprised 6 g/L tartaric acid, 40 mL/L 1 N NaOH, 100 mg/L potassium metabisulphite and a proportion of ethanol that raised from 0 to 13% in the first 12 days of extraction. This concentration was reached by adding every two days 2 mL of ethanol absolute (12 mL total) to simulate alcoholic fermentation. The extracts were centrifuged (15 minutes, 13000 rpm) and an aliquots of the supernatant (400 µL) were dried under vacuum at 20°C. Pellets were stored at -20°C.

3.2.5. Exhaustive extraction of anthocyanins and tannins

Total anthocyanins were extracted from the skins of 20 berries by soaking the peeled skins in 100 mL methanol for 24 h, then storing the extracts at –20°C (Mattivi et al., 2006). Total tannins were extracted from the skins and seeds of 20 berries grounded separately to a fine powder before extracting 1 mg of the sample in 1 mL 70% (v/v) acetone in water, for 24 h in dark room (Downey et al., 2003). Skin and seed extracts were then centrifuged (15 minutes,

13000 rpm) and two 400 µL aliquots of the supernatant were dried under vacuum at 20°C. Pellets were stored at -20°C.

3.2.6. Anthocyanin determinations

Total and extractable anthocyanins were separated by HPLC as described by Mattivi et al. (2006), using a Waters 1525 instrument equipped with a diode array detector (DAD) and a reversed-phase column (RP18 250 x 4 mm, 5 µm) with a pre-column (Phenomenex, Castel Maggiore, BO, Italy). The concentration was determined by measuring absorbance at 520 nm. A calibration curve was established using a malvidin-3-glucoside standard (Sigma-Aldrich, ST. Louis, MO, USA).

3.2.7. Tannin determinations

Total and extractable skin and seed tannins were measured by HPLC with the equipment described above. The tannin content was determined by acid-catalyzed cleavage in the presence of excess phloroglucinol (Kennedy and Jones, 2001). The separation of monomer subunits and cleaved proanthocyanidins was carried out following the two different HPLC methods proposed by Downey et al. (2003). The concentrations of free monomers and hydrolyzed terminal subunits were determined from standard curves prepared with commercial standards of catechin, epicatechin, epicatechin-gallate and epigallocatechin (Extrasynthese, Genay, France) by measuring absorbance at 280 nm (Downey et al., 2003). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy and Jones, 2001).

The seed tannin content was assigned to free monomers, terminal subunits and extension subunits, whereas the skin tannin content was assigned to terminal subunits and extension subunits. The mean degree of polymerization (mDP) was calculated by summing terminal and extension subunits and dividing by terminal subunits (Downey et al., 2003).

The anthocyanin and tannin concentrations were expressed as mg per kg of berries (mg kg^{-1}), in order to take into account variations in berry weight that modify the concentration of these compounds during ripening.

3.2.8. Skin CWM preparation

The four subsamples collected in each sampling date for the skin CWM analysis, were combined and CWM was isolated following the procedure proposed by Vries et al. (1981). Briefly, skins of 100 berries were peeled, cleaned with milliQ water and lyophilized with a Drywinner (Heto-Holten, Allerød, Denmark). Skin were then grounded to a fine powder before boiling in milliQ water for 5 minutes to inactivate enzymes. The samples were homogenized with a D-Series Benchtop Homogenizer (PRO Scientific, Oxford, CT USA)

and were centrifuged in a Eppendorf Centrifuge 5804 (Eppendorf, Hamburg, Germany) to remove water. The solid residue was then washed several times with 70% ethanol (30 minutes at 30°C each time), to remove alcohol soluble solids and phenols, and finally with absolute ethanol and acetone to promote also a faster drying of the cell walls. CWM powders were stored in the dark at room temperature.

3.2.9. Analysis of CWM composition

Uronic acids were determined in the sulfuric acid cell wall hydrosilate by the colorimetric 3,5-dimethylphenol assay after cell walls pretreatment (30 °C, 1 h) with aqueous 72% sulfuric acid followed by hydrolysis with 1 M sulfuric acid (100 °C, 3 h). Pure galacturonic acid was used as a standard. The proteins and total phenolic compound content of the cell wall material were determined after extraction with 1 M NaOH (100 °C, 10 min) by the colorimetric Coomassie Brilliant Blue assay and by the colorimetric Folin–Ciocalteau reagent assay, respectively. Bovine serum albumin (BSA) fraction V and pure gallic acid were used as standards, respectively. The total glucose was determined using a kit for glucose enzymatic analysis from R-biopharm (Darmstadt, Germany) after pretreatment (30 °C, 1 h) with aqueous 72% sulfuric acid, followed by hydrolysis with 1 M sulfuric acid (100 °C, 3 h). Hydrolysis using only 1 M sulfuric acid (100 °C, 3 h) was used to determine noncellulosic glucose. Cellulosic glucose was obtained by difference between the total glucose and non-cellulosic glucose content. The acid-insoluble residue obtained after pretreatment and hydrolysis was used to estimate the content of lignin (Klason lignin).

3.2.10. Binding reaction between commercial tannin and CWM

The interactions between CWM and proanthocyanidins was studied using Merlot skin CWM from both seasons combined with a seed enological tannin (TanReactive, supplied by Agrovin S.A., Alcazar de San Juan, Spain), and it was followed the procedure proposed by Castro-López et al. (2016). Briefly, the enological tannin was dissolved in a model solution at a concentration of 2 g/L and it was then combined with CWM at the final concentration of 13 g/L. After the binding reactions the recovered tannins were analyzed by HPLC using the method proposed by Downey et al. (2003) and by size exclusion chromatography (SEC) following the method described by Kennedy and Taylor (2003) with the adaptation proposed by Castro-López et al. (2016).

3.2.11. Statistical analysis

Data were analyzed by longitudinal data analysis using the mixed procedure available in SAS v9.0 (SAS Institute, Inc., Cary, NC, USA), with compound symmetric (cs) as covariance structure.

3.3. RESULTS AND DISCUSSION

The evolution of total and extractable flavonoids and the properties of skin CWM were studied on Merlot berries sampled in the last phases of ripening. Exhaustive extraction with methanol and acetone were carried out to analyze the total content of anthocyanins and tannins, while a model hydroalcoholic solution was used to obtain the extractable fraction. The analyses of CWM composition and of the interactions with a commercial seed tannin were performed for a better understanding of the role that CWM plays on phenolic maturity.

3.3.1. Berry development and composition

Very different climatic conditions characterized the two years from April to September (Figure 1), since in 2014 precipitations were higher than in 2015 (462 mm vs 281 mm), and temperature lower in the average of the period (19.5°C vs. 20.9 °C). Veraison date was not affected by the diverse climate: in 2014 it occurred on 1 of August and in 2015 on 2 of August. The opposite climatic conditions of the two years explains the advance in ripening and sampling registered in 2015 compared to 2014. In the warmer season the berry sampling started 22 days earlier and berries showed higher sugar concentration than in the cooler (Table 1). Soluble solids raised in the last 20 days of ripening, with higher accumulation rate and final concentration at harvest in 2015. In the last part of ripening berry and seed weights did not change in each year. Skin weight remained stable in 2014, while a small increase was noted in 2015.

Figure 1. Average air temperature and precipitations from April to September in the years 2014 (A) and 2015 (B). Bars indicate the mm of rainfall; lines with dots indicate temperatures.

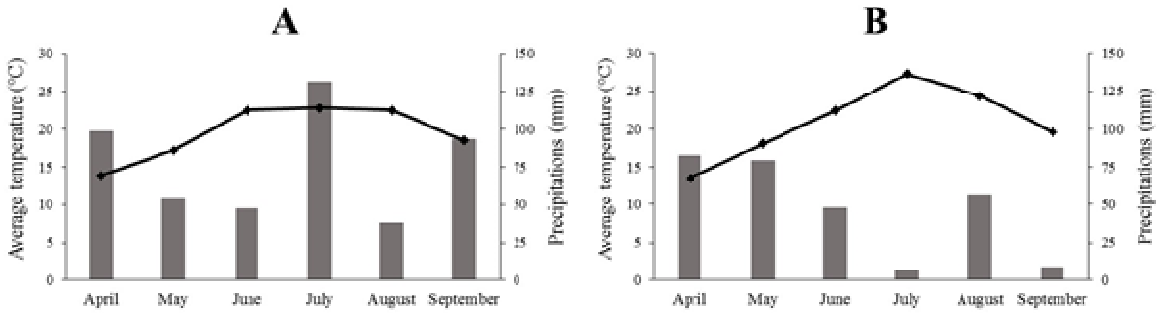


Table 1. Soluble solids concentration and mean weights of berry, skin and seed during ripening in the years 2014 and 2015.

Days after veraison	Soluble solids (°Brix)	Berry weight (g)	Skin weight (g/berry)	Seed weight (g/berry)
47 - 2014	21.6 b	2.60	0.290	0.103
58 - 2014	22.0 ab	2.58	0.297	0.104
66 - 2014	22.2 a	2.47	0.276	0.102
Significance	**	ns	ns	ns
26 - 2015	22.7 b	1.98	0.269 b	0.083
37 - 2015	25.1 a	1.94	0.322 a	0.090
46 - 2015	25.6 a	1.96	0.327 a	0.096
Significance	*	ns	*	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

3.3.2. Anthocyanin and tannin analysis

The concentration and characteristics of total and extractable flavonoids (anthocyanins and tannins) for both years are shown in Table 2. The level of both total and extractable anthocyanins increased in the last phases of ripening, in the two years of the study. In 2014 the concentration of total skin tannins showed small fluctuations while the extractable portion raised only after the first sampling date. In the following season, no variation was found on both total and extractable skin tannins. The increase of extractable anthocyanins found in both years seems more correlated with the higher amount in berries (total) rather than higher extraction caused by the degradation of skin cell structure during ripening (Río Segade et al., 2008). On the contrary, skin tannins did not showed a clear trend, since it is well known their behavior could be affected by many factors such as variety, cultural practices and environmental conditions (Harbertson et al., 2002; Bindon et al., 2014; Hernández-Hierro et al., 2012; Fournand et al., 2006). The composition of anthocyanins and skin tannins did not show any change between sampling date (Table 3 and 4).

Although seed monomer subunits decreased in the last phases of ripening (data not shown), total and extractable seed tannins showed only minor variations, in concordance with literature that reports a sharp decline soon after veraison and minor fluctuations until the point of harvest (Ristic and Iland, 2005; Pastor del Rio and Kennedy, 2006). Looking at our results of Merlot seed tannin, the oxidative crosslinking of polymers and the formation of

Table 2. Total and extractable anthocyanins, skin and seed tannins (mg kg $^{-1}$) during ripening in the years 2014 and 2015.

Days after veraison	Total					Extractable				
	Anthocyanins	Skin tannin	mDP	Seed tannins	mDP	Anthocyanins	Skin tannin	mDP	Seed tannins	mDP
47 - 2014	1034.7 b	872,5 b	9.31	1633,9	7.23	390.8 b	378,8 b	6.20	776,3	4.66
58 - 2014	1140.6 a	1001,9 a	9.69	1699,7	6.38	377.0 b	429,2 a	7.22	779,9	4.36
66 - 2014	1122.1 a	910,7 ab	9.95	1689,4	6.90	432.1 a	437,7 a	6.23	805,1	4.69
Significance	*	*	ns	ns	ns	*	*	ns	ns	ns
26 - 2015	1605.9 b	1016,3	8.46	1720,6	4.93	480.7 b	473,8	5.33	1141,5	4.13
37 - 2015	1781.4 a	1009,5	7.58	1854,6	5.65	519.3 b	448,6	5.77	1139,9	4.19
46 - 2015	1795.6 a	994,8	7.30	1847,8	5.59	591.6 a	480,4	5.65	1087,1	4.21
Significance	*	ns	ns	ns	ns	*	ns	ns	ns	ns

Different letters within a column indicate significant differences, in each year. Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.
mDP, mean degree of polymerization.

branched polymers more resistant to hydrolysis that are linked to the decrease of seed tannins after veraison (Cadot et al., 2006; Downey et al., 2003) seems to have negligible effects in the last 20 days of ripening. The lack of variation in the composition of seed tannins in the last phases of ripening of the two years of study supports these considerations (Table 5).

Table 3. Composition (%) of total and extractable anthocyanins during ripening in the years 2014 and 2015.

Days after veraison	Total anthocyanins			Extractable anthocyanins		
	Glycosilate	Acetate	Cumarate	Glycosilate	Acetate	Cumarate
47 - 2014	72.5	15.6	11.9	78.7	14.9	6.4
58 - 2014	71.9	16.2	11.9	78.1	15.4	6.5
66 - 2014	72.9	15.7	11.4	75.2	16.0	8.8
Significance	ns	ns	ns	ns	ns	ns
26 - 2015	72.5	15.7	11.8	76.3	16.6	7.1
37 - 2015	71.9	16.2	11.9	75.7	16.5	7.7
46 - 2015	72.3	15.5	12.3	76.4	15.7	7.9
Significance	ns	ns	ns	ns	ns	ns

ns, not significant.

Table 4. Composition (%) of total and extractable skin tannins during ripening in the years 2014 and 2015.

Days after veraison	Total skin tannins				Extractable skin tannins			
	C	EC	EGC	ECG	C	EC	EGC	ECG
47 - 2014	11.4	68.0	17.1	3.4	15.6	67.7	13.5	3.1
58 - 2014	11.2	66.8	18.4	3.6	13.2	62.3	21.2	3.3
66 - 2014	10.7	68.3	17.5	3.4	15.2	64.1	17.6	3.1
Significance	ns	ns	ns	ns	ns	ns	ns	ns
26 - 2015	12.9	69.9	14.5	2.7	18.5	65.1	15.0	1.5
37 - 2015	13.8	70.5	13.4	2.3	17.1	66.3	15.4	1.2
46 - 2015	13.9	70.3	13.5	2.3	17.4	65.8	15.4	1.4
Significance	ns	ns	ns	ns	ns	ns	ns	ns

ns, not significant; C, catechin; EC, epicatechin; EGC, epigallocatechin; ECG, epicatechin-gallate.

Table 5. Composition (%) of total and extractable seed tannins during ripening in the years 2014 and 2015.

Days after veraison	Total seed tannins			Extractable seed tannins		
	C	EC	ECG	C	EC	ECG
47 - 2014	20.9	55.1	24.1	31.5	58.5	10.0
58 - 2014	22.1	54.6	23.2	29.8	57.9	12.2
66 - 2014	21.3	56.1	22.6	29.2	55.8	15.0
Significance	ns	ns	ns	ns	ns	ns
26 - 2015	31.1	50.8	18.0	37.0	54.9	8.1
37 - 2015	29.9	51.7	18.4	35.0	56.8	8.2
46 - 2015	29.1	52.3	18.6	35.0	56.9	8.0
Significance	ns	ns	ns	ns	ns	ns

ns, not significant; C, catechin; EC, epicatechin; ECG, epicatechin-gallate.

The extractability of skin and seed flavonoids, calculated as the percentage of the extractable portion relative to the total amount, did not show any difference in the three sampling dates of each year of the research (Table 6). Previous studies reported that anthocyanin and tannin extractability have no specific developmental trend, but they change in response to variety and cultural conditions (Bindon and Kennedy, 2011; Fournand et al., 2006; Bindon et al., 2014).

Table 6. Extractability (%) of anthocyanins, skin tannins and seed tannins during ripening in the years 2014 and 2015.

Days after veraison	Anthocyanin exctractability	Skin tannin extractability	Seed tannin extractability
47 - 2014	37.8	43.6	47.5
58 - 2014	33.0	42.7	46.2
66 - 2014	38.6	50.0	47.8
Significance	ns	ns	ns
26 - 2015	30.0	47.0	67.0
37 - 2015	29.5	44.9	63.1
46 - 2015	33.0	49.7	59.5
Significance	ns	ns	ns

Different letters within a column indicate significant differences.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

The mDP of the total tannin resulted higher than that of the extractable portion (Table 2), in concordance with the results found by Allegro et al. (2016). The difference of mDP values is probably due to the higher extraction efficiency of acetone than that of hydroalcoholic solution (Bautista-Ortín et al., 2013; Pastor Del Rio and Kennedy, 2006; Bautista-Ortín et al., 2012). No change of mDP was observed in the last part of ripening probably because in this period tannin structure does not undergo important mutations. Bordiga et al. (2011) reported similar results in a study conducted on six varieties: in the last two sampling dates, performed when berries reached respectively 16 and 20 °Brix, no change of mDP was observed, while soon after veraison skin tannin mDP increased and seed mDP declined.

In the last part of ripening of our Merlot berries, the concentration of extractable anthocyanins increased, while extractable tannins showed minor variations on concentration and no change in composition. These results explain only partially the changes that are expected to be involved in the phenolic maturity: the higher level of extractable anthocyanins at harvest could be linked to the greater wine color achievable with riper grapes, but the reduction of astringency that is expected during the course of ripening does not find any correspondence with the small changes observed on tannins.

3.3.3. Analysis of skin CWMs and interactions with enological seed tannin

Considering the lack of knowledge on the relationship between progression of ripening and reduction of astringency, it was investigated the CWM concentration and composition of Merlot berry-skins sampled in the last 20 days of ripening and the role of these CWM in removing tannins from a wine-like solution.

Skin CWM concentration and composition did not show any statistical difference in the last part of ripening (Table 7), but in both years slight tendencies appear with regards to skin CWM quantities, uronic acids (pectins) and proteins that seem to increase, while cellulose decreased. Also, previous studies reported only minor variation in the concentration of the main constituent of CWM before harvest, even if the turnover of polysaccharides and proteins could change the properties of CWM (Ortega-Regules et al., 2008b; Vicens et al., 2009).

The CWM isolated during ripening was allow to interact with a commercial seed tannin. Phloroglucinolysis analysis showed that during the last phases of ripening, skin CWM increased its affinity for the commercial seed tannin as the tannins remained in solution decreased significantly (Table 8).

Table 7. Concentration and composition of CWM during ripening in the years 2014 and 2015.

Days after veraison	CWM (mg/ g of berries)	Uronic acids (mg/ g of CWM)	Protein (mg/ g of CWM)	Cellulose (mg/ g of CWM)	Non-cellulose glucose (mg/ g of CWM)	Polyphenols (mg/ g of CWM)	Lignin (mg/ g of CWM)
47 - 2014	4,75	324.0	101.0	168.7	6.7	14.6	384.9
58 - 2014	5,40	352.8	102.7	159.9	5.9	13.4	365.3
66 - 2014	5,41	360.6	106.5	155.4	6.3	13.4	400.4
Significance	ns	ns	ns	ns	ns	ns	ns
26 - 2015	5,45	268.9	103.0	128.3	8.4	14.2	477.1
37 - 2015	5,53	292.3	106.3	117.3	9.4	15.8	458.9
46 - 2015	5,83	309.5	110.3	108.1	9.1	12.7	450.3
Significance	ns	ns	ns	ns	ns	ns	ns

Different letters within a column indicate significant differences in each year. Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

Table 8. Results of the interaction between enological seed tannin and CWM of different ripening stages in the years 2014 and 2015 (phloroglucinolysis analysis).

	Tannin in solution (mg/ L)	mDP	ECG (mg/ L)
Commercial seed tannin	798.4 a	2.04 a	104.4 a
Interaction with CWM of 47 DAV 2014	688.4 b	1.84 b	72.9 b
Interaction with CWM of 58 DAV 2014	651.0 c	1.84 b	70.3 b
Interaction with CWM of 66 DAV 2014	574.4 d	1.80 b	62.4 c
Significance	*	*	*
Commercial seed tannin	798.4 a	2.04 a	104.4 a
Interaction with CWM of 26 DAV 2015	624.3 b	1.90 b	72.1 b
Interaction with CWM of 37 DAV 2015	634.3 b	1.89 b	72.3 b
Interaction with CWM of 46 DAV 2015	566.3 c	1.89 b	63.8 c
Significance	*	*	*

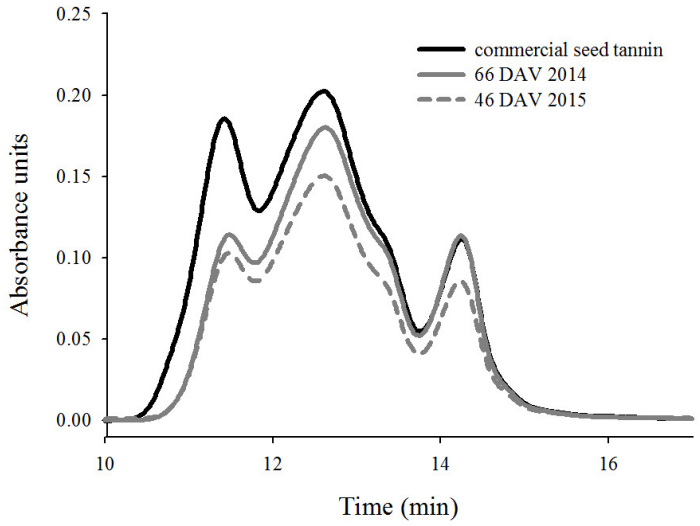
Different letters within a column indicate significant differences, in each year.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.
mDP, mean degree of polymerization; ECG, epicatechin-gallate.

To better understand these interactions and given that the results of the phloroglucinolysis analysis are incomplete, since not all the proanthocyanidins in solution are depolymerized by phloroglucinol, a size exclusion chromatography (SEC) analysis was also conducted. This analysis allowed us to explore how the skin cell walls reacted with the different fractions of

the seed tannin sample (high, medium and low molecular weight fractions) and the differences between the capacity of interaction of the most mature grapes skin cell walls in both years. Graphics of SEC analysis (Figure 2) confirmed that CWM adsorbed preferentially proanthocyanidins of high molecular weight (those whose maximum appeared at lower retention time in the SEC graphic) and strengthened the results of phloglucinolysis analysis about mDP of the tannins remained in solution. CWM interacts selectively with high molecular mass proanthocyanidins (Bautista-Ortín et al., 2016b; Bindon et al., 2010a) because the higher the dimension of the proanthocyanidin is, the higher the number of reactive sites that allow the binding is (Haslam, 1998). Cell walls from 2015 grapes also retained important quantities of medium molecular weight proanthocyanidins while not all or only small amounts of those of low molecular weight were bound, respectively in 2014 and 2015. Differences observed by SEC between samples of the two years are larger than those observed with the phloroglucinolysis analysis, the 2015 samples retaining larger amounts of tannins. We may speculated that these results are due to the higher level of maturity of berries sampled in 2015, since a previous study conducted on Cabernet Sauvignon reported an increasing affinity of skin CWM for tannins as ripening progressed, even when only minor chemical changes occurs on CWM (Bindon et al., 2012). The authors suggested that the increase of CW porosity accompanied by maturation allows the incorporation of higher quantities of tannins. Among these tannins, some of them could be tannins resistant to phloroglucinol depolymerization (i.e. oxidized tannins), accounting for the differences observed between the SEC and phloroglucinolysis analysis.

The reduction of the concentration of galloylated units noted with phloglucinol seems to be a consequence of the variation in the size of the galloylated form, rather than a specific affinity of the CWM as also suggested by Bindon et al. (2010b). It is important to note that in red wine the interaction between CWM and tannins could be much more complicated than in model solution, since CWM binds also anthocyanins, subtracting adsorption site to tannins that as a result become more extractable (Bautista-Ortín et al., 2016a). Therefore it comes to light that is very difficult to predict the final concentration of phenolic compounds in wine: the extractability from the skin is influenced by the presence of the CWM in the same tissue and precipitations are caused by the CWM solubilized during vinification (Bindon et al., 2016).

Figure 2. Comparison of the size exclusion chromatogram of the commercial seed tannin before and after the interaction with CWM of the years 2014 and 2015.



3.4. CONCLUSIONS

The variations of anthocyanins that occurs during the last 20 days of ripening and the changes of skin CWM affinity for tannins can play an important role in the final phenolic maturity of Merlot grape. In the two years of study, total and extractable anthocyanins rose, and that could allow to obtain higher colored wine with the progression of ripening. On the other side, the minor variations of skin and seed tannins doesn't give any information on the modifications of sensory attributes, such as the reduction in astringency that is desired for the production of premium red wines. However, some interesting suggestions rose looking at the dynamic affinity of skin CWM for seed tannins: CWM favored the adsorption of more galloylated tannins and with higher mDP, the class of compounds reported to be involved in the perception of astringency and in the sensation of roughness (Vidal et al., 2003), in the most mature grapes. In other words, the reduction of astringency that is expected with the progression of ripening could be related to the increasing affinity of the CWM for the tannins responsible of this sensation, rather than changes in tannin structure. Following this hypothesis, increasing quantities of astringent tannins could be removed from must or wine by precipitation with CWM.

This research shows new insights in the study of the phenolic maturity that should be deepen by further investigation taking into account the role of viticultural variables and enological techniques on the interactions between CWM and tannins.

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4. EFFECT OF SUNLIGHT EXPOSURE ON BERRY COMPOSITION AND WINE SENSORY ATTRIBUTES IN *Vitis vinifera* L. CV. GRECHETTO GENTILE

4.1 INTRODUCTION

Cluster sunlight exposure is affected by many factors such as trellis system, vine vigor and canopy management. The viticultural technique most used worldwide to modify cluster microclimate is the leaf removal of the basal part of the shoots, which alters dramatically light interceptions of cluster and, according to genotype, time and intensity of application, may have different impact on yield, grape composition and cluster sanity.

Early works reported that defoliation of the cluster zone was traditionally applied from fruit-set to veraison and generally improved microclimate condition of clusters, increasing the degree of light exposure and decreasing *Botrytis cinerea* infection (Gubler et al., 1991; Zoecklin et al., 1992; Percival et al., 1994a,b). Several authors, mainly reporting the effects of shading on black berry color, agreed that the increase of light interception enhanced the flavonoid content of grapes (Crippen and Morrison, 1986; Iland, 1988; Hunter et al., 1991; Zoecklein et al., 1992; Dokoozlian and Kliewer, 1996; Matus et al., 2009;) particularly in cooler viticultural regions where no excessive temperatures were reached (Jackson and Lombard 1993). On the contrary, in warm regions defoliation could determine excessive berry temperature that may have negative impact on the accumulation of anthocyanins (Mori et al., 2005; Mori et al., 2007; Movahed et al., 2016).

Leaf removal applied before flowering lowers the source of photosynthates inducing a reduction of fruit-set that leads to minor yield and looser clusters (Poni et al., 2006) and usually imply an overall enhancement of sugar content together with an increase of anthocyanins concentration in black berry cultivars (Intrieri et al., 2008; Poni et al., 2009; Diago et al., 2010; Tardaguila et al., 2010; Filippetti et al., 2011; Palliotti et al., 2011; Gatti et al., 2012; Gatti et al., 2015).

The modification of bunch light exposure can also affect volatile compounds. Arnold and Bledsoe (1990) reported that leaf removal from fruit-set to harvest reduced the vegetal aroma of Sauvignon blanc wines and several studies demonstrated the negative correlation between light incidence on clusters of Cabernet franc and Merlot, and the content of methoxypyrazines, compounds responsible of the herbaceous flavor (Scheiner et al., 2010; Sivilotti et al., 2016).

While the effect of cluster light exposure on the concentration of proanthocyanidins in grape and wine is not clear (Joscelyne et al., 2007; Kemp et al., 2011), there is agreement in the

literature that the exposition of clusters to sunlight increases flavonol accumulation in berries (Downey et al., 2006) also supported by the expression of the gene encoding the flavonol synthase in the skins (Downey et al., 2004; Pastore et al., 2013). A number of papers described the growth of flavonols following leaf removal on black-berry variety (Price et al., 1995; Diago et al., 2012; Figueiredo et al., 2013; Sternad et al., 2013; Moreno et al., 2015; Feng et al., 2015; Pastore et al., 2017), while few is known about the effect of sun exposure on flavonols in white-berry varieties.

Flavonols are present in the berry skin bound to various sugar (glycosides) and the most abundant are quercitin-3-O-glucoside and quercitin-3-O-glucoronide (Cheynier et al., 1986). Kampferol and isoramnetin are present at lower level, while myricetin, laricitin and syringetin are detected only in black-berry variety (Mattivi et al., 2006).

Flavonols play an important role in the co-pigmentation of red wines (Boulton, 2001; Lambert et al., 2011) while it has not yet been cleared their role on sensory properties. Preys et al., (2006) reported a relation between flavonols concentration and the bitter taste of wines, while Hunfangel and Hoffman (2008) described grape flavonols as velvety astringent but not bitter. Surveys conducted on berries of red and black currant (*Ribes rubrum* and *Ribes nigrum*) showed that flavonols contribute to the sensation of astringency (Schwartz and Hofmann, 2007; Sandell et al., 2009), while in a study on black-tea infusion (*Camellia sinensis*) appeared that flavonols has the ability to amplify the bitterness of other compound such as caffeine (Scharbert and Hofmann, 2005).

Since few studies were conducted on the role that increased berries light exposure plays on white grape composition, with particular regards to the phenolic compounds and the tastes they elicit on wines, it was decided to investigate these issues setting up a trial on the white berry cv. Grechetto gentile. This variety is cultivated in Bologna area (Italy) for the production of DOCG and DOC (appellations of origin) Pignoletto wines and its sensory profile is characterized by a slight bitterness and astringency that in some cases exceeds becoming unpleasant. In the actual context of climate change, the aim of the present trial is to investigate possible relationships between increasing cluster light exposure with the resulting berry flavonoid composition, and astringency and bitterness traits in corresponding wines. Grechetto gentile cv. may represent a model variety for this topic with the forecast that the emerging results could be take into account also for others white berry cultivars with similar compositional traits.

4.2 MATERIALS AND METHODS

4.2.1. Plant material and yield components

The study was conducted in the 2014 and 2015 seasons in a 30-years-old non irrigated vineyard of *Vitis vinifera* L. cv. Grechetto gentile, grafted onto Kober 5BB rootstock, located in Valsamoggia, Bologna, Italy (latitude 44°28'N; longitude 11°07'E). Vines were spaced 1.5 m within the row and 3.5 m between the rows and trained to a vertically shoot positioned (VSP) cane pruning system. Each vine was winter-pruned leaving one cane of 14 nodes. During the growing season, the number of shoots was kept uniform by shoot thinning. Shoots were trimmed twice, in June and July, and pest management was carried out according to Emilia-Romagna region standard practices.

In two uniform rows, 40 plants were chosen and randomly assigned to the following treatments: non-defoliated control (CK) and defoliation at stage BBCH 75 – pea sized berry (DEF). Defoliation was applied on 26 June 2014 and 30 June 2015, and consisted in the removal of all main leaves and lateral shoots around the clusters.

At harvest (23 September 2014 and 15 September 2015) the yield of the tagged plants was weighted and the number of cluster counted. The incidence of cluster rot and sun-burned berries was assessed by estimating the surface with visual symptoms.

4.2.2. Berry temperatures and light incidence on cluster

Berry temperature of 8 tagged clusters were recorded between stage BBCH 77 (berries beginning to touch) and harvest, using a microprobes connected to a datalogger (GMR Strumenti, Firenze, Italy) installed on the row. Probes were inserted into the subcuticular layers of the mesocarp of CK and DEF berries, placed on both sides of the canopy. Temperatures were recorded at one-hour interval. Light incidence on cluster was evaluated with a pyranometer (Skye Instruments, Llandrindod Wells, UK) measuring PAR (photosynthetic active radiation) around clusters at 10:00 AM of a full sunny day, and it was expressed as the percentage of incident light on cluster on the maximum irradiance measured out of the vineyard.

4.2.3. Leaf area measurement

After harvest 20 representative shoots per treatment were removed from plants within the blocks and the area of main and lateral leaves were measured with a LI-3100 A (Li-cor, Lincoln, Nebraska USA). The leaf area of each vine was calculated multiplying the average leaf area of the 20 shoots by the number of shoots per vine.

4.2.4. Berry sampling

At harvest three samples of 20 berries were sampled from each tagged plant for the following determinations: a) must biochemical parameters; b) skin and seed tannins; c) skin flavonols. The berries for the determinations of must biochemical parameters were immediately processed, while the remaining samples were frozen and stored at –20°C.

4.2.5. Biochemical analysis of musts

Must parameter samples were analyzed to determine the soluble solids concentration using a temperature-compensating Maselli R50 refractometer (Maselli Misure, Parma, Italy). The must pH and titratable acidity were measured using a Crison Titrator (Crison Instruments, Barcelona, Spain).

4.2.6. Tannins analysis

Tannins were extracted from the skins and seeds of 20 berries grounded separately to a fine powder before extracting 1 mg of the sample in 1mL 70% (v/v) acetone in water, for 24 hours in dark room (Downey et al., 2003). Skin and seed extracts were then centrifuged (15 minutes, 13000 rpm) and two 400 µL aliquots of the supernatant were dried under vacuum at 20°C. Pellets were stored at -20°C.

The determinations of tannins were performed with a HPLC Waters 1525 equipped with a diode array detector (DAD) and a reversed-phase column (RP18 250 x 4 mm, 5 µM) with a pre-column (Phenomenex, Castel Maggiore, BO, Italy). The content of these compounds was determined by acid-catalyzed cleavage in the presence of excess phloroglucinol (Kennedy and Jones, 2001). The separation of monomer subunits and cleaved proanthocyanidins was carried out following the two different HPLC methods proposed by Downey et al. (2003). The concentrations of free monomers and hydrolyzed terminal subunits were determined from standard curves prepared with commercial standards of catechin, epicatechin, epicatechin-gallate and epigallocatechin (Extrasynthese, Genay, France) by measuring absorbance at 280 nm (Downey et al., 2003). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy and Jones, 2001). The seed tannin content was assigned to free monomers, terminal subunits and extension subunits, whereas the skin tannin content was assigned to terminal subunits and extension subunits. The mean degree of polymerization (mDP) was calculated by summing terminal and extension subunits and dividing by terminal subunits (Downey et al., 2001).

4.2.7. Flavonol analysis

Flavonols were extracted from the skins of 20 berries by soaking the peeled skins in 100 mL methanol for 24 h and were determined by HPLC after acid hydrolytic cleavage of the

flavonol glydosides (Mattivi et al., 2006). The HPLC instrument was equipped as described above and the concentrations of quercitin, kaempferol and myricetin aglycons were determined from standard curves prepared with commercial standards of these compounds (Extrasynthese, Genay, France), by measuring absorbance at 370 nm.

The content of flavonols and tannins were expressed as mg per kg of berries (mg kg^{-1}), in order to compare the concentrations in grape with those of the resulting wines.

4.2.8. Microvinifications

At harvest, grapes of each treatment were divided into two lots of about 40 kg each and were vinified separately (4 microvinifications per year) at the experimental winery of ASTRA (Tebano, Ravenna, Italy). Since one of the main goal of this research was to better understand the relation between the presence of tannins and flavonoids in wine and the sensations of astringency and bitterness, it was decided to develop a particular vinification protocol that might be able to emphasize differences of phenolic tastes. After grapes were destemmed and crushed, a cold pre-fermentative maceration was performed to enhance the extraction of flavonoids into the must, that otherwise could result insignificant (Mattivi et al., 2006). Then, as variations in the content of ethanol and organic acids determines different perception of astringency and bitterness (Gawel et al., 2013), it was decided to remove this variability between samples by homogenizing the sugar content of the musts before alcoholic fermentation and the acidic profile of the wines before bottling. The rest of the operations resembled a standard protocol used for the vinification of white grapes. After the cold pre-fermentative maceration, crushed berries were softly pressed and the recovered musts were sulphited by adding 50 mg/L of SO_2, as potassium metabisulfite, and kept at 8°C for 24 hours for clarification. Musts were then inoculated with 20 mg/L of a commercial yeast strain (Zymaflore® VL2, Laffort, Bordeaux, France) for the alcoholic fermentation that took place at 18°C. After alcoholic fermentation, wines were sulphited by adding 30 mg/L of SO_2, cooled at 8°C for 24 hours and racked in stainless steel containers until bottling. At bottling 20 mg/L of SO_2 were added.

4.2.9. Chemical analysis and sensory evaluation of wines

The determinations of wine tannins were performed using the MCP protocol proposed by Sarneicks et al. (2006). The analysis of alcohol content, residual sugars, pH, volatile acidity and organic acids were conducted in the ASTRA laboratory (Tebano, Ravenna, Italy), following the official method of the International Organization of Vine and Wine (OIV, 2017), while the analysis of flavonols and idrossicinnamic acids were carried out in the

laboratory of the "Fondazione E. Mach – IASMA" (San Michele all'Adige, Trento, Italy), following the protocol proposed by Mattivi et al. (2006) and an internal protocol respectively. Wines of each vintage were evaluated 3 months after bottling. Quantitative descriptive analysis (QDA) of wines were carried out at ASTRA sensory laboratory by a group of 20 panellists, (12 female and 8 male) for 2014 wines and (11 female and 9 male) for 2015 wines, with long term experience in white wine sensory evaluation. Tasting sessions were conducted in separate boots, at 21°C ambient temperature and samples (40 mL) were served in standard ISO 3591 glasses, labelled with different letters. Panellists scored the perceived intensity of 9 attributes previously selected, on a 10 cm unstructured linear scale. The left-side end of the scales was "low intensity" and the right-side end was "high intensity".

4.2.10. Statistical analysis

A combined analysis of variance over years was performed using the mixed procedure available in SAS v9.0 (SAS Institute, Inc., Cary, NC, USA). Treatment comparisons were analyzed using the Tukey test with a cut off at $P \leq 0.05$.

4.3. RESULTS AND DISCUSSION

The effects of defoliation on grape technological parameters and phenolic composition were investigated over two consecutive years on Grechetto berries. Sensory evaluations of the resulting wines, were performed to study the relation between wine phenolic composition and the sensations of astringency and bitterness.

4.3.1. Environmental condition, light incidence and berry temperature

Vintages of 2014 and 2015 were characterized by different climatic conditions: considering the period from July to September, precipitations were 262.9 mm in 2014 while only 71.2 mm in 2015, and the average air temperature was 21.2°C in the first year of the experiment and 23.7°C in the second (Figure 1).

As expected, clusters of defoliated vines resulted highly exposed to sunlight while the incidence of light was low on CK (Table 1). In 2015 was noticed higher cluster exposure than in 2014. The higher irradiance following defoliation increased average berry temperature, the number of hours in which berry temperature exceeded 30°C and the number of hours in which berry temperature raised over 35°C. In the hot and dry season of 2015, these parameters showed higher values compared to those of 2014.

Figure 1. Average air temperature and precipitations from April to September in the years 2014 (A) and 2015 (B). Bars indicate the mm of rainfall; lines with dots indicate temperatures.

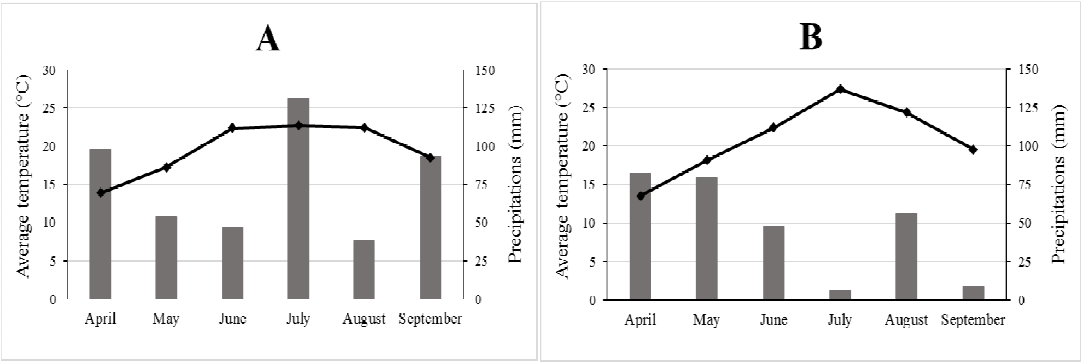

Table 1. Light and berry temperatures of non-defoliated (CK) and defoliated (DEF) grapes in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Light incidence on cluster (%)	4.4 b	61.4 a	12.4 b	67.3 a	*	ns
Average berry temperature (°C)	20.0 b	21.2 a	23.3 b	24.3 a	*	ns
Maximum berry temperature (°C)	32.4 b	42.4 a	39.0 b	43.2 a	*	ns
Berry temperature above 30°C (h)	40 b	160 a	286 b	395 a	*	ns
Berry temperature above 35°C (h)	0 b	8 a	4 b	108 a	*	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

4.3.2. Leaf area, yield component and grape composition

Defoliation removed all the leaves in the basal nodes of the shoots reducing considerably main and lateral leaf area (Table 2). In 2014 lateral leaf area resulted higher than in 2015, probably because the abundance of rainfall in the first season enhanced the growth of lateral shoots. Similarly, an overall increase of the pruning wood was observed in the first year of the experiment, without differences between treatments.

Rot incidence on cluster was lowered by defoliation and this effect was much more evident in 2014, when the precipitations in the last part of ripening favored the propagation of this pathogen on CK grapes. As reported by Percival et al. (1994a,b), the improvement of spray infiltration and the reduction of cluster wetness that can be achieved with defoliation, could

result effective in reducing cluster rot. Negligible percentage of sun-burned berries were found only on defoliated cluster in 2015, probably caused by the high temperatures that berry reached.

Defoliation did not influenced crop load, cluster and berry weights (Table 2), as previously reported when defoliation of the basal part of the shoots was applied in post bloom (Bledsoe et al., 1988; Feng et al., 2015; Mosetti et al., 2016).

Sugar concentration at harvest was not affected by leaf removal because all the values of fruit-to-leaf ratio (Table 3) are much higher than 0.7-1.4, which is the optimal range for an adequate ripening (Kliewer and Dokoozlian, 2005). In 2014 defoliation raised must pH and lowered titratable acidity, probably because the higher berry temperatures registered in berries of defoliated vines caused the reduction of malic acid (Lakso and Kliever, 1975; Bledsoe et al., 1988; Gatti et al., 2015). In 2015 pH and acidity resulted respectively higher and lower than the previous year, but no difference appeared between CK and DEF, because the hot climate condition in 2015 allowed the achievement of the threshold high temperatures values also in CK berries (286 hours above 30°C), which could have been determined a reduction of malic acid as happened in defoliated berries.

Table 2. Vegetative parameters and yield components of non-defoliated (CK) and defoliated (DEF) vines in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Main LA (m^2 / vine)	4.38 a	2.21 b	4.10 a	2.15 b	ns	ns
Lateral LA (m^2 / vine)	9.11 a	6.29 b	7.24 a	5.71 b	**	ns
Cluster rot incidence (%)	6.70 a	1.50 b	0.45 a	0 b	*	ns
Sun-burned incidence (%)	0	0	0 b	0.45 a	*	ns
Cluster (n° / vine)	23.4	23.6	24.5	25.1	ns	ns
Yield/vine (kg)	4.18	4.65	4.49	4.54	ns	ns
Cluster weight (g)	177.9	185.8	183.4	181.9	ns	ns
Berry weight (g)	2.15	2.08	1.98	2.00	ns	ns
Pruning wood (kg / vine)	3.63	3.19	2.31	2.21	*	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

Table 3. Grape composition and leaf-to-fruit ratio of non-defoliated (CK) and defoliated (DEF) vines in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Soluble solids (°Brix)	21.3	22.1	24.6	23.8	***	**
pH	3.21 b	3.32 a	3.60	3.58	***	*
Titratable acidity (g/L)	10.70 a	8.31 b	5.36	5.58	***	*
Leaf-to-fruit ratio (m^2 / kg)	3.49 a	1.98 b	2.68 a	1.77 b	ns	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns not significant.

4.3.3. Skin and seed phenolic compounds

The present study compared the results of non-defoliated control (naturally shaded) with those of sun-exposed berries after leaf removal carried out at pea-sized berry. The defoliation did not affected total skin tannins (Table 4) as found by Sivilotti et al. (2016), but only the concentration of terminal subunits, as found by Yu et al. (2016). The concentration of extension subunits, which represents the largest part of skin tannins, was not affected by defoliation. Similar results were found by Ristic et al. (2010) in the comparison between berries naturally shaded by foliage and berries highly exposed to sunlight via leaf removal and particular shoot disposition.

Previous studies investigated the role of light on the fate of skin tannins by comparing artificial shading treatments with non-defoliated control, and an overall decrease of these compounds was observed in the shaded berries of Pinot noir and Shiraz (Cortell and Kennedy, 2006; Ristic et al., 2007). Comparing the results of our work with those of the above mentioned studies, appears that the absence of light induced by artificial shading can have a detrimental effect on skin tannins, but that the increment of light interception on cluster does not stimulate any additional skin tannins biosynthesis. In our study also the temperature increased due to the higher solar irradiance (following defoliation), but it did not affected the accumulation of skin tannins, as previously evidenced altering berry temperature by forced convection on cluster of Merlot grape (Cohen et al., 2012).

Table 4. Skin tannin concentration, mDP and composition of non-defoliated (CK) and defoliated (DEF) berries in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Terminal subunits (mg / kg berries)	77.6 b	85.6 a	68.4 b	85.0 a	ns	ns
Extension subunits (mg / kg berries)	1509.7	1517.0	1049.9	1187.6	ns	ns
Total tannins (mg / kg berries)	1587.3	1602.6	1118.2	1272.6	ns	ns
mDP	20.5 a	18.7 b	16.4 a	15.0 b	**	ns
Catechin (%)	6.4	7.2	7.6	8.4	ns	ns
Epicatechin (%)	43.3	43.8	53.4	53.8	**	ns
Epigallocatechin (%)	47.6	46.3	36.9	35.7	**	ns
Epicatechin-gallate (%)	2.7	2.7	2.1	2.1	ns	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; ns not significant.

Skin tannin mDP resulted lower in DEF treatment, since in the calculation of mDP the denominator (terminal subunits) was higher and the numerator (total tannins) unchanged. The composition of skin tannins was not affected by defoliation but in the hotter season (2015) were found higher percentages of epicatechin counterbalanced by lower level of epigallocatechin.

Defoliation decreased the concentration of seed tannin monomer subunits in both years, while no difference was found on the concentration of terminal and extension subunits (Table 5). The concentration of total seed tannins resulted lower in DEF berries only in 2015, when the values of defoliated and control berries reached higher values compared to those of 2014. Defoliation did not affect the composition of seed tannins nor their mDP. The effect of sun exposure on seed tannins is not clear: Ristic et al. (2010) did not find any statistical difference between exposed and naturally shaded cv. Shiraz berries, while Yu et al. (2016) reported a slight increase following leaf removal in only one of the two years of study. No effect of artificial shading was noted in concentration and composition of cv. Shiraz berries seed tannins (Downey et al., 2004).

Table 5. Seed tannin concentration, mDP and composition of non-defoliated (CK) and defoliated (DEF) berries in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Monomer subunits (mg / kg berries)	230.8 a	147.2 b	320.3 a	175.3 b	**	ns
Terminal subunits (mg / kg berries)	160.5 b	195.1 a	229.8 a	200.5 b	**	**
Extension subunits (mg / kg berries)	872.3	940.1	1065.6	1024.5	**	ns
Total tannins (mg / kg berries)	1263.6	1282.4	1615.7 a	1400.3 b	**	**
mDP	6.45	5.92	5.71	6.15	ns	ns
Catechin (%)	23.1	23.3	26.3	24.1	ns	ns
Epicatechin (%)	54.9	52.5	52.0	52.1	ns	ns
Epicatechin-gallate (%)	22.1	24.3	21.7	23.8	ns	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; ns not significant.

The free forms of myricetin, quercitin and kaempferol were detected after acid hydrolysis (Table 6). In 2014 myricetin was found only in traces on control berries – it was reported to be absent in white berry varieties (Mattivi et al., 2006) - but defoliation strongly stimulated the synthesis of this flavonol compound. Also quercitin and kaempferol increased drastically after leaf removal and a 30-fold increase of total flavonols was observed. In 2015 the rise of flavonols after defoliation was less intense because CK showed higher values than those of the previous year, while DEF lower. The more intense light incidence on control cluster reported in 2015 compared to that of 2014 (Table 1), which was probably caused by the seasonal climatic conditions and by the lower growth of lateral shoots in the second year (Table 2), could have stimulated the synthesis of flavonol, explaining the higher concentration at harvest. Considering the defoliation treatment, the lower level of flavonols found in 2015 compared to that of 2014, is probably due to the higher temperature to which berries were subjected in the second year of trial. In fact, some authors (Degu et al.,2016; Mohaved et al., 2016) reported that high temperatures had a detrimental effect on the concentration of flavonols.

Table 6. Flavonols concentration and composition of non-defoliated (CK) and defoliated (DEF) berries in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Quercitin (mg / kg berries)	2.70 b	71.82 a	15.38 b	42.14 a	**	**
Kampferol (mg / kg berries)	0.26 b	30.37 a	4.16 b	17.99 a	**	**
Myricetin (mg / kg berries)	0.03 b	2.73 a	2.11 b	5.66 a	**	**
Total flavonols (mg / kg berries)	2.99 b	104.9 a	21.65 b	65.78 a	**	**
Myricetin (%)	0.6 b	2.6 a	10.1	8.7	**	ns
Quercitin (%)	92.5 a	68.8 b	71.8 a	64.2 b	**	ns
Kampferol (%)	6.9 b	28.6 a	18.1 b	27.0 a	**	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; ns not significant.

The results of defoliation on the white-berry cv. Grechetto gentile confirmed the overall increase of flavonols found also in previous studies conducted on different black-berry varieties (Feng et al., 2016; Moreno et al., 2015; Pastore et al., 2017), even if the rise of flavonol found in 2014 was much higher than those reported in literature. In control berries the percentage of quercitin is above the 70%, which represents a common value for many white-berry varieties (Mattivi et al., 2006), but defoliation modified the flavonol profile by the increase of the percentage of kaempferol counterbalanced by the decrease of quercitin. The percentage of myricetin rose in 2014, while no difference was noted in 2015.

4.3.4. Chemical composition and sensory attributes of wines

Wines were produced by grapes of control and defoliated vines with the aim to enhance the knowledge of the relation between phenolic compounds and the sensation of astringency and bitterness. In order to achieve this goal, it was necessary to exclude changes in the content of alcohol and organic acids, as differences in these compounds modify the sensations induced by phenols (Gawel et al., 2013).

Since sugar concentrations of the musts were standardized before fermentation by adding glucose, no difference were detected in the alcohol content of the wines of the same years (Table 7). Similarly, no differences were found in pH and in the content of each acid as the concentration of tartaric and malic acid were standardized before bottling. As expected changes observed in grape composition due to diverse climatic condition of the two years,

determined differences in wine: in 2015 the alcohol content was higher than that of 2014 and reached 15% v/v, while acidity, in particular malic acid, in the second year resulted much lower than the previous.

The phenolic compounds detected in wines were tannins, hydroxycinnamic acids and flavonols. The analysis of tannins did not show any difference between CK and DEF wines, and the absence of changes are coherent with the similar contents detected in the skin. Seed tannins should not be present in our wines because the condition at which the cold pre-fermentative maceration was conducted (low temperature, absence of alcohol and limited duration) should avoid their extraction (Bautista-Ortín et al., 2012). Hydroxycinnamic acids did not show any difference. The only changes regarding wine phenolic compounds were found on flavonols: DEF wines showed in both years higher concentrations than CK, resembling the differences found in grape.

The results of the organoleptic analysis are reported in Table 8. In 2014 color resulted more intense in DEF than CK, while the vegetal aroma was higher in control wines. In the same year DEF wines showed higher level of astringency and bitterness while no difference in the sensorial properties was found between wines of the following vintage.

The aim of this work was to explore more precisely the relations between different phenolics and the sensory attributes they elicit, such as astringency and bitterness (Singleton et al., 1975). Our results shows that these sensations are linked with the presence of flavonols: in both years these compounds are the only phenolics found at different concentrations, and in the first vintage wines with higher level of flavonols resulted more astringent and bitter. The lack of difference in the taste of 2015 wines, can be explained by the findings of Gawel et al. (2013), who reported that the phenolic tastes are more evident at low pH and moderate alcohol level. In our study, changes in taste were found only when wine pH was near 3.20 and alcohol content around 13%, not when wines reached higher values of pH and alcohol, as happened in 2015.

Table 7. Composition of the wines obtained with non-defoliated (CK) and defoliated (DEF) grapes in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Alcohol (% v/v)	13.3	13.3	14.7	14.7	**	ns
Residual sugars (g / L)	< 1	< 1	< 1	< 1	ns	ns
Total dry extract (g / L)	22.2	21.4	19.9	19.5	ns	ns
pH	3.21	3.24	3.68	3.60	**	ns
Volatile acidity (g / L)	0.25	0.20	0.41	0.50	**	ns
Tartaric acid (g / L)	1.60	1.45	0.80	0.80	**	ns
Malic acid (g / L)	5.10	4.85	2.45	2.50	**	ns
Lactic acid (g / L)	0.32	0.32	0.30	0.30	ns	ns
Citric acid (g / L)	0.41	0.36	0.30	0.30	*	ns
Tannins (mg / L)	38.6	44.3	47.6	54.7	ns	ns
Hydroxycinnamic acids (mg / L)	46.6	52.9	36.7	43.7	ns	ns
Flavonols (mg / L)	0.65 b	1.15 a	0.38 b	1.75 a	ns	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; ns not significant.

It is well known that astringency and bitterness are elicited by phenolics but it is still very difficult to explain the taste of each compound, also because it was demonstrated that phenolic compound such as tannins and hydroxycinnamic acids have a synergistic effect on the perception of their taste (Ferrer-Gallego et al., 2014). In our study higher astringency and bitterness were observed in wines with higher concentration of flavonols and similar concentration of tannins and hydoxycinnamic acids, but only when pH and alcohol content were moderate. Considering the role that flavonols play on enhancing the taste of other phenolic compounds (Scharbert and Hofmann, 2005), we can speculate that the difference in the concentration of flavonols, although of small magnitude, could have increased the perception of astringency and bitterness of tannins and hydoxycinnamic acids.

Table 8. Perceived intensity of sensorial traits of the wines obtained with non-defoliated (CK) and defoliated (DEF) grapes in the years 2014 and 2015.

Parameter	2014		2015		Year effect	Year x treatment interaction
	CK	DEF	CK	DEF		
Color	4.05 b	5.47 a	5.01	5.06	ns	**
Floral aroma	3.90	4.15	4.09	3.95	ns	ns
Fruity aroma	3.99	3.97	4.09	3.87	ns	ns
Vegetal aroma	3.77 a	3.40 b	3.04	3.02	*	**
Acidity	4.44	4.63	3.89	3.81	*	ns
Sapid taste	4.43	3.99	3.86	4.27	ns	**
Body	4.28	4.35	4.75	4.76	*	ns
Astringency	3.52 b	3.90 a	3.12	3.10	**	ns
Bitterness	3.15 b	3.55 a	3.31	3.32	ns	ns

Different letters within a column indicate significant differences in each year.
Asterisks indicate significance at: * $P < 0.05$; ** $P < 0.01$; ns not significant.

4.4. CONCLUSIONS

The increase of cluster light exposure after defoliation induced the decrease of grape acidity in the coldest season, and the rise of flavonols in both years. Vinifications were conducted following a particular protocol designed to emphasize differences of phenolic tastes, and wine obtained with defoliated grapes resulted more astringent and bitter in the first season, when pH and alcohol were moderate. Our work describes that the increase of light incidence on cluster of cv. Grechetto gentile can intensify undesired sensations in wine. Further researches are needed to confirm the role that flavonols and other phenolic compounds play on the sensation of astringency and bitterness and it will be important to verify these finding also on other white-berry cultivars, in particular those worldwide cultivated.

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5. CONCLUSIONS

The researches carried out during the three years of the PhD provided detailed information about the effects of delayed pruning on cv. Merlot and described the consequences of cluster sunlight exposure on cv. Grechetto gentile. In both experiments, the main point of interest was the study of the influences on berry flavonoids, and in order to deep the knowledge of the changes that flavonoids undergo during ripening, was also investigated the role of skin cell wall material on phenolic maturity.

The study of the delayed pruning suggested that, in the condition of the experiment, this technique was able to reduce sugar concentration at harvest with acceptable loss of yield, when applied in the first of the two phenological phases tested. The main point of interest regarding the effects on phenolic compounds was deeply investigated and the results obtained with the extractions using the wine-like solution, proved that the delay of pruning had no detrimental effect on the concentration and composition of anthocyanins and tannins at harvest. When the treatment was carried out later, the negative impact on yield was so severe that becomes not convenient to adopt this technique. Further investigations on the storage reserves are needed to clarify the effects of this technique on bud fertility: carbohydrate and nitrogen stored in the permanent organs of the vines, which play an important role on the floral differentiation of the developing shoots and so on the yield performances, might have been reduced by the development of high number of shoots, which were then removed by the late pruning.

The investigations on the phenolic maturity of Merlot berries was shared with the Enology team of the University of Murcia and this collaboration was essential for the achievement and the success of the trial. In fact, the interactions between a commercial seed tannin and the skin cell wall materials of berries sampled during the last phases of ripening, showed new insights on the phenolic maturity, since the adsorption of the tannins considered more involved in the perception of astringency and in the sensation of roughness, increased until the point of harvest. On the other hand, among all the berry flavonoids analyzed during ripening, only anthocyanins changed, showing an increase in both years. Therefore, while higher color is achievable with riper grapes, the expected reduction of astringency during maturity seems more correlated to the increasing activity of the cell wall material rather than the minor variations in concentrations and compositions, that skin and seed tannins undergo in the last part of ripening.

Finally, the survey conducted on the white berry cv. Grechetto gentile pointed out the implications of high solar irradiance on grape composition and described the consequences

on the phenolic tastes of the resulting wines. The results found on berry flavonoids and the sensory evaluation of the wines lead to find a relation between the increased concentration of flavonols found in berries and wines of the highly light exposed treatment, and the more intense perception of astringency and bitterness of the resulting wines. These results appears interesting both for describing the effects that can be driven by climate change on white berry varieties and for enhancing the knowledge of the relation between phenolic compound and tastes of wine.

The set of results of the present PhD enhanced the knowledge on the behavior of grape flavonoids under the current global warming scenario and proposed innovative approaches in the study of the phenolic maturity. Both scientific findings and practical information are now available for researchers of Viticulture and Enology and for the actors of the wine industry, with the sincere wish that this work could represent an incentive for further studies.

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