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Researches on berry composition in red grape: agronomical, biochemical and molecular approaches

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1.0 GENERAL INTRODUCTION

1.1 Dynamic of grape berry growth

According to botanical classification grape fruit (*pericarp*) is classified as a berry. It is a simple fruit, consisting of skin (*exocarp*), flesh (*mesocarp*) and a thin internal epidermis layer which separate flesh from seeds (*endocarp*). Exocarp represents at ripening the 5-20% of berry fresh weight and it is constituted by one epidermal layer, in the exterior part, coated with cuticle and epicuticular waxes (pruin). Internally in the skin, there are 11-12 ipodermal layers with collenchimatic cells and idioblasts. Mesocarp is made up by 25-30 parenchimatic cells layers. Endocarp is constituted by one very thin epidermal layer and 2-3 collenchimatic cells layers (Failla, 2007).

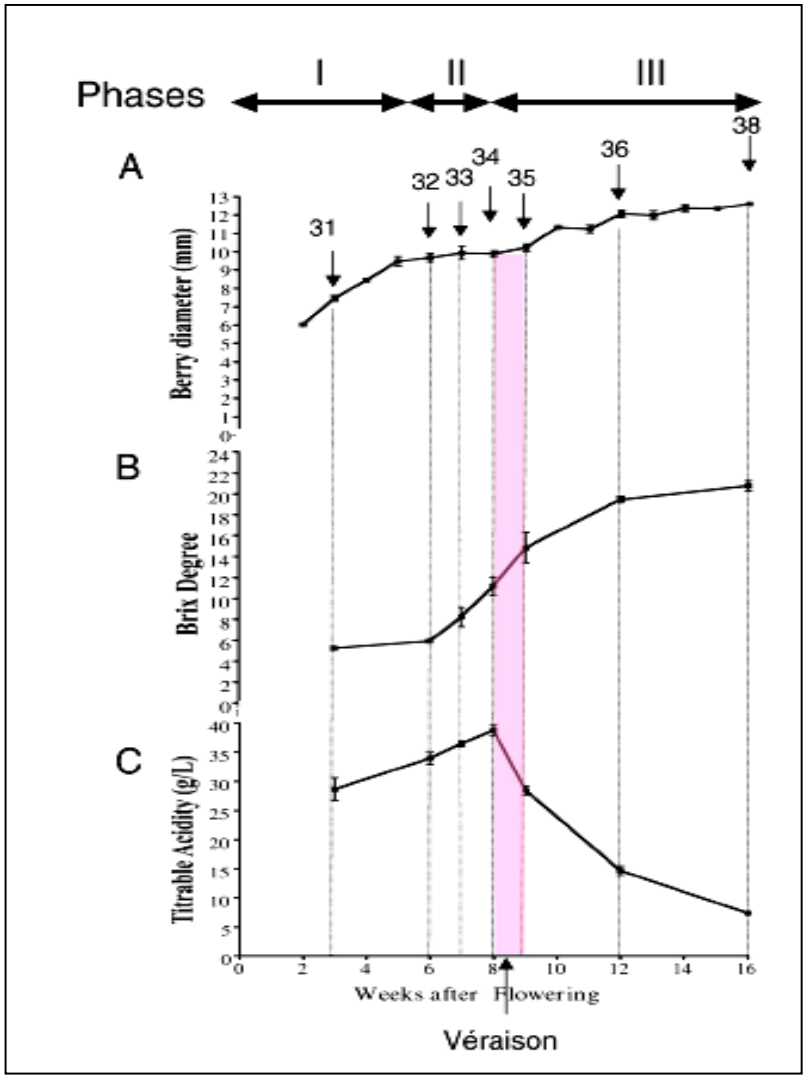


Figure 1. Changes in grape berry diameter (mm) and composition (Brix Degree and titrable acidity, g/L) during berry development (Deluc *et al.*, 2007).

Grape berry growth is described by a double-sigmoid curve, which can be divided into three major phases, each one involving changes in berry morphology and composition (Fig.1). In the first phase (I), which begins immediately after bloom, berry growth is due to rapid cell division and subsequent expansion. At this phase, berry seeds begin to develop and the main biochemical changes involve the increase in organic acid (mainly malate and tartrate), which reaches the maximum at the end of phase I. Tannins and hydroxycinnamates are synthesized at this stage. In the second phase (II, lag phase) there is no increase in berry size, seeds development go through and embryo reaches maturity. At the end of this stage glucose and fructose begin to accumulate. Véraison, the onset of berry ripening, marks the start of the last phase (III). This phase is characterized by a new cellular expansion, determining an increase in berry size and at this stage berry also begins to soften. The main biochemical changes involve colour development, which

starts towards anthocyanin accumulation and chlorophyll degradation in the skin of red grapes. At the same time sugars content increase, organic acid concentration decline, with subsequent pH increase, and flavor compounds and volatile aroma (derived from terpenoids) are synthesized.

At molecular level, a wide reprogramming of trascriptome and metabolome has to occur to promote berry growth. Recently Deluc *et al.*, 2007, have characterized all the changes in the trascriptome of Cabernet Sauvignon during berry development (Fig.2). Approximately 60% of total transcripts were differentially expressed in the three different stages of berry growth. Genes for biogenesis of cellular components, transport regulation and energy are over expressed in phase I. In phase II there is a large number of transcripts involved in transcription, storage proteins synthesis and metabolism; in phase III was observed an increase of transcripts for protein fate and synthesis and for cell cycle and DNA processing.

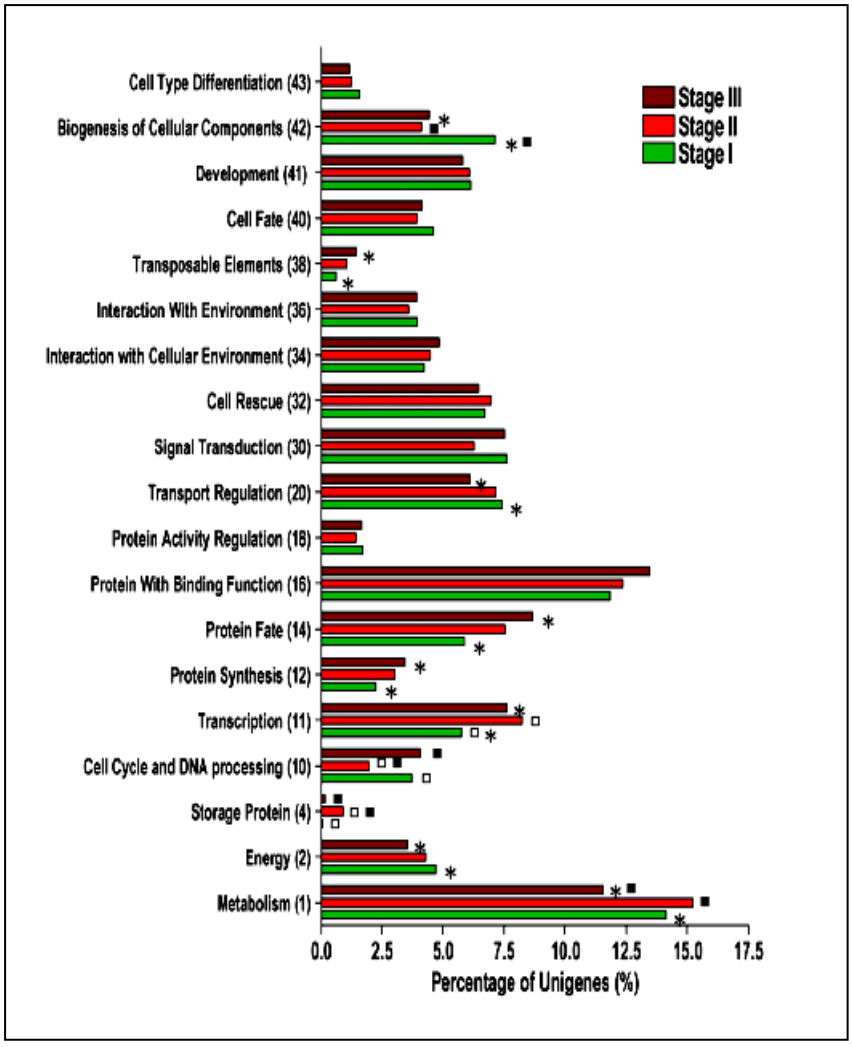


Figure 2. Distribution of annotated unigenes, according to their functional categories, with a two-fold or greater change in abundance within the three main phases of berry development (Deluc *et al.*, 2007)

Many endogenous and exogenous factors influence berry growth.

Among the endogenous factors growth factors play surely a key role. Application of exogenous ethylene at 8 weeks after anthesis cause an increase in berry growth but a decrease in average cell size (Hale *et al.*, 1970). Auxines role in berry development is controversial. Some authors reported that auxines promote cell division and expansion and that their concentration reaches the maximum in phase I of berry growth (Cawthon and Morris, 1982). Gibberellic acid concentration is high just after anthesis, but decrease until the beginning of phase II, where there is a second peak of active gibberellin compounds. Gibberellins could have a role in cells enlargement (Coombe, 1971).

Among exogenous factors, berry development is closely related to water availability in the soil. Fruit volumetric growth is in fact primarily due to water accumulation and during berry

development is necessary a coordination between water and solute transport to maintain cells turgor. Water stress leads generally to smaller fruits (Roby *et al.,* 2004). Also light and temperature, which are strictly linked, affect berry growth. Experiments conducted under controlled conditions have demonstrated that berry growth increase when increase light exposure (Dookozlian and Kliewer, 1996) and this suggests that probably light stimulates cell division or cell expansion in grape berries (Dookozlian, 2000). These results can't be confirmed in field conditions, where, when light increase, are often involved higher temperature and subsequent berry dehydration (Bergqvist *et al.*, 2001). Temperature influences in fact both grape cell division and expansion. The optimum temperature for berry growth is among 20°-25°C. Stage I of berry growth is the most sensible to high temperature: temperature reaching value of 35°C reduces growth rate and berry size at harvest (Dookozlian, 2000). Among macronutrients, potassium seems to be essential for grape berry growth and development. After véraison, an increase in potassium concentration in the berry is observed and it is compatible with its role in regulation of osmotic potential, turgor maintenance and growth control (Conde *et al.*, 2007).

1.2. Berry ripening and berry composition

Dynamic of berry ripening is extremely variable among different varieties, among one year from another for the same variety, among different clusters on the same vine and vineyard and among different berries on the same cluster. The nature of the signal that promotes the beginning of berry ripening seems to be related to the same plant endogenous and exogenous factors, as previously seen for berry ripening. Among growth factors, it has been demonstrated that pre véraison berries exposed to abscisic acid accelerate the onset of ripening (Coombe, 1992). On the contrary, even if grape is considered a non-climacteric fruit, it has been reported that ethylene inhibitors applied at 4,5,6,7 weeks after anthesis delay widely berry ripening (Hale *et al.*, 1970). In the same way, treatments of berries with a synthetic auxine-like compound (BTOA) caused a delay in the onset of berry ripening (Davies *et al.*, 1997). Water stress during berry ripening alters berry composition, causing generally an increase in sugars and in skin anthocyanin and tannin (Roby *et al.*, 2004). Timing of water deficit is important, because if it occurs at post-véraison sugars could decrease (Keller *et al.*, 2006). Low light causes a decrease in the rate of berry ripening, with a reduction in sugar and phenolic compounds content and an increase in acidity (Smart *et al.*, 1988). Regards to the temperature, the specific number of heat units (degree days, DD), that determines rate of berry ripening, is typical of each variety. In general low temperatures delay ripening, even if excessively high temperature after véraison may lead to the same result (Dokoozlian, 2000).

As it was seen, the dynamic of berry ripening is fundamental to determine final berry composition and as a consequence grape quality. Sugars, organic acids and phenolic compounds are the main biochemical components of grape berry.

1.2.1 Sugars

Sugar accumulation in grape berries is an important phenomenon which impact on alcohol amount in wine. At the beginning of berry development, sugars are produced by the green berries, while subsequently berry becomes a typical "sink" organ and uses carbohydrates produced by photosynthesis of leaves. Sucrose produced by leaves is then transported for long distance to berries into the phloem. From véraison xylem flow is almost impeded, hence water may reach berries via phloem with sugars. Sucrose is loaded into the phloem by either a symplastic or apoplastic mechanism. In the first mechanism, plasmodesmata are involved; in the second mechanism energy is required and sucrose moves across membranes towards sugars transporters.

Functionally, we distinguish two types of sugars transporters: symporters, which transport sugars in the same direction of the proton gradient, and antiporters, which transport sugars against the proton gradient. Upon its arrival in the phloem of the berry, sucrose may be unloaded. The mechanism of unloading sucrose from the phloem is less clear, even if there are evidences of symplastic and apoplastic mechanisms of unloading. Both disaccharide (DST) and monosaccharide transporters (MST) have been found in plants (Williams *et al.*, 2000). Sucrose, the predominant sugar transported in the phloem, accumulates in grape berry in form of glucose and fructose. So far, the complete cDNA sequences of six proteins homologue to plasma membrane hexose transporters (VvHT1, VvHT2, VvHT3, VvHT4, VvHT5, VvHT6) have been cloned. Functionally, only VvHT1 has been characterized: it is a high affinity H^+-dependent symporters, which could transport different monosaccharides. VvHT1 is expressed both in grape leaves and berries. In berries, mRNA for VvHT1 is abundant before véraison and in the late phase of berry ripening (Vignault *et al.*, 2005). VvHT1 protein levels are high only when berries are green because the protein is not present in ripening berries. Since at the end of berry ripening the glucose/fructose ratio is approximately equal to 1, the cleavage of sucrose may be catalysed mainly by invertase. Invertases are enzymes that produce one molecule of glucose and one molecule of fructose from one molecule of sucrose. Different invertase isoforms are localized in the cell wall (insoluble invertases), cytoplasm and vacuole (soluble invertases). Role of cell wall invertase may be to promote sucrose unloading from the phloem in order to maintain the sucrose concentration gradient and therefore sink strength. GIN1 and GIN2 are two vacuolar invertases cloned from grape berries. Their expression level reaches its maximum at the beginning of berry growth and declines when sugar accumulation starts (Boss and Davies, 2001).

Various parameters, including light, water and ion status, hormones and plant health regulate sugar accumulation in grape berry towards a complex mechanism (Conde *et al.*, 2007). Sugars transporters are tightly controlled and there are several levels (transcriptional or post-transcriptional mechanisms) at which regulation might occur.

Main role of sugars in grape berry is contribute to provide the carbon skeleton of many compounds (such as organic acids or amino acids) and to be energy sources for cells. By now it is

scientifically accepted that sugars play also an important role as signalling molecules in control of growth and development (Rolland *et al.*, 2006).

1.2.2 Organic acids

Tartaric and malic acids generally represent from 69 to 92% of all organic acids in grape berries (Kliewer, 1966). Small amounts of citric, succinic, lactic and acetic acids are also present in the berry.

While tartaric acid is synthesized until véraison, malic acids levels vary highly during berry ripening. Green berries, together with leaves, are the main grape organs able to produce organic acids (Dookozlian, 2000).

Tartaric acid concentration decrease after véraison is mainly due to a dilution effect, since berry volume increase and amount of tartrate remains constant. So far, no enzymes degrading tartaric acid have been ever found in grape.

In contrast, the decrease in malic acid amount at the beginning of ripening is associated with respiration and enzyme degradation as well as dilution effect. Malic acid may be therefore transformed to hexoses or used as energy source. Enzymes responsible for malate synthesis or catabolism exist in citosolic and mitochondrial isoforms and are malate dehydrogenase and NAD-malic enzymes (Conde *et al.*, 2007).

Metabolic changes occurring during grape berry development, together with light and temperature, are the main parameters controlling the malate synthesis/degradation rate (Taureilles-Saurel *et al.*, 1995). Grape berries malic acid concentration at harvest is negatively correlated with temperature during the ripening period. It has been demonstrated that the optimum temperature range for acids synthesis is between 20°C and 25°C and that, with increasing temperature, synthesis of malic acid decrease and catabolic rate accelerate (Kliewer *et al.*, 1967).

Even the potassium amount in grape berries could avoid tartrate and malate salts formation and therefore it could cause a reduction of the total acid concentration (Iland and Coombe, 1988).

1.2.3 Phenolic compounds

1.2.3.1 Non-flavonoids

Phenolic compounds of the grape are divided between the non-flavonoids and flavonoids groups.

Hydroxycinnamic acids, benzoic acids and stilbenes belong to the non-flavonoids group (Waterhouse, 2002). The hydroxycinnamates present in grape berries are derivatives of caffeic, p-coumaric and ferulic acids, which are present both in the skin hypodermal cells and in the flesh (Adams, 2006). The form in which they are usually present is esters of tartaric acids. Hydroxycinnamic acids, that are the main phenolic compounds in white wine, are produced in the

grape berry before véraison; then the synthesis declines and total amount remains constant. Caftaric acid is the predominant hydroxycinnamates in grape (170 mg/kg) (Kennedy *et al.*, 2006).

Benzoic acids are a minor component in grape berry composition. Six derivatives of benzoic acids: (gallic acid, protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, vanillic acid and syringic acid) have been found in grape berry. Gallic acid is the major benzoic acid present in grape berry and it is present both in skin and seeds. Gallic acid, protocatechuic acid and gentisic acid accumulate at the beginning of berry development; then the synthesis increases reaching a maximum at 80-90 days after full bloom. In contrary, the concentration of p-hydroxybenzoic, acid, vanillic acid and syringic acid varies slightly during berry ripening (Chen *et al.*, 2006).

Stilbenes are found in the skin of the grape berry and in vegetative tissues. Although stilbenes are present in low quantity both in berries and wine, they have aroused a lot of interest because of their effects on human health, in particular for what concern resveratrol (Bavaresco and Fregoni, 2001). The key enzyme in stilbenes biosynthesis is stilbene synthase (STS), which is part of a large multigene family: in grapevine actually 20 genes for STS have been found. STS catalyzes the last step of the well-characterized phenylalanine/polymalonate biosynthetic pathway that brings to the formation of *trans*-resveratrol (Delaunois *et al.*, 2009). Resveratrol could exist in different forms: in monomeric unit (as dimethylated derivatives, *trans*-pterostilbene, and as in form of 3-O-*β*-D-glucoside, *trans*- and *cis*-piceid) or in dimer units (viniferins). Stilbene biosynthesis can be constitutive or activated in response to pathogens attack or abiotic elicitors (for example UV radiation, aluminium chloride, ozone, wounding). Constitutive biosynthesis of resveratrol increases in berry grape from véraison to ripening phase. In particular, it has been demonstrated that resveratrol glucosides are preferentially produced in absence of biotic or abiotic elicitors, while *trans*-resveratrol represents the inducible form (Gatto *et al.*, 2008).

1.2.3.2 Flavonoids

All flavonoids share a common chemical structure represented by a C15 skeleton (C6-C3-C6, Fig.3). Depending on the position of the linkage of the aromatic ring (B ring) to the benzopyrano moiety (C ring), we can distinguish the flavonoids (2-phenylbenzopyrans), the isoflavonoids (3-benzopyrans) and the neoflavonoids (4-benzopyrans). Based on the degree of oxidation and saturation of the C-ring, the flavonoids may be divided into 8 subclasses (Fig.4). The B ring can further be hydroxylated, ortho-dihydroxylated or vicinal-trihydroxylated. Other flavonoids modifications include methoxylation, *O*-glycosilation of

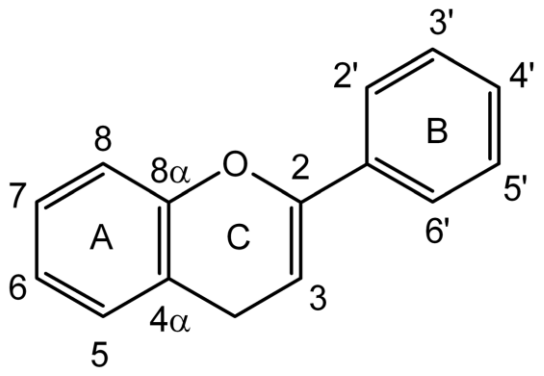


Figure 3. Chemical structure of flavonoid molecule (Aron and Kennedy, 2008).

hydroxyl groups or C-glycosilation of flavonoid skeleton carbon atom. Moreover flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules (Aron and Kennedy, 2008).

Many physiological roles are attributed to flavonoids in plants: they are able to protect plant from UV radiation; they can act as attractants for pollination and seed dispersal or as deterrents for herbivores. Besides, flavonoids can act as phytoalexins in plant defence against pathogens (Downey *et al.*, 2006).

In grape are commonly detected different classes of flavonoids: anthocyanins, flavonols and flavan-3-ols (tannins or proanthocyanidins) (Fig.5).

Flavonoids are localized both in berry skin and seeds. In particular, in hypodermal cells of the skin we can found tannins, flavonol, monomeric flavan-3-ols and, in red grape, anthocyanins.

Anthocyanins are pigments and represent the major class of flavonoids of the skin of red grape varieties. The anthocyanins commonly found in grape include delphinidin, cyanidin, petunidin,

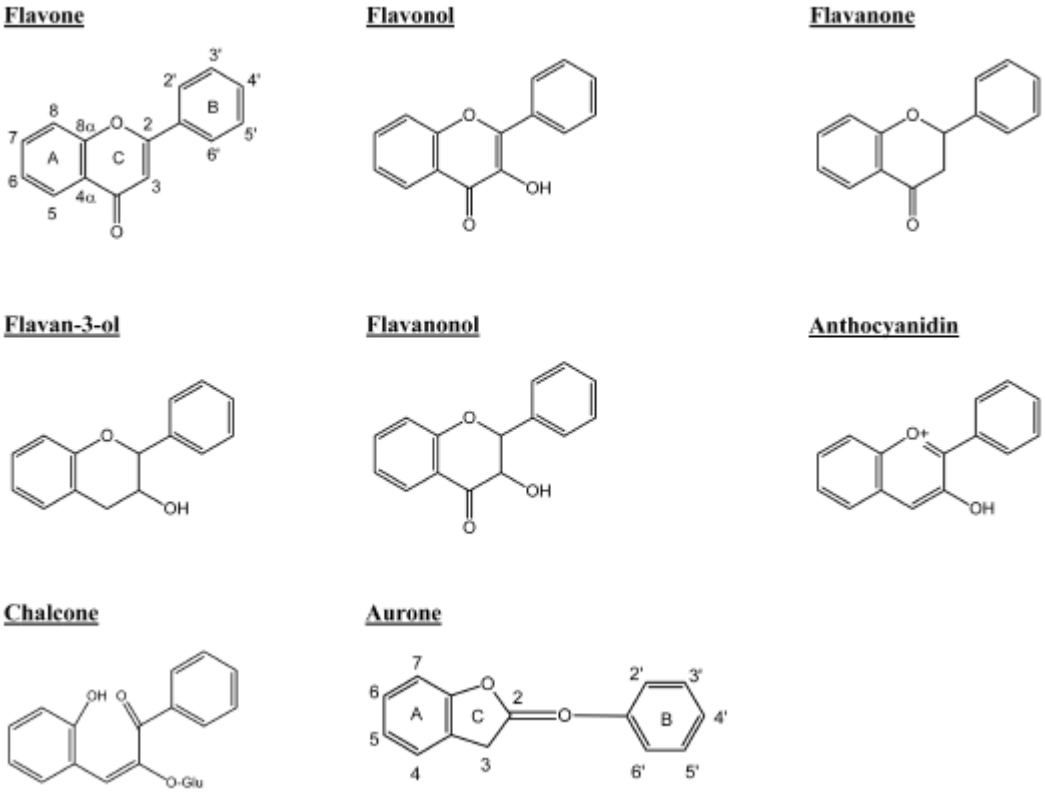


Figure 4. Chemical structure of flavonoid subclasses (Aron and Kennedy, 2008).

peonidin and malvidin-3-O-glucosides, 3-(6-acetyl)-glucosides and 3-(6-*p*-coumaroyl)-glucosides. In certain grape varieties have been found even traces of peonidin and malvidin 3-(6-caffeoil)-glucoside. Malvidin-3-O-glucoside is the most abundant anthocyanin present in grape.

Monomeric flavan-3-ols (catechin, epicatechin, epigallocatechin) are present in the skin at lower concentrations, while they are present at high concentrations in form of polymers within tannins

(proanthocyanidins). Tannins are polymers of different condensed monomers: epicatechin and epigallocatechin constitute the chain of the polymers ("extension" subunits); catechin is present as "terminal" units (Braidot *et al.*, 2008). The mean degree of polymerisation (mDP), which represents the mean length of the tannin chain, is of about 28 in berry skin (Bogs *et al.*, 2005).

The last component of flavonoids in the skin is represented by flavonols, in particular glucosides, galactosides and glucuronides of quercetin, kaempferol and myricetin. In some varieties there are also little amount of isorhamnetin, laricitrin and syringetin (Mattivi *et al.*, 2006).

Flavonoids in the seeds accumulate not only in the external part, but even in the inner layers. Grape seeds are the primary source of flavan-3-ols and tannins in grape berries. Total tannin content is in fact higher in seeds than in skin even if seed tannins have a lower mDP. Even the composition of seed tannins is different comparing to the skin one: in seed tannins generally epigallocatechin is not present and epicatechin gallate is the major constituent of "extension" subunits (Adams, 2006).

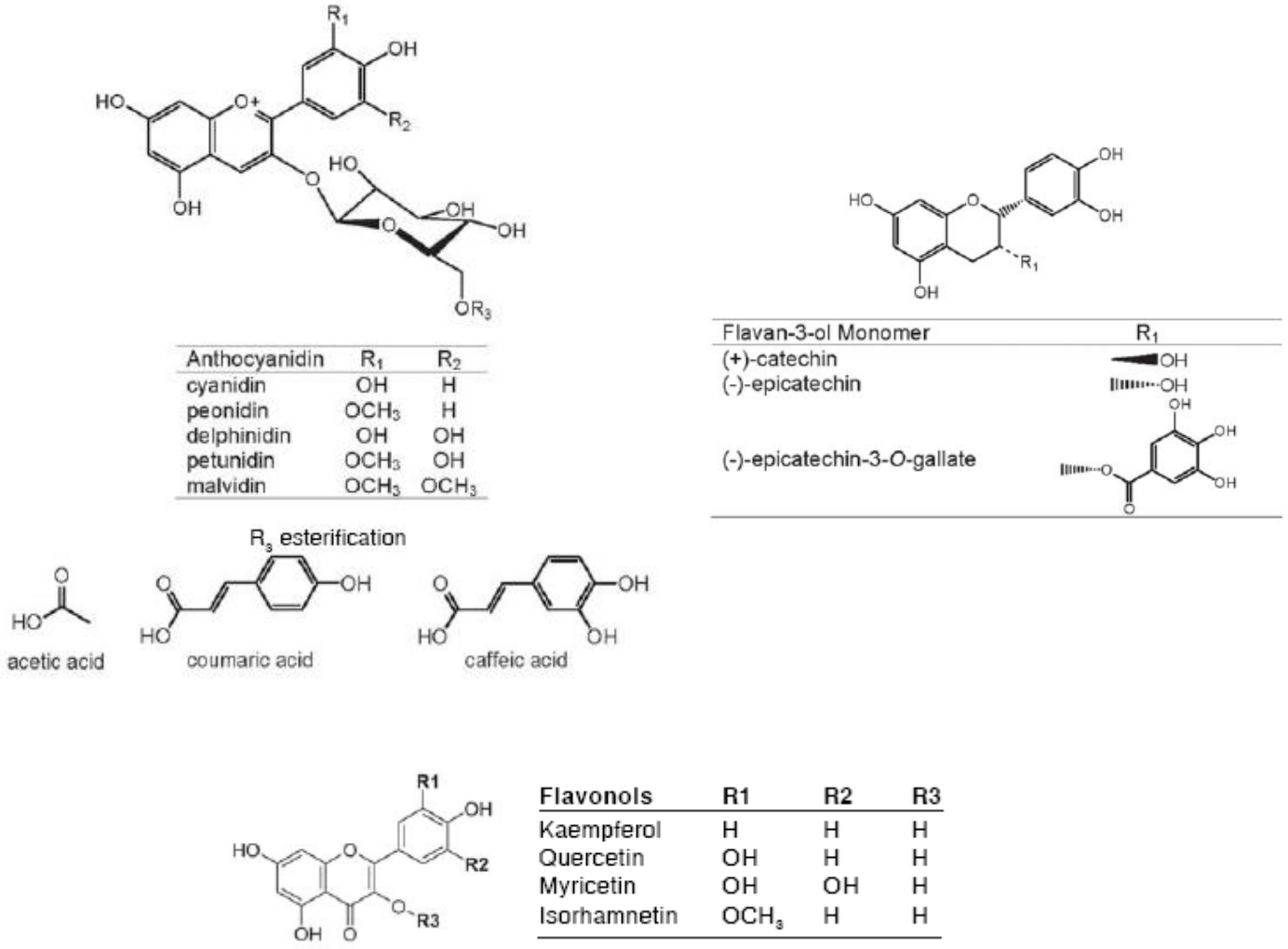


Figure 5. Chemical structure of the three main classes of flavonoids present in red grape berry (Kennedy *et al.*, 2005).

1.3 Biosynthesis of flavonoids in red grape berry

Flavonoids are synthesized within the general phenylpropanoid pathway. From the same precursors and towards specific branches, these pathways bring to the synthesis of anthocyanins, flavonols and proanthocyanidins. The enzymes involved in flavonoid biosynthesis are associated at the cytoplasmic surface of the endoplasmic reticulum and represent a multienzyme complex.

1.3.1 Synthesis of the early products of the phenylpropanoid pathway

PAL is a key enzyme, ubiquitous in plants, which catalyzes one of the first steps in phenylpropanoids biosynthesis, the hydrolysis of ammonia from phenylalanine to produce cinnamic acid. Cinnamic acid is the precursor of all phenolic compounds. Recently in grape was found the subcellular localization of PAL: PAL is present in the cell walls, secondarily thickened walls and parenchyma cells of the berry tissues (Chen *et al.*, 2006). This enzyme, that is a member of a large gene family, has been cloned in many plant species. In tobacco plants, two PAL isoforms have been found: PAL1 that is associated with the endoplasmic reticulum and PAL2 that is able to diffuse through the citosol (Achnine *et al.*, 2004). In grape are probably present 15-20 PAL isoforms (Sparvoli *et al.*, 1994). Ritter and Schulz (2004) have elucidated the crystal structure of the PAL enzyme at high resolution, exploiting its homology with the enzyme Histidine Ammonia Lyase (HAS). PAL has a tetrameric D2-symmetric structure in which the four subunits are tightly linked. For what concern PAL molecular architecture, it is a α-helical protein (52%) with the 5% of the residues organized in β-strands. The mobile N-terminal peptide of 24 amino acids may anchor the enzyme at other cell components. The active centre of PAL is represented by a 4-methylidene-imidazole-5-one (MIO) group, which is derived post-translationally from a tripeptide segment in two autocatalytic dehydration reactions. MIO group is responsible for the unusual nonoxidactive deamination reaction of PAL.

The cinnamic acid produced by PAL is converted to *p*-coumaric acid towards the action of cinnamate 4-hydrolase (C4H). C4H belongs to the cytochrome P450 monoxygenases family. The enzymes of this family are able to catalyse exothermic reactions that are irreversible. Many cDNAs encoding C4H were isolated from different plants (**http://drnelson.utmem.edu/CytochromeP450.html**). In grape C4H have not yet been cloned, but it's known that it is present in the chloroplasts and in the nucleus of berry tissues (Chen *et al.*, 2006).

One molecule of *p*-coumaric acid produced by C4H is condensed with an acetil-CoA molecule to form *p*-coumaroil-CoA that is the precursor of all the flavonoids. The enzyme which catalyses this reaction is the 4-coumarate:coenzyme A ligase (4CL). 4CL is localized in grape in the secondarily thickened walls and in the parenchyma cells of the berry pulp vascular tissue (Chen *et al.*, 2006). 4CL belongs to a multigene family. In *Arabidopsis thaliana* there are four members in 4CL family (At4CL1-4). Of the four isoforms only At4CL3 is involved in flavonoids biosynthesis (Hamberger and Hahlbrock, 2004), while the other isoforms participate in lignin production. In grape two sequences coding for 4CL have been cloned in Cabernet Sauvignon

(http://compbio.dfci.harvard.edu/tgi/cgibin/tgi/mapTCs.pl?map=map00940&species=grape&gi_dir=vvgi).

It's very interesting to note that the activities of PAL, C4H and 4CL are really correlated and change concomitantly (Chen *et al.*, 2006).

1.3.2 Specific steps towards flavonoid biosynthesis: structural genes involved in flavonols, flavan-3-ols and anthocyanin biosynthesis

The first enzyme involved specifically in flavonoids biosynthesis is calchone synthase (CHS)(Fig.6). CHS catalyzes the condensation of one molecule of *p*-coumaroil-CoA with three molecules of malonyl-CoA to produce naringenin chalcone. CHS, as other enzymes involved in the phenylpropanoid metabolism like STS, belongs to a large superfamily of proteins, the family of type III Polykethide Synthase. Until now, three different isoforms of CHS have been found in grape. CHS1 and CHS2 are two different isoforms that are very similar both at nucleotide (92.4% of homology) and at amino acid level (96.7% of homology). The major differences in the nucleotide sequences are present in their 3' and 5' untranslated regions and both the proteins encoded by these two isoforms are constituted by 393 amino acid residues. The third isoform (CHS3) is different from CHS1 and CHS3, showing only the 89% of homology. Many differences have been found even in the 3' and 5' untranslated region of CHS3, comparing this region with the ones of the other two isoforms. CHS3 encoded for a protein of 389 amino acids. In berry skin of the red cultivars Cabernet Sauvignon and Merlot mRNAs for CHS1, CHS2 and CHS3 were detected in the early stage of berry development (green berries). These levels decrease until véraison and then, after véraison, there is a new increase of these mRNAs accumulation. Among the three different isoforms has been demonstrated that CHS3 accumulates mainly in berry skin of red cultivar after véraison, while there are no differences between the amount of CHS1 and CHS2 in red or white cultivars (Chardonnay) (Goto-Yamamoto *et al.*, 2002). The three dimensional structure of CHS was solved from alfalfa (*Medicago sativa*) in the past few years. CHS maintains an αβαβαβ protein fold that is typical of all the members of the enzyme superfamily of type III Polykethide Synthase (Ferrer *et al.*, 1999).

Naringenin calchone is converted to flavanones (naringenin) to form the flavanoid core towards a stereo-specific ring closure catalysed by chalchone isomerase (CHI). It is believed that existed only one locus encoding for CHI in grapevine and this is compatible with the ability of its substrate to isomerise non enzimatically and with the fact that mutations in CHI are in fact very leaky. Recently, instead, two isoforms of CHI were identified (Jeong *et al.*, 2004). Jez *et al.*, (2000), have crystallized the CHI enzyme in alfalfa. CHI is a functional monomer of 220 amino acid residues that adopts an open-faced β-sandwich fold. The active site of the enzyme is characterized by an apolar environment and by different amino acids that are located in a large β sheet and in a layer of α-helices. On the basis of the CHI sequence, that doesn't display any detectable homology with other proteins, seems that this enzyme is unique to the plant kingdom.

Naringenin is then hydroxylated at position 3 by flavanone 3-hydroxylase (F3H) to form dihydrokaempferol, the flavonols precursor.

Dihydrokaempferol can be substrate of three different enzymes which directly bring to the production of the flavonol kaempferol towards the action of flavonol synthase (FLS), or to the biosynthesis of dihydroquercetin and dihydromyricetin towards the action respectively of flavonoid-3'-hydroxylase (F3'H) and flavonoid-3'5'-hydroxylase (F3'5'H).

These enzymes catalyse the hydroxylation of the B ring of dihydrokaempferol in 3' or 3'5' positions. F3'H activity results in the production of cyanidin-like anthocyanins, while F3'5'H action brings to the production of delphinidin anthocyanins species. The cloning of both F3'H and F3'5'H genes from grape has shown that in grape are present two copies of F3'H and several copies of F3'5'H in a single chromosome (Bogs *et al.*, 2006; Castellarin *et al.*, 2006). In flowers of *Petunia Hybrida* has been shown that F3'H and F3'5'H can act both on the substrates and products of F3H (Stolz *et al.*, 1985; Menting *et al.*, 1994).

As previously described, FLS is the key enzyme in the biosynthesis of flavonols. This enzyme employs dihydrokaempferol, dihydroquercetin and dihydromyricetin as substrates to synthesize the flavonol aglicones. FLS is a 2-oxoglutarate dependent dioxygenase, which requires 2-oxoglutarate and ferrous iron for catalytic function. This protein family is characterized by two sets of four β-sheets arranged in an anti-parallel manner. In this rigid structure are housing two conserved motifs essential for binding ferrous iron and 2-oxoglutarate. Protein sequence alignment of FLS form various species, among which *Vitis Vinifera* FLS, indicated 5 conserved residues, which are present at different sequence positions and determine enzymatic activity (Chua *et al.*, 2008). In Shiraz flower were identified two distinct cDNA encoding for FLS: VvFLS1 and VvFLS2, which have an overlapping region of 425 nucleotides with the 79% of homology at sequence level. As VvFLS2 expression does not change during berry development, it is probable that VvFLS1 is the enzymatic isoform responsible for flavonols synthesis in berry, even if it is also expressed in many other grape organs (leaves, tendrils, pedicels, buds and inflorescence) (Downey *et al.*, 2003).

VvFLS1 directly competes for its substrates with the enzyme dihydroflavonol 4-reductase (DFR), which catalyses the first step in the conversion of dihydroflavonols to proanthocyanidins or anthocyanins. This point of the biosynthetic pathway is a key regulatory point because it determines the direction towards flavonols or proanthocyanidins and anthocyanins synthesis.

DFR is in fact responsible for the NADPH-dependent reduction at the 4 position of the C ring of dihydroquercetin and dihydromyricetin to give respectively leucocyanidin or leucodelphinidin. The sequence of DFR contains most of the motives present in the short-chain dehydrogenase/reductase (SDR) superfamily and the 3D description of the DFR-$NADP^+$-dihydroquercetin ternary complex has confirmed it (Petit *et al.*, 2007). In a recent study has been demonstrated that the active site of grape DFR can host the flavonols quercetin or myricetin in a similar way to the dihydroflavonol

dihydroquercetin. In presence of flavonols, two molecules bind at the same time the catalytic site altering its geometry and leading to the inactive state of the enzyme (Trabelsi *et al.*, 2008).

Leucocyanidin and leucodelphinidin are, in turn, substrates to the next step of the proanthocyanidin and anthocyanin biosyntheses, which involve either anthocyanidin reductase (ANR) and leucocyanidin reductase (LAR) or leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT).

In grape, LAR converts leucocyanidin in the flavan-3-ol (+)-catechin with a NADPH-dependent reduction. ANR acts instead on the products of the enzyme LDOX, which catalyzes the cyanidin and delphinidin production from leucoanthocyanidins. Then ANR uses these substrates to synthesize

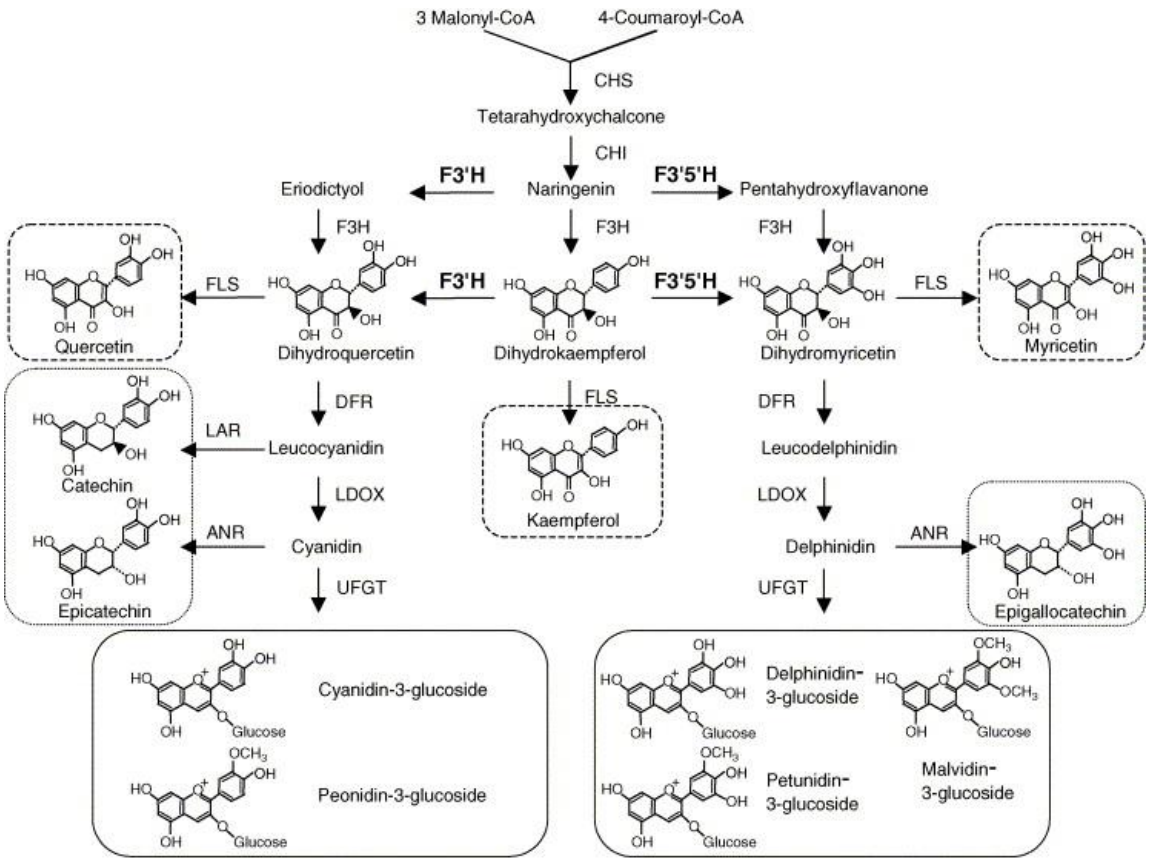


Figure 6.Diagrammatic representation of the flavonoids biosynthesis pathway. Enzyme abbreviations are as given in the text (Jeong *et al.*, 2006).

respectively (-)-epicatechin and (-)-epicatechin-3-O-gallate (Gagné *et al.*, 2009).

As FLS, also LDOX belongs to a family of 2-oxoglutarate-dependent oxygenase (Nakajima *et al.*, 2006). In a recent work has been demonstrated that in *Arabidopsis Thaliana*, LDOX could have also a FLS activity in vivo (Stracke *et al.*, 2009). Southern blot experiments conducted on *Vitis Vinifera* cv. Red Globe have shown that it is present only a single copy of LDOX gene in grapevine (Gollop *et al.*, 2001).

The anthocyanidins, cyanidin and delphinidin, produced towards the action of LDOX are then stabilized through the addiction of a glucose residue at the 3 position of the C ring. This reaction is catalysed by the enzyme UFGT and allows the anthocyanins transport into the vacuole.

UFGT is the key enzyme in anthocyanin biosynthesis, because it is expressed only in red skin cultivars. It was isolated for the first time as a partial cDNA clone and sequence comparison of the corresponding gene from other species has shown only the 65% of homology (Sparvoli *et al.*, 1994). UFGT seems to be present in grape genome as a single copy and it is interesting to note that its promoter and coding regions have been found also in white cultivar and are similar to the sequences present in their red-skin sports (Kobayashi *et al.*, 2001). The 3-*O*-glycosilation of anthocyanin is almost always a prerequisite for further modification, as methylation and acylation.

Methoxyl groups are found in three of the main anthocyanins (peonidin, petunidin and malvidin-3-*O*-glucoside). This reaction brings to more stable compounds for the methylation of the reactive hydroxyl groups and the enzymes responsible are *O*-methyltransferases (OMTs). The first partial purification and characterization of a methyltransferase catalyzing the methylation of cyanidin-3-glucoside to peonidin-3-glucoside was performed on a cell suspension of *Vitis vinifera* cv Gamay Freaux. This enzyme is S-adenosyl-L-methionine (SAM)-dependent and it was shown that it has a high substrate specificity, reacting only with cyanidin and not with delphinidin (Bailly *et al.*, 1997). More recently it has been described the identification and the biochemical characterization of a novel Mg^{2+}-dependent anthocyanin O-methyltransferase (AOMT) from grapevine. This enzyme belongs to the type 2 OMTs, a group of lower molecular mass (23–27 kD) cation dependent OMT. AOMT showed a preference for 3', 5'methylation compared to 3'methylation and its transient expression in tobacco (*Nicotiana Benthamiana*) resulted in a higher accumulation of malvidin-3-rutinoside compared to delphinidin-3-rutinoside (Hugueney *et al.*, 2009).

Acylation of anthocyanins consists in the addition of an aliphatic acetyl group or an aromatic *p*-coumaroyl group to the 6' position of the 3-*O* -glucoside. The enzymes responsible of this modification are anthocyanin and flavonoid acyltransferases, which are classified into two different categories (aliphatic and aromatic acyltransferases) on the basis of the acyl-donor specificity. Acylated anthocyanins show a low sensivity to pH changes because are more soluble in water and lead to higher anthocyanins structure stability protecting the sugar molecule against degradation by glucosidase (Welch *et al.*, 2008).

Although the flavonoid biosynthetic pathway takes place in the cytoplasm, once the biosynthesis and the additional modifications are complete, the most part of the products are delivered into and stored in distinct cellular compartments as it happens for many other secondary metabolites.

1.4 Subcellular compartmentation and transport mechanisms of flavonoids in red grape berry

While the biosynthetic pathway of flavonoids has been well studied, it is not still possible completely understand the flavonoids transport in plant because several mechanisms could coexist and overlap.

In red cultivars, flavonoids are localized and stored at cellular level mainly in the cell wall and in the vacuoles. Microscopic observations have shown the presence of anthocyanins in a non-complex form in the vacuoles, in the first external layers of the skin hypodermal tissue. In particular anthocyanins have been found in spherical pigmented inclusions, membrane-delimited, known as anthocyanic vacuolar inclusions, which contain also tannins and organic compounds. Moreover anthocyanins are also present in the cytoplasm into vesicles called anthocyanoplasts, which are probably responsible for their transport into the vacuole. Flavan-3-ols and tannins are instead localized both in the vacuoles and in the cell wall of berry skin. Inside the vacuole, tannins are present in the form of free tannins, while in the internal face of tonoplast and in the cell wall they are linked respectively to proteins and to polysaccharides. The composition of tannins from the cell wall compared to those localized in the internal part of the cell is quite similar, even if in the cell wall the mDP is 2-3 times higher than that of the vacuole and tonoplast. Regards to flavonols, with fluorescence induction towards different reagents, it has been demonstrated their localization into the cell wall of spruce needles (Hutzler *et al.*, 1998).

Further the flavonoids transport mediated by a complex vesicle trafficking system, it was supposed also a ligandin transport model, which involves tonoplast primary and secondary active transporters (Grotewold and Davies, 2008). The proposed mechanisms were studied, at the beginning, mainly in *A. Thaliana*. The first kind of active transporters belong to the ATP-binding cassette (ABC) proteins, which share a common ATP-binding domain. The involvement of the ABC proteins in flavonoids transport was suggested for the first time in the *bronze2* (*bz2*) mutant of maize, defective in the accumulation of anthocyanin in the vacuole (Marss *et al.*, 1995). The *bz2* gene encodes for a glutathione S-transferase (GST) and, as ABC proteins have a preference for gluthatione conjugates and their transport activity is stimulated in presence of glutathione, the involvement of ABC proteins in the vacuolar transport of anthocyanin was presumed (Yazaki, 2006). Recently five GSTs were purified from *Vitis Vinifera* L. Cv. Gamay Fréaux cell suspensions.

The GSTs are all soluble or membrane associated dimers, which catalyse the conjugation of the tripeptide glutathione (γ-Glu-Cis-Gly) to a variety of electrophilic compounds. On the basis of intron structure and protein sequence alignments, three of the five sequences cloned were classified in type τ GST (VvGST2, VvGST3 and VvGST1), while the remaining two in type ϕ GST (VvGST1 and VvGST5). In particular, the transcriptional profiling of VvGST1 and VvGST4 was strongly positive correlated with some key genes in anthocyanins biosynthesis pathway and complementation

studies in *bz2* maize mutants confirmed their important role in anthocyanins transport, also in increasing the turnover of intermediates (Conn *et al.*, 2008).

Secondary active transport involves, instead, a proton gradient-dependent transport, depending on a preexisisting H^+ gradient across the vacuolar membrane generated by vacuolar ATPase and vacuolar H^+-pyrophosphatase (Klein *et al.*, 1996). In *Vitis Vinifera* L. Cultivar Shiraz Gomez *et al.* (2009) reported the identification of genes encoding proteins with high sequence similarity to the Multidrug And Toxic Extrusion (MATE) family and among them *antho*MATE1 (AM1) and *antho*MATE3 (AM3) seem to be the most probable candidates as anthocyanin transporters. AM1 and AM3 are expressed mainly in the berry and they are primarily localized in the tonoplast. Different experiments revealed that they are vacuolar H+-dependent transporters specialized in acylated anthocyanins transport. This suggests the existence of alternative mechanisms involved in the transport of glucosylated anthocyanins (Gomez *et al.*, 2009).

Another secondary active transporter, homologue to mammalian bilitranslocase (BTL), has been described both for red (Braidot *et al.*, 2008a) and white grape berries (Bertolini *et al.*, 2009). This protein is a membrane protein of about 31 kDa and, towards immunohistochemical identification, an anti-BTL antibody allows localizing BTL-homologue in the skin of red grape berry and also in vascular bundle of berry pulp. The analysis of the BTL-homologue expression profile in different tissues during ripening has suggested that this protein could have a key role not only in anthocyanin accumulation, but also for intermediate metabolite translocation during berry development, in particular in physiological conditions associated with a low transport efficiency as during the late ripening stage (Braidot *et al.*, 2008b).

1.5 Flavonoid gene expression during berry development

The pattern of flavonoid gene expression is very different among different organs and among the different phases of berry ripening. Data about the trend in gene expression of some structural genes of flavonoid biosynthesis in berry skin compared to flesh, have been published by Boss *et al.* in 1996 (a) for red cultivar Shiraz. Northern blot analysis of berry skin RNA have shown that the majority of genes involved in flavonoid biosynthesis are just expressed from flowering to 2-4 weeks after full bloom and then after véraison. Only the expression of the gene UDP Glc-Flavonoid 3-O-glucosyl Transferase (UFGT) is strictly linked with véraison phase. In berry flesh all the genes analyzed are expressed at lower levels compared to skin except for Phenylalanine Ammonia Lyase (PAL) and UFGT, whose expression was not detectable in the flesh at any stage of the analysis (Boss *et al.*, 1996a). Similar experiments conducted in white cultivars have shown that in white berries UFGT is never expressed (Boss *et al.*, 1996b). The early expression of the genes from CHS to LDOX and FLS, LAR and ANR could be explained by the accumulation of flavonols and tannins in the young berry. Regards to the genes encoding anthocyanin modifying enzymes, it was demonstrated that the gene VvF3'H-1 (the only functional isoform identified) is expressed in berry skin of cultivar

Merlot throughout ripening, even if its expression decreases 8–10 weeks after blooming. VvF3'H-1 transcripts increase soon after, at the onset of véraison and shows a secondary peak of expression one week before harvest, concurrently at the peak of anthocyanin content. VvF3'5'H-1 is instead weakly expressed during the first stages of berry ripening and the peak of expression is reached when all berries have turned red. Transcripts of the isoforms VvF3'5'H-2-long and VvF3'5'H-2-medium appear at the onset of véraison and lasted till full ripening, while the isogene VvF3'5'H-2-short was constitutively expressed throughout the ripening (Castellarin *et al.*, 2006).). In cv Shiraz both F3'H and F3'5'H transcripts were detected at flowering and in the early stages of berry ripening, but their expression reaches a maximum one week before and week after véraison respectively (Bogs *et al.*, 2006). Transcriptomic analyses on Shiraz and Nebbiolo grape berries, harvested at different ripening stages, have shown that in these varieties the expression of AOMT follows the same profile. AOMT is not expressed before the onset of ripening, but, after véraison, there is a strong increase in the amount of transcripts during the first weeks and then the expression decreases and remains constant until harvest (Hugueney *et al.*, 2009). In pre- and post véraison stages berries of cultivar Shiraz was studied the transcription of the five isoforms of GST genes identified by Conn *et al.* (2008). The interesting thing is that the expression of GST is linked not only to berry ripening, but also it seems to be dependent on the kind of isoform. In fact expression of *Vv*GST2, *Vv*GST3 and *Vv*GST4 was found to be until 60-fold higher in post véraison berries compared to green berries, while for *Vv*GST1 and *Vv*GST5 the increase was lower. *Vv*GST1 was instead the most strongly induced in suspension cell cultures in conditions which enhance anthocyanin accumulation. These differences in the transcription profile could be due to interspecies variation or differential response of the five isoforms to specific conditions.

1.6 Regulatory genes of the flavonoid biosynthesis pathway

1.6.1 Regulation of anthocyanin biosynthesis

Among the three classes of flavonoids present in grapevine, the regulation of anthocyanins biosynthesis has been the most studied thanks to the numerous available colour bud sports, which involve alterations in the red or purple berry anthocyanin content. Bud sports are usually infrequent changes in phenotype affecting shoots of woody perennials, but colour bud sports are common and examples in horticultural plants are widespread. Alterations in the anthocyanins of a plant can be in fact very distinct and easily observed and generally have no detrimental effect on the viability of the plant. The molecular basis underlying the appearance of bud sports are not yet well understood.

The study of the genes regulating structural genes expression in anthocyanin biosynthesis was at first done in maize, petunia and snapdragon (Davies and Schwinn, 2003) and then full characterized in *Arabidopsis* (Broun, 2005). From this researches emerged that three different types of transcription factors play a key roles in regulation of anthocyanin biosynthesis: MYB, bHLH

(basic Helix-Loop-Helix) and WDR (WD40 repeats), even if the WD40 proteins function seem to be only to assist MYB and bHLH proteins.

MYB genes are present in plant genomes as different families, which have evolved after duplications of their DNA binding domain. In the DNA binding domains of MYB genes there are 100-160 residues, depending on the number of imperfect repeats (R) of the N-terminal region. The MYB R2R3 subfamily is the most abundant in plants (Martin and Paz-Ares, 1997). The first experience on MYB genes in grapevine was done on the cultivar Kyoho, which belongs to *Vitis labruscana* (*V. Labrusca x V. Vinifera*). With cDNA of ripening berries was constructed a cDNA library and then, using random primers designed on maize MYB conserved sequences, eight different grape MYB homologue sequences were identified. Among these, only four species of MYB gene can be really identified: MYBA, MYBB, MYBC, and MYBD. The whole cDNA sequences of MYBA and MYBB showed the existence of three different species for MYBA (VlMYBA1-1, VlMYBA1-2 e VlMYBA2) and two for MYBB (VlMYBB1-1 e VlMYBB1-2), which are different in the nucleotide sequence. Transcription analyses revealed that VlMYBA is only weakly expressed before véraison, but immediately after the beginning of anthocyanin accumulation and berry softening, VlMYBA expression rapidly increases. VlMYBB expression was instead detected in all the berry ripening stages analyzed even if also VlMYBB expression increases after véraison. Regards to VlMYBC and VlMYBD, these genes seems to be few linked to berry véraison because the first one is expressed at high levels just at the beginning of ripening and at véraison the transcription decreases; in the case of VlMYBD, the expression was detected only in the early stages of berry ripening. The tight relationship between VlMYBA and berry véraison and anthocyanin accumulation was further confirmed by expression analyses which revealed that VlMYBA transcripts, differentially from the others VlMYB genes identified, were detected only in berry tissues. The introduction of VlMYBA1-1, VlMYBA1-2 e VlMYBA2 in embryos causes the appearance of reddish-purple spots and so the authors concluded that VlMYBA was the transcription factor regulating UFGT expression (Kobayashi *et al.*, 2002). The molecular basis of the regulation of UFGT expression were then confirmed also for *Vitis Vinifera* and it was shown that VvMYBA was probably responsible of the ancestral differentiation between red and white cultivar. The white-skinned varieties Italia and Muscat of Alexandria have, in fact, a GRET1 retrotransposon inserted in both the alleles of the promoter region of VvMYBA1 gene and this causes the inactivation of its expression. The recovery of the ability to accumulate anthocyanin by the cultivar Ruby Okuyama, an Italia red-skinned sport, was in fact attributed to the missing of one unfunctional allele caused by the excision of the GRET1 retrotransposon (Kobayashi *et al.*, 2004). Further researches on Malian and Shalistin, two new grape cultivars, which have arisen as buds sports of Cabernet Sauvignon, demonstrated that in grapevine exists a berry colour locus in which are associated the VvMYBA1 and VvMYBA2 genes, either capable to activate UFGT (Walker *et al.*, 2006), and that, in white-skinned cultivar, the presence of GRET1 in VvMYBA1 is often accompanied by two non-conserved mutations (one amino acid substitution and one frame shift)

that leads to a smaller and unfunctional VvMYBA2 protein. From this studies emerged that in the grape pigmented skin berry varieties have to exist at least one functional VvMYBA1 allele, redVvMYBA1 (rVvMYBA1), and one functional VvMYBA2 allele, redVvMYBA2 (rVvMYBA2) (Walker *et al.*, 2007).

In nature, the colour of grape berry ranges from the lightest pink to the darkest purple tones according to the amount and quality of anthocyanin accumulated in the berry skin. This suggests a polygenic control of anthocyanin accumulation in *Vitis Vinifera*, despite evidence that the primary determination of anthocyanin production in berries appeared to be controlled by a single dominant locus (Barrit and Einset, 1969). As a result, many authors try to find out the presence of polymorphisms in the allelic profile of rVvMYBA genes. Recently, on a Shiraz x Grenache F1 pseudo-testcross (represented by 191 individuals) was mapped a single QTL responsible for up to 62% of the anthocyanin content variation. Among the 68 unigenes identified, a cluster of four VvMYBA genes (VvMYBA1, VvMYBA2, VvMYBA3, VvMYBA4) was considered essential to determine the genetic bases of the quantitative variation of anthocyanin content in berry skin. Five polymorphisms in three gene belonging to this cluster (one retrotransposon in VvMYBA1, 3 Single Nucleotide Polymorphisms one in VvMYBA1, one in VvMYBA2 and one in VvMYBA3 and one 2bp indel in VvMYBA2) were in fact enough to account for 84% of the observed anthocyanin quantitative variation on a core collections of 141 genotypes (Fournier-Level *et al.*, 2009).

After the identification of VvMYBA1, the first expression analyses of this gene in grapevine were done by Jeong *et al.* (2004) on conditioned berry with the aim to alter anthocyanin accumulation. The change in expression levels of VvMYBA1 coincided with the changes in the expression levels of other tested anthocyanin biosynthetic enzyme genes (CHS1, CHS2, CHS3, CHI1, CHI2, F3H1, F3H2, DFR, LDOX and UFGT) and also with the accumulation of anthocyanins in the berry skins. This suggests that the product of VvMYBA1 gene may control the transcription of not only UFGT, but also of other structural genes, whose expression increase after véraison. Remains still unclear how could be regulated, at transcriptional level, the genes from the pathway in pre-véraison stages. Recently other two MYB genes (VvMYB5a and VvMYB5b) have been cloned from grape berry cDNA (Deluc *et al.*, 2006; Deluc *et al.*, 2008). Different promoters of grapevine genes encoding enzymes of the flavonoid pathway were used to test the ability of VvMYB5a and VvMYB5b to activate them. The results indicated that either VvMYB5a and VvMYB5b can enhance the expression of grape structural genes involved in the common flavonoid pathway (VvCHI, VvF3'5'H, VvLDOX) together with the expression of specific genes involved in proanthocyanidin biosynthesis, but their action produce no effects on UFGT expression. In berry, VvMYB5a expression is high in the early stages of berry development and then decreases rapidly to a very low level after véraison. The expression of VvMYB5b follows, instead, a different pattern: in whole berries it appears expressed throughout all the development with a decrease at véraison and a strong accumulation of the transcripts two weeks after véraison. Among the different berry tissues, both the genes are expressed mainly in the

skin (Deluc *et al.*, 2006; Deluc *et al.*, 2008). Taken together, these information indicate a probable direct involvement of VvMYB5a and VvMYB5b in the transcriptional control of genes of the flavonoid pathway in the early stages of grape berry development.

To date, no grapevine bHLH or WDR proteins involved in anthocyanin accumulation have been isolated. Many authors reported the presence of a WDR protein expressed exclusively in the pigmented berry tissues (Ageorges *et al.*, 2006) and the MYB genes necessity, in transient expression system assays, of the presence of a bHLH protein to induce promoter activities (Walker *et al.*, 2007; Deluc *et al.*, 2008).

1.6.2 Regulation of flavonols and proanthocyanidins biosynthesis

Three R2R3-MYB transcription factors (AtMYB12/PFG1, AtMYB11/PFG2 and AtMYB111/PFG3) involving in flavonols biosynthesis regulation have been recently described in *Arabidopsis* (Merthens *et al.*, 2005; Stracke *et al.*, 2007). Using the conserved R2R3 repeated region of AtMYB12 gene, two similar sequence (VvMYBF1 and VvMYBF2) were isolated in the *Vitis Vinifera* genomic sequence. VvMYBF1 was successively characterized and analysed in cv. Shiraz and Chardonnay. In Shiraz berry skins, VvMYBF1 expression was highest four weeks before véraison, then decreased and two smaller peaks were detected around véraison and six weeks later. This expression profile well correlates either with VvFLS1 expression, but only partially with flavonol accumulation in Shiraz berry skins. Towards transient expression experiments using Chardonnay grape suspension were identified the structural genes of the flavonoid pathway activated by the VvMYBF1 gene. VvMYBF1 is able to induce the promoters of VvFLS1, confirming its specificity for flavonols synthesis, and also the promoters of AtCHS and VvCHI, suggesting that it can activate the genes providing substrates for flavonols synthesis. Surprisingly VvMYBF1 induces also the activation of VvLDOX gene, suggesting an alternative pathway to produce flavonols in plants via the DFR activity. As just demonstrated in *Arabidopsis* for AtMYB12, also VvMYBF1 does not require the presence of a bHLH protein for the activation of the genes, from them regulated (Czemmel *et al.*, 2009).

The mechanisms involved in the fine spatiotemporal regulation of proanthocyanidins biosynthesis remain still unclear for grape berry. In *Arabidopsis Thaliana* at least six transcription factors (belonging to MYB, bHLH, WD40, WRKY, zinc finger and MADS box proteins) were found to regulate this pathway (Lepiniec *et al.*, 2006). In grapevine berry the transcription factors VvMYB5a (Deluc *et al.*, 2006), VvMYB5b (Deluc *et al.*, 2008) and VvMYBPA1 (Bogs *et al.*, 2007) were identified as involved in the proanthocyanidins biosynthesis pathway. In particular, both VvMYB5b and VvMYBPA1 are able to activate VvANR and VvLAR1 genes, while VvMYB5a does not activate the VvANR promoter. Recently, an additional transcription factor (VvMYBPA2) for the regulation of proanthocyanidin biosynthesis has been identified (Terrier *et al.*, 2009). The expression profile of these regulatory genes is very different at level of berry developmental stage and at level of berry

tissue localization and these results could explain the existence of different transcription factors with apparent redundant function.

1.7 Factors affecting flavonoids biosynthesis

Biosynthesis of flavonoids is affected by many endogenous (as genetic variation, plant growth regulator and developmental processes) and exogenous factors (as environmental conditions, soil type, water and nutritional status, microbial interactions, pathogenesis and wounding). Among these factors, site and season seems to have the greatest influences on the flavonoid content (Downey *et al.*, 2006). Separating the effect of each parameter on the biosynthesis of each flavonoid class is very difficult. In addition, it has to be considered that the same factor could be, in different conditions, an enhancer or an inhibitor of the same step of flavonoids biosynthesis. Following, it will be described the effect of three primary factors affecting flavonoids biosynthesis on anthocyanins and flavonols amount and composition: sugars, light and temperature.

1.7.1 Factors affecting anthocyanins amount and composition: sugars, light and temperature

1.7.1.1 Sugars effect on anthocyanin amount and composition: flavonoids gene sugars-induced

The relationship between sugars and anthocyanins accumulation was early proposed by Pirie and Mullins (1977), which initially established that sugars skin content of red grapes could have a regulatory role in anthocyanins production. Few years later, Roubelakis-Angelakis and Kliewer (1986) found in vitro an increase in PAL activity, following sugars treatment. The sucrose-induced production of anthocyanins was at first demonstrated in Petunia and *Arabidopsis* (Tsukaya *et al.*, 1991; Ohto *et al.*, 2001), where it was found the up-regulation of CHS, after sucrose administration. Solfanelli *et al.*, (2006) reported successively in *Arabidopsis* the up-regulation of almost all structural genes involved in the pathway and also of the MYB regulatory gene PAP1 after sucrose treatments. The concomitant accumulation of flesh soluble solids and skin anthocyanins in red grape varieties, suggested also a possible sugars-induced anthocyanin accumulation during berry ripening. The first experiences on *V. Vinifera* cell suspensions confirmed that sucrose treatment promotes anthocyanin biosynthesis (Vitrac *et al.*, 2000). At the beginning, it was suggested that cell response in terms of anthocyanin accumulation, could be linked only to the high osmotic potential triggered by high sugars levels (Do and Cormier, 1991); many studies have instead shown that sugars are not only carbohydrates sources, but also acts as signal molecule. Until now, in grape it has been demonstrated the up-regulation sugars-induced (in particular by both glucose and fructose) of different structural genes of anthocyanin pathway as CHS, DFR and LDOX (Gollop *et al.*, 2001; Gollop *et al.*, 2002) and more recently F3H (Zheng *et al.*, 2009). Interestingly, DFR and LDOX genes possess sucrose boxes in their promoter region, which seems to be responsible of the sugars induction. The sensitivity of grape berries to sugars varies during fruit development, being highest at stage of véraison. The sugar-sensing mechanism operating in grape are poorly understood.

Probably the sugar signal is transmitted via a transduction involving variation of Ca^{2+} concentrations, kinases and phosphatases (Vitrac *et al.*, 2000). In particular, in the case of F3H, it was suggested a key role of a Hexokinase dependent pathway in controlling the expression of F3H RNA level (Zheng *et al.*, 2009).

1.7.1.2 Light and temperature effects on anthocyanin amount and composition

It has often being difficult to separate the effect of light and temperature in the field. The approaches used to investigate into the effects of light on anthocyanins biosynthesis involving the application of a range of shading or exposuring cluster treatments, which resulted in different exposure levels of fruit and sometimes of leaves (with concomitant impact on photosynthesis). The application of these treatments on grape bunches involves also modification in their temperature. In general, an increasing vine temperature may accelerate the rate of some metabolic process, even if in grape it has been demonstrated that above 30°C, many metabolic pathway stop or are significantly reduced. In general, a low light cluster light exposure caused a significant decrease in anthocyanin content. In contrast, the anthocyanin amount does not increase linearly with the increase of sunlight exposure, as a consequence of the combined cited temperature effect. However cluster position on the east or wesr side of the vine seems to be a key factor in determining the result of increased light on anthocyanin biosynthesis (Bergqvist *et al.*, 2001). Spayd *et al.*, 2002 monitored the temperature of east-exposed (receiving only morning sun) and west-exposed (receiving afternoon sun) clusters, demonstrating higher temperature for cluster on the west-side of the canopy. To distinguish the temperature effect from the light one, west-exposed clusters were cooled to the temperature of shaded clusters and shaded clusters were then heated to the temperature of west-exposed clusters. The skin of west-exposed clusters showed the lower anthocyanins levels, but the measured increase in skin anthocyanins, when west-exposed clusters were chilled, suggesting a primary temperature role. This was confirmed when it was also observed that Shiraz bunches closed into lightproof boxes, which excluded light without modifying temperature, presented at harvest an anthocyanin content comparable to normally exposed fruits (Downey *et al.*, 2004). It has been suggested that phenols biosynthesis could be controlled by light quality, in particular by the photochemical effects of red and UV radiations (Pereira *et al.*, 2006). In the case of grape anthocyanins, this cannot still be confirmed since it was reported that skin of Merlot berries, shielded by UV-barriers from bunch closure stage to harvest time, showed a similar anthocyanin content to control clusters (Spayd *et al.*, 2002). Very interesting seems to be the light effect on anthocyanin composition. Shifts towards a higher proportion of 3'-substituted anthocyanins (based on cyanidin and peonidin) were associated to artificial shading treatments (Downey *et al.*, 2004; Bucchetti, 2004), but also to full cluster light exposure, obtained with different canopy managements (Guidoni *et al.*,2004; Bucchetti *et al.*, 2007; Filippetti *et al.*, 2009). In addition, a decrease in 3'- substituted anthocyanins was obtained by screening the 98% of UV radiation (Spayd *et al.*, 2002). Malvidin 3-O glucoside seems to be the less sensitive to lower light

exposure levels. A shift from nonacylated glucosides to coumaroyl glucosides was also associated to cluster shading (Haselgrove *et al.*, 2000).

Temperature effect seems to be particularly significant after véraison and, as previously seen, it can be affirm that high diurnal temperature generally decrease anthocyanin amount (Yamane *et al.*, 2006; Mori *et al.*, 2007a). Very interesting is also the effect of different night temperature on anthocyanin accumulation. The heat night, in fact, contributes to decrease anthocyanin content (Mori *et al.*, 2005). The temperature effect on anthocyanin composition has been studied in potted Pinot Noir vines located in a phytotron. The results indicated that the amount of all 3'5'-substituted anthocyanin, except malvidin 3-O glucoside, was reduced by high temperature (Mori et al., 2007b).

1.7.1.3. Light influence on anthocyanin gene expression

Photoreceptors as phytochromes, cryptochromes and phototropin are ubiquitous in fruits. In *Arabidopsis* the light induction of flavonoid biosynthesis is very complex and involves phytochromes and cryptochromes, respectively photoreceptors of red/far-red and UV/blue light. First researches on importance of light induction in flavonoids biosynthesis focused only on CHS, demonstrating that light induces accumulation of CHS transcripts (Jenkins, 1997). Recently, light regulatory units (LRUs) sufficient for light responsiveness, were identified in the promoters of *Arabidopsis* CHS, CHI, F3H genes (Hartmann et al., 2005). Experiments successively conducted on seedlings subjected to light treatment, showed that light induces the immediate expression of CHS and F3H genes, and after also transcripts accumulation of DFR and LDOX, specific genes in anthocyanin biosynthesis, was detected. In addition, an induction light-mediated was demonstrated for two flavonoid biosynthesis regulatory MYB genes, PAP1 and PAP2 (Cominelli et al., 2007). The effects on anthocyanins biosynthesis of light at different wavelength have been shown that UV-A light seems to induce a lower effect in stimulating gene expression compared to white and blue light, even if all transcripts of CHS, F3H, DFR and LDOX were induced. UV-B exposure induces CHS in a similar way to UV-A light, but does not affect DFR and LDOX expression. In the case of F3H, it was detected only a transient accumulation of transcripts after UV-B treatment (Cominelli et al., 2007). The researches on light influence on anthocyanins biosynthesis genes were conducted in grapevine with different approaches. When in seedlings and cell cultures anthocyanin gene expression is induced by light *in vitro*, almost all genes appear to be constitutively expressed (Sparvoli et al., 1994). Also in grape, DFR and LDOX possess in their promoter region light regulatory units (Gollop et al., 2001; Gollop et al., 2002). The light intensity reductions in field condition results in different responses of anthocyanin gene expression, which seem to be cultivar dependent. Experiments conducted on Cabernet Sauvignon bunches in which light intensity was reduced, resulting in a down-regulation of almost all grape structural genes (CHS, CHI, F3H, DFR, LDOX, DFR, UFGT) and MYBA1 regulatory gene (Jeong et al., 2006). On the contrary the artificial shading of Shiraz or Sangiovese bunches does not affect the global anthocyanin gene expression,

which was similar to exposed bunches or caused only a delay in their activation (Downey et al., 2004; Bucchetti et al., 2004).

1.7.1.4 Temperature influence on anthocyanin gene expression

It has been hypothesized that temperature-mediated alteration of membrane fluidity may be the primary temperature sensing mechanism in higher plants. This suggests that sensors located in microdomains of membranes are capable of detecting physical phase transition, eventually leading to conformational changes and/or phosphorylation/dephosphorylation cycles, due to changes in temperature. Plant response to high temperature involve so morpho-anatomical, physiological and biochemical changes, which sometimes have to occur very rapidly. The molecular response to heat stress involve both the induction of enzymes involved in oxidative stress (as Peroxidase, Superoxide Dismutase, Ascorbate Peroxidase, Catalase) and Heat Shock Proteins (HSP, Wahid et al., 2007). As previously seen, anthocyanin biosynthesis is inhibited by higher temperature and this is confirmed by molecular studies. Yamane et al., 2006, showed, in the red table grape cultivar Aki Queen, that high temperature decreased mRNA accumulation of structural anthocyanin biosynthesis genes and MYBA1. In Pinot Noir vines, instead, the decrease in anthocyanin content due to high temperature treatment cannot to be linked to a decrease in anthocyanin biosynthesis, as no significant differences in gene expression were found. These results led the authors to suggest that the reduction in anthocyanin biosynthesis may be due to enzyme inhibition or anthocyanin degradation rather than a transcriptional effect (Mori et al., 2007a).

1.7.2 Light effect on flavonols biosynthesis

Differentially from what previously seen for anthocyanin, it is well known that temperature had little to no effect on flavonols biosynthesis. The higher flavonol content of sunexposed berries could so be attribute only to light effect. Flavonols are synthesized to screen UV radiation and their role is compatible with the induction by a light stress. Many authors reported the rapid flavonols increase following cluster exposure and stimulation by UV-light (Spayd et al., 2002; Downey et al., 2004; Pereira et al., 2006). Regards berry flavonol composition, some authors reported only few effects of light in altering proportion of different flavonols (Spayd et al., 2002; Tarara et al., 2008), even if myricetin 3-glucoside seems to be the less affected (Pereira et al., 2006). Further confirmations of what seen about light requirement for flavonol biosynthesis, were given observing that the expression both of FLS, the key enzyme responsible of flavonols synthesis, and its regulatory gene, VvMYBF1, rapidly increase as a consequence of cluster exposure (Downey et al., 2004; Czemmel et al., 2009).

1.8 Cultural practices improving grape berry composition

Despite the fact that the knowledge about the molecular mechanisms which regulate final grape berry composition is continuously enriched by new researches and by the sequencing of whole grape genome (Jaillon et al., 2007), in viticulture traditional approaches to improve berry

composition are often used. As a consequence of many complex factors as the higher heterozigosity, which is a typical characteristic of fruit tree, the rigidity of wine production legislations and the market exigencies, it was usually preferred to employ some cultural practices, which easily and directly could modify the parameters of interest. Among all the cultural practices applied in grapevine, summer pruning acts on the modification of the source/sink balance and/or on the alteration of clusters microclimate. In particular, defoliation of shoots at different stages of berry development and ripening and cluster thinning have been demonstrated to be very efficient in improving berry composition, even if they work through different mechanisms of action. Defoliation consists, in fact, on the removal of total or only a part of shoots basal leaves, and, eventually, on the removal of the laterals which develop from the same basal shoots nodes. Cluster thinning acts, instead, directly on reduction of vine production. In both cases, the results obtained by the application of defoliation or cluster thinning are strictly linked to the different stage in which they are applied.

1.8.1 Pre-bloom defoliation

Pre-bloom defoliation is applied at stage of fully developed inflorescences (single flowers separated; phenological stage 17, according to Eichorn and Lorenz, 1977). Generally, six-eight main basal leaves and the laterals present at the same nodes, are removed, manually or mechanically. Many studies reported that source limitations occurring at the onset of flowering can cause a sharp reduction in berry set percentage (Coombe, 1959; Caspari and Lang, 1996). With pre-bloom defoliation a similar result can be obtained, since photosynthetically active leaves, which represent the primary source of carbohydrates for the developing inflorescences, are removed. Due to pre-bloom defoliation, clusters morphology is induced to change and, at harvest, clusters of defoliated vines present a low number of berries, berries are smaller and, as a result, bunches are in general looser compared to those of non defoliated vine. Besides, clusters of defoliated vine are full exposed to light and well-airy during all development and ripening. This approach has a positive effect in decreasing rot infections and it is very effective in those varieties which are characterized by excessively tight bunches and high productivity. Experiences conducted in different red varieties as Sangiovese (Poni *et al.*, 2006; Intrieri *et al.*, 2008; Filippetti *et al.*, 2009) Barbera, Lambrusco Salamino (Poni *et al.*, 2009), Pinot Nero and Nero d'Avola (Di Lorenzo, 2005) have been demonstrated that early defoliation can also improve berry composition. In particular, early defoliation is able to induce a general increase in soluble solids concentration and pH and a decrease in titrable acidity (often due to the lower malic acid concentration). Among flavonoids, until now, only studies on anthocyanins have been published and the results indicate that early defoliation acts on promoting anthocyanin biosynthesis and on influencing their final composition, with an increase in 3'-substituted anthocyanins as cyanidin and peonidin 3-O glucoside (Filippetti *et al.*, 2009). According to Poni *et al.*, 2006 and Intrieri *et al.*, 2008, the effect of pre bloom defoliation on improving berry composition could be attributed to the increase in leaf/area crop

weight ratio. The increase in leaf area/crop weight ratio seems primary related to the reduction in yield, via an effect induced by reduced fruit-set, as previously seen. The initial decrease in total leaf area caused by defoliation promotes, in the vine, the activation of a compensation mechanism through an increase in main leaves and/or laterals growth. This compensation has to occur very rapidly because it has been demonstrated that, around fruit set, the recovery is just completed (Poni *et al.*, 2008). As a consequence, at harvest, defoliated vines present generally total leaf area values comparable to the non defoliated ones. Berry sugars content is strictly linked to source/sink balance and so to leaf area/crop weight ratio. Young leaves, directly exposed to sunlight, are the only source of carbohydrates for ripening berries. In particular, Quinlan and Weaver (1970) demonstrated that defoliation promotes an acceleration in carbohydrates translocation towards clusters. Furthermore, the capacity, induced by defoliation, of photosynthetic compensation of the retained grapevine leaves results in higher net CO_2 exchange rates for defoliated vines compared to non defoliated ones (Poni *et al.*, 2006; Chanisvili *et al.*, 2004) Finally, it has to be considered that, as a result of the strong compensatory laterals regrowth, it could be observed a higher contribution of younger leaves in the canopy composition of defoliated vines (Poni *et al.*, 2006; Poni *et al.*, 2009).

All these mechanisms together may be considered as responsible of the enhancement of carbohydrate content for berry ripening and so of the increase in sugars content observed in defoliated vine berries. The change in cluster microclimate induced by early defoliation seems, instead, to be more responsible than the effect of modifications in source sink balance for the decrease in tritable acidity observed in defoliated vine berries. Increased bunch exposure and subsequent berry temperature increase might, in fact, trigger an acceleration in the process of malic acid degradation (Kliewer and Schultz 1964). The enzymatic activity of the Malic Enzyme (ME), which is able to degrade malic acid, is, in fact, promoted by higher temperature, while Phosphoenolpyruvate (PEP) carboxylase (one malic acid producing enzyme) activity is inhibited in the same conditions (Lakso and Kliewer, 1975). The observed effect of pre-bloom defoliation on increasing anthocyanins amount is very complex to explain, as, how previously seen could be attributed to a combined effect of sugars increase and microclimate modifications.

1.8.2 Véraison defoliation

Defoliation at the beginning of véraison is frequently used in viticulture with the aim to improve the local microclimate of the fruiting area. Véraison defoliation is applied on high density canopies to increase light exposure and air circulation around the bunches, with positive effects on berry composition and lower incidence of rot. Véraison defoliated vine presented, differently from what previously seen in the case of pre-bloom defoliation, a leaf area/crop weight ratio similar or lower than non defoliated vines. In fact, removal of main and laterals basal leaves of each shoot at beginning of véraison does not affect the global photosynthetic apparatus, since the removed leaves are more than 120 days old and they represent low active source organs. Besides, at véraison also clusters formation is just completed and as a results, following véraison defoliation, only few effects

are observed in terms of berry sugars content for different red varieties as Nebbiolo (Guidoni *et al.*, 2004), Barbera (Guidoni *et al.*, 2007) and Cabernet Sauvignon (Matus *et al.*, 2009). As previously seen for pre-bloom defoliation, the increased temperature associated to higher cluster exposure, induces an acceleration in malic acid degradation, which implies lower titrable acidity and higher pH for véraison defoliated berries (Guidoni *et al.*, 2004; Guidoni *et al.*, 2007; Kozina *et al.*, 2008).

Considering flavonoids biosynthesis and in particular anthocyanin amount, the results of véraison defoliation seem to be very linked to cultivar and to the climatic conditions of the season. Results of an increase in total anthocyanin were reported by many authors in the case of Cabernet Sauvignon (Matus *et al.*, 2009; Hunter *et al.*, 1991), Pinot, Nero d'Avola (Di Lorenzo, 1995) and Nebbiolo (Guidoni *et al.*, 2004). In other cases, as observed in Barbera (Guidoni *et al.*, 2007) or in Merlot (Pereira *et al.*, 2006), anthocyanins of véraison defoliated vine berries decreased after the treatment (Guidoni *et al.*, 2007). The relationship between anthocyanin composition and véraison defoliation has also been demonstrated to be cultivar dependent. Sometimes, in fact, véraison defoliation promotes a general increase in all anthocyanins, as in Cabernet Sauvignon (Matus *et al.*, 2009), while in the case of Barbera (Guidoni *et al.*, 2007) and Merlot (Pereira *et al.*, 2006)it has been showed a variety dependent selective increase of grape anthocyanins. Among the other two classes of flavonoids, only recently it was evaluated the impact of véraison defoliation on berry flavonols content, showing in all cases an increase in flavonols content in defoliated vine (Pereira *et al.*, 2006; Matus *et al.*, 2009). In particular, kaempferol 3-O glucoside and quercetin 3-O glucoside were the most significant flavonols induced by véraison defoliation, in berry skin of cultivar Merlot (Pereira *et al.*, 2006).

1.8.3 Cluster thinning

Cluster thinning is in general applied to prevent overcropping in cases of varieties characterized by excessive bud fertility or in climate conditions which promote a too much high floral bud induction. In these conditions, when it could occur an imbalance between productivity and vine vegetative growth, cluster thinning is performed to obtain a leaf area/yield ratio around 0.8-1.2 m^2/kg. Vines that fell within these values were considered well balanced and capable to fully ripen (Kliewer and Weaver, 1971; Intrieri and Filippetti, 2000; Kliewer and Dookozlian, 2005). The result of cluster thinning is strictly dependent on the date in which it is applied. Cluster removal early in the season, as around bloom, is very risky and may not lead to the desired result. The presence of fruits, in fact, stimulates the leaf photosynthesis rate in grapevine, and so the reduced sink size might lead to a feedback regulation of the leaf photosynthesis rate, causing a delay in berry ripening and a reduction in berry and wine quality (Naor *et al.*, 1997). Besides, many authors reported an increased berry weight for cluster thinned vines when thinning was performed around fruit set (Kliewer and Weaver, 1971; Weaver and Pool, 1972; Dokoozlian and Hirtschfelt, 1995). If cluster thinning is instead performed too late in the season, as when there is no more shoots growth, photosynthesis remains unchanged and surplus photoassimilates could bring to an excessive vine

vigor with negative effects on canopy microclimate (Smart *et al.* 1990). Therefore, the optimal cluster thinning timing is when shoots growth has slowed and cellular division is completed, as occurred at véraison, so that photoassimilates could be direct prevailingly towards bunches (Keller *et al.*, 2005). In addition to the timing of cluster thinning, also the amount of fruit removed may be important. This aspect have to be evaluated each season on the basis of the climate conditions and of the vine productivity. In fact, a field study on mid-crop level Cabernet Sauvignon reported any improvement in berry composition following neither 33% nor 66% cluster thinning performed two weeks after bloom (Ough and Nagaoka, 1984). In a study conducted over three years, it has been demonstrated that, until medium-low productivity levels (around to 12 t/ha), cluster thinning does not involve consistent differences in fruit composition of field-grown Sangiovese. In contrast, in vines that are overcropped, 33% of cluster removal at véraison is more efficient than 66%, in improving berry composition (Filippetti *et al.*, 2007). Improvement in berry composition following cluster thinning could be attributed to the modification in leaf area/crop weight ratio as it does not affect local cluster microclimate. When leaf area/crop weight ratio reaches values around or over 1 m^2/kg, berries of cluster thinned vines are generally characterized by higher soluble solids content (Filippetti *et al.*, 2007; Intrieri *et al.*, 2005; Keller *et al.*, 2005; Guidoni *et al.*, 2002; Guidoni *et al.*, 2008). Kliewer and Weaver, 1971 reported in fact a positively correlated relationship between soluble sugars and the increase in leaf area/crop weight ratio. Cluster thinning could possibly also increase pH and lower titrable acidity (Jackson and Lombard, 1993). Regards to flavonoids, it has been demonstrated that in general anthocyanins total amount increase with the increase of leaf area/crop weight ratio. Also anthocyanin composition is affected by cluster thinning, as both in Sangiovese and Nebbiolo an increased 3'-substituted composition, which depending on the varieties could be explained by an increase in cyanidin 3-O glucoside or peonidin 3-O glucoside respectively (Filippetti *et al.*, 2007; Guidoni *et al.*, 2008).

1.9 Aims of the research

It is well known that the best grape quality can occur only through the achievement of optimal source/sink ratio. Vine balance is in fact a key parameter in controlling berry sugar, acidity and secondary metabolites content (Howell, 2001; Vanden Heuvel et al., 2004).

Despite yield reduction and quality improvement are not always strictly related, cluster thinning is considered a technique which could lead to improvement in grape sugar and anthocyanin composition (Dokoozlian and Hirschfelt, 1995; Guidoni *et al*., 2002).

Among several microclimatic variables which may impact grape composition, the effect of cluster light exposure and temperature, which probably act in synergistic and complex way, has been widely explored showing positive even sometimes contradictory results (Spayd et al., 2001; Tarara et al., 2008). Pre-bloom and véraison defoliation are very efficient techniques in inducing cluster microclimatic modification. Furthermore pre-bloom defoliation inducing a lower berry set percentage may act also on yield reduction (Intrieri et al., 2008; Poni et al., 2009).

On these basis the aim of the first experiment of the thesis was to verify in cv Sangiovese the effects on ripening and berry composition of management techniques which may increase source/sink ratio and /or promote light incidence on berries throughout grape ripening.

An integrated agronomic, biochemical and microarray approach, aims to understand which mechanisms are involved in berry composition and may be conditioned in the berries during ripening in vines submitted to three treatments.

In particular the treatments compared were:

a) cluster thinning (increasing in source/sink ratio)

b) leaf removal at véraison (increasing cluster light exposure)

c) pre-bloom defoliation (increasing source sink ratio and cluster light exposure).

Vine response to leaf removal at véraison was further evaluated in the second experiment on three different varieties (Cabernet Sauvignon, Nero d'Avola, Raboso Piave) chosen for their different genetic traits in terms of anthocyanin amount and composition.

<u>EXPERIMENT 1</u>: physiological, molecular and biochemical response of cv. Sangiovese (*Vitis Vinifera* L.) to early defoliation (pre-bloom), late defoliation (véraison) and cluster thinning.

2.0 MATERIAL AND METHODS

2.1 Plant material and leaf area measurements

The trial was conducted in 2008 and 2009 on adult Sangiovese (Vitis Vinifera L.) vines, clone 12T grafted to SO4, in a vineyard in Bologna, Italy (44°30'N, 11°24'E), with North–South oriented rows. Vine spacing was 1 m x 2.8 m and the training system was a vertical shoot positioned spur pruned cordon (12 buds per vine), with cordon height at 1.0 m above ground and a canopy height of about 1.3-1.4 m. Pest management was conducted according to local practice of Regione Emilia Romagna. Shoots were manually trimmed when most started to outgrow the top wire. This occurred on Julian Day (JD) 192 in 2008 and JD 160 in 2009. Nine vines per treatment in three blocks were selected in a single uniform row and each vine was randomly assigned to the following treatments: a) control (C), no treatments; b) pre-bloom defoliation (Early Defoliation, ED), hand defoliation of six basal leaves at stage 17 (inflorescence fully developed, single flowers separated according to Eichorn and Lorenz, 1977; JD 147 in 2008 and JD 141 in 2009); c) véraison defoliation

Fig. 1. Different treatments applied to Sangiovese vines in the experiment. A. Early defoliation (ED) at pre-flowering stage; B. Cluster Thinning (CT) at véraison; C. Late Defoliation (LD) at véraison.

(Late Defoliation, LD), hand defoliation of six basal leaves at véraison (JD 212 in 2008 and 210 in 2009); cluster thinning (CT), removal of 50% of total cluster per vine at véraison (JD 211 in 2008 and 210 in 2009) (Fig.1).

Each vine in trial was uniformed per bud load and cluster number at flowering. In the defoliation treatments, any laterals growing in the 6 basal node shoot zone were also removed. Leaf area removed in the ED and LD treatments and after shoot trimming were measured with a leaf area meter (LI-3000A, Li-Cor Biosciences, Lincoln, Nebraska, USA), maintaining separate the main and laterals contributions. To estimate leaf area before defoliation treatments per vine and final leaf area per vine, a linear relationship between shoot length and leaf area for at least 15 shoots per treatment was achieved. Each shoot per vine was then measured and the shoot length used to estimate leaf area towards the previously described linear relationship. Two separate correlations for main and laterals were established (Table 1).

	Year	Source of shoots	Linear relationship used to calculate leaf area
Before ED	**2008**	**Vines out of trial**	**Principal: y=14.591x (R^2=0.90)**
			Laterals: y=9.3734 (R^2=0.94)
	2009	**Vines out of trial**	**Principal: y=12.227x (R^2=0.91)**
			Laterals: y=5.7372 (R^2=0.82)
Before LD	**2008**	**Vines out of trial**	**Principal: y=16.353x (R^2=0.91)**
			Laterals: y=14.765x (R^2=0.97)
Harvest	**2008**	**C vines**	**Principal: y=16.212x (R^2=0.70)**
			Laterals: y=14.834x (R^2=0.70)
		ED vines	**Principal: y=12.093x (R^2=0.74)**
			Laterals: y=16.932x (R^2=0,95)
		LD vines	**Principal: y=10.306x (R^2=0.80)**
			Laterals: y=17.74x (R^2=0.91)
		CT vines	**Principal: y=13.441x (R^2=0.71)**
			Laterals: y=15.844x (R^2=0.89)
Harvest	**2009**	**C vines**	**Principal: y=19.605x (R^2=0.64)**
			Laterals: y=23.772x (R^2=0.91)
		ED vines	**Principal: y=13.577x (R^2=0.79)**
			Laterals: y=15.748x (R^2=0.96)

Table 1. Equations of the linear relationship used in 2008 and 2009 years to calculate leaf area per vine.

In 2009 the linear relationship was established only before the treatment of pre-bloom defoliation and not before véraison defoliation. At harvest, leaf area of LD and CT vines was estimated using the linear relationship obtained for C vines.

2.2 Temperature monitoring

Skin berry temperature was monitored in 2008 and 2009 in 4 selected clusters in a C vine and in a ED vine. For each of this two treatments, temperature data were collected from the stage 33 (beginning of bunch closure, berries touching, according to Eichorn and Lorenz, 1977; JD 182 in 2008 and JD 181 in 2009) to harvest (JD 266 in 2008 and JD 261 in 2009) towards eight T-type thermocouples (RS components, MI, Italy) positioned in the sub-cuticolar tissues of the berry skin (Fig.2). Four thermocouples were positioned on two different clusters, two in the east side and two on the west side of the cordon. For each side, one thermocouple was inserted in a berry located in the external part of the cluster and the other in the internal part. Each probe was then connected to a CR10X data logger (Campbell Scientific Ltd., Leicestershire, United Kingdom), registering temperature data every 15 minutes.

Fig. 2. Thermocouples positioning in the sub-cuticolar tissues of berry skin at the stage of green berries.

2.3 Berry sampling

In 2008 and 2009 berries were sampled weekly starting from the beginning to the end of véraison and then every two weeks until harvest. In 2009 the samples from harvest were kept separately per vine. For each treatment, samples of 18 berries were taken from three vines of each block and bulked to make one unique sample in order to monitor berry ripening. These berries were so immediately crushed and used to perform analyses of total soluble solids concentration (°Brix), pH and titratable acidity (TA).

For biochemical and molecular analyses an additional sampling at time of thermocouple positioning was effectuated. For HPLC analyses of skin anthocyanins and flavonols 20 berries

belonging to vines of the same treatment and block, were stored in a -20°C freezer until analysis was carried out.

Only in 2008, a sample of 30 berries, belonging to vines of the same treatment and block, was dissected directly in the field, immediately frozen in liquid nitrogen and stored at -80°C for subsequent microarray analyses.

2.4 Monitoring of berry ripening

The must obtained after crushing the samples taken for monitor berry ripening, was immediately filtered through a strainer and a drop was employed for °Brix analysis by a temperature-compensating CR50 refractometer (Maselli Misure Spa, PR, Italy). Five ml of the same must were then diluted 7 times with bi-distilled water and used to perform a titration reaction by a Crison Compact Tritator (Crison, Barcelona, Spain) with 1N, 0.5 N or 0.25 N NaOH (Sigma-Aldrich, St. Louis, MO, USA), according to the stage of berry ripening, to obtain pH and TA data (expressed as g/L of tartaric acid equivalents).

2.5 Evaluation of clusters morphology and analyses at harvest

At harvest, two clusters per vine (one situated in the east and one situated in the west side of the cordon) were individually picked, weighed and measured for cluster length and width to estimate compactness. Compactness was also visually estimated on each cluster using code OIV 204 (Organisation international de la vigne et du vin, 1983).

Each berries of these clusters was then grouped into six berry size categories defined as *a*≤ 0.6 g; *b*=0.6-1.2 g; *c*=1.2-1.8g; *d*=1.8-2.4g; *e*=2.4-3.0g; *f*≥3.0g. For each size categories, a maximum of 15 berries were immediately put in liquid nitrogen and stored in a –80°C freezer to carry out berry skin thickness measurements by a Universal Testing Machine (UTM) TAxT2j Texture Analyzer (Stable Micro Systems Surrey, UK) equipped with a HDP/90 platform and a 25 kg load cell. For berry skin thickness measurements, a piece of skin of ~0.25 cm^2 was removed at the lateral side of each sampled berry and then it was carefully positioned on the UTM platform to prevent folds in the skin. Berry thickness analysis were performed as reported in Letaief *et al.*, (2008). The remaining berries of each size categories (maximum 20 for class) were then separately crushed and soluble solids were determined on the juice with a handheld refractometer.

The remaining clusters on each vine were picked and individually weighed. For each cluster compactness was then evaluated towards measurements of cluster length and width and visually estimated using code OIV 204 (Organisation international de la vigne et du vin, 1983). Besides, a visual evaluation of sunburn and Botrytis damage (% for each cluster) was carried out.

2.6 HPLC analyses of anthocyanins and flavonols.

The berries sampled for HPLC analysis were weighed immediately before the extraction of anthocyanins and flavonols. The skin was then handly carefully removed from the pulp and

immediately immerged in 100 ml of HPLC-grade methanol (Carlo Erba Reagents, Milano, Italy). The extraction was carried out in the dark for 24 hours and the extract was separated from the skin residues and conserved at -20°C until HPLC analysis. To analyze the total content of each flavonol aglycone, an aliquot of 5 ml of methanolic extract was completely dried under vacuum. To achieve the acid hydrolyzation of flavonols glucosides, the pellet was resuspended in 2.5 ml of methanol and 2.5 ml of 2M trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) in milliQ water. The reaction was conducted at 100°C in a boiling hot water bath, with a condenser, for 2 hours. The reaction product was then completely dried under vacuum and the pellet obtained resuspended in 1 ml of methanol until HPLC analysis.

HPLC separation and quantification of anthocyanins and flavonols were performed on a Waters 1525 instrument equipped with a diode array detector (DAD), using a reversed-phase column Phenomenex (Castel Maggiore, BO, Italy) RP18 250 mm x 4 mm (5 µM), with precolumn. Either for anthocyanins and flavonols, the eluents used were the following: A = $HClO_4$ 0.3% in milliQ water; B = methanol.

For anthocyanin analysis, the methanolic extract was filtered through 0.22 µm, 13 mm PTFE syringe-tip filters (Teknokroma, Barcelona, Spain) and an aliquote of 270 µl was diluted to a final volume of 1 ml with the A eluent. The binary gradient was the same as reported in Mattivi et al., (2006), with suitable modifications. The time for column equilibration was 5 minutes and the injection volume was 60 µl. Separation of the main free anthocyanins was obtained at 43°C and they were quantified at 520 nm with a calibration curve with malvidin-3-glucoside chloride (Sigma-Aldrich, St. Louis, MO, USA).

Hydrolyzed flavonols samples were filtered through 0.45 µm, 13 mm PTFE syringe-tip filters (Teknokroma, Barcelona, Spain) directly into liquid chromatography vials. Flavonols aglicones were separated at 40°C through the linear gradient as reported in Mattivi et al., (2006). The time for equilibration column and the injection volume were the same used for the HPLC-DAD analyses of anthocyanins. Quercetin, myricetin and kaempferol were quantified at 370 nm with the corresponding external standard purchased from Extrasynthèse (Genay, France).

2.7 Statistical analyses

For each year, statistical analysis was carried out using the mixed General Linear Model (GLM) procedure of the SAS statistical package (SAS Institute, Cary, North Carolina, USA). Treatment comparison was performed by *t-test*. Visual ratings of sunburn damage and Botrytis damage percentage such as anthocyanins and flavonols percentage data were subjected to square root transformation prior to analysis.

2.8 Microarray analyses.

2.8.1 RNA extraction and RNA evaluation

Microarray analysis was carried out in collaboration with the working group of prof. Pezzotti (University of Verona, Italy). Sampling was effectuated as previously described, .Berries collected for microarray analyses were dissected in the field, immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses. On the basis of biochemical results microarray analysis was performed on three selected stages of berry ripening: beginning of véraison (stage II), end of véraison (stage IV) and harvest (stage VI). For each sampling three biological replicates were analyzed, for a total of 36 hybridizations.

Total RNA was isolated from ~200 mg of the powdered berry tissue (seeds were previously removed) using a Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. One µl of each RNA sample was measured spectrophotometrically to determine RNA quantity and 260/280 and 260/230 ratio, using a NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was then further checked by evaluation on an Agilent 2100 Bioanalyzer using RNA-6000 Nano Assay Reagent Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Samples exhibiting $A_{260}/A_{280} < 1.8$ or $A_{260}/A_{230} < 1.8$ or degradation after Bioanalyzer analysis were not carried through labeling and hybridization.

2.8.2 Synthesis of cDNA

Ten µg of total RNA for each sample were used to perform first cDNA synthesis using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Life Technologies, Australia). Components were combined according to what reported in Table 2.

Component	Amount
RNA	**10 µg**
oligo DT primer (50 pmol/µL)	**2 µl**
DEPC water	**to volume**
Total	**11 µl**

Table 2. Amount of different components combined in the first step of the first cDNA synthesis.

Samples were then heated to 70°C for 10 minutes in a thermocycler, briefly spinned and placed in an ice-water slurry for t10 minutes. To each sample were added the components as described in Table 3. After placing samples in a thermocycler set at 42°C for 2 minutes, 2 µl of Superscript II were added and the samples incubated at 42°C for 60 minutes (total reaction volume: 20 µl). To carry out the second strand cDNA synthesis components indicated in Table 4 were added to the first stand reactions.. The samples were then incubated at 16°C for 2 hours. After the addition of 2

µl of 5U/µl of T4 DNA polymerase to each reaction, the samples were incubated at 16°C for further 5 minutes. Ten µl of EDTA 0.5M were then added to the samples placed on ice and the samples stored overnight at -20°C.

Component	Volume
Reaction volume from the previous step	11 µl
5X First Strand Buffer	4 µl
0.1 M DTT	2 µl
10 mM dNTP Mix	1 µl
Total	18 µl

Table 3. Volume of different components combined in the second step of first cDNA synthesis.

Component	Volume
Reaction volume from the first strand reactions	20 µl
DEPC water	91 µl
5X Second Strand Buffer	30 µl
10 mM dNTP Mix	3 µl
10 U/µl DNA ligase	1 µl
10 U/µl DNA Polymerase I	4 µl
2 U/µl RNase H	1 µl
Total	150 µl

Table 4. Volume of different components combined in the first step of second cDNA synthesis.

2.8.4. RNase A Cleanup and cDNA precipitation and evaluation

To the second strand cDNA reactions 1 µl of RNase A (4mg/ml) was added and the samples were then incubated at 37°C for 10 minutes. After an extraction with an equal volume (163 µl) of phenol :chloroform : isoamyl alcohol, the samples were centrifuged at 12,000 x g for 5 minutes. CDNA was precipited from the upper aqueous layer adding in order 16 µl of 7.5M ammonium acetate, 7 µl of 5

mg/ml glycogen and 326 µl of ice-cold absolute ethanol. Samples were then centrifuged at 12,000 x g for 20 minutes and the pellet washed twice with 500 µl of ice-cold 80% ethanol (v/v). After drying pellet in a SpeedVac (Thermo Fisher Scientific, Wilmington, DE, USA), it was rehydrated with 20 µl of sterile water. To establish cDNA quality, 1 µl of each sample was load to a NanoDrop1000 Spectrophotometer and then analyzed using the Agilent Bioanalyzer. As previously described for RNA, cDNA samples exhibiting $A_{260}/A_{280} < 1.8$ or $A_{260}/A_{230} < 1.8$ or degradation after Bioanalyzer analysis were not carried through labeling and hybridization.

2.8.5. Sample labeling

The cDNA labeling was performed using a NimbleGen One-Color DNA labeling kit (Roche, Nimblegen Inc., Madison, WI, USA). To 1µg of cDNA were added Cy3-random primers (9mers) and nuclease-free water to a final volume of 80 µl. After denaturation in a thermocycler at 98°C for 10 minutes and quick-chill in an ice-water bath for additional 10 minutes, to the samples were added 20 µl of dNTP/Klenow master mix prepared as specified in Table 5.

The samples were incubated at 37°C for 2 hours in a thermocycler with heated lid, protected from light. The reaction was stopped after addition of 10 µl of 0.5M EDTA (Sigma-Aldrich, St. Louis, MO, USA). In order to precipitate Cy-3 labeled cDNA, to each tube were added 11.5 µl of 5M NaCl (Sigma-Aldrich, St. Louis, MO, USA) and 110 µl of isopropanol (Sigma-Aldrich, St. Louis, MO, USA).

Reagents	Volume
10 mM dNTP Mix	**10 µl**
Nuclease-free water	**8 µl**
Klenow fragment (3'->5' exo-) 50U/µl	**2 µl**
Total	**20 µl**

Table 5. dNTP/Klenow master mix composition.

After a centrifugation at 12,000 x g for 10 minutes, the pellet was rinsed with 500 µl of 80% ice-cold ethanol and dried in a SpeedVac on low heat. The pellet was then rehydrated in 25 µl of nuclease free water and 4 µg of Cy-3 labeled cDNA dried to be hybridized.

2.8.6. Hybridization and washing

The microarray chip used in this experiment was produced by the technology of oligo synthesis on chip by the Roche NimbleGen Company. On a single slide it was possible to analyze

simultaneously 12 samples (Fig.3). For each predicted gene were designed 4 different probes spanning the entire gene sequence. Each gene probe is 60mer oligonucleotide and in each slide are

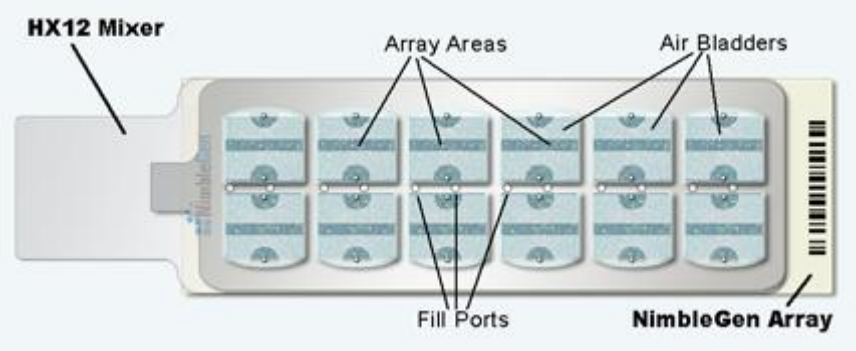


Fig. 3. Image of a HX12 Mixer-slide assembly.

present 12 x 135000 probe sets, representing 29550 predicted genes on the basis of the 12X genome of Vitis Vinifera sequenced by the French–Italian Public Consortium for Grapevine Genome Characterization (Jaillon et al., 2007) plus additional positive and negative controls. With each array it was so possible to analyze 29550 genes, corresponding to the entire grape transcriptome. Before hybridization, to each Cy-3 labeled cDNA sample, were added 3.3 µl of Sample Tracking Control (STC) reagent. STC allows to recognize the identity of each sample and to rule out the possibility of samples mixing during hybridization. To each sample were added 8.7 µl of hybridization solution prepared following the manufacturer's protocol. This solution contains also an alignment oligo, which helps the alignment of a grid for data acquisition . The slide was assembled with the mixer, following the instructions supplied by the company, the mixer-slide assembly (Fig. 3) was placed in the slide bay of the Hybridization System, which was pre-heated for at least three hours at 42°C. The hybridization reaction was made at 42°C for 20 hours. After the hybridization occurred, each mixer-slide assembly was washed using a NimbleGen Wash Buffer Kit, following the instructions provided by the manufacturer. The mixer was carefully removed and the slide was washed three times with Wash Solution I, II and III for 2 minutes, 1 minutes and 15 seconds, respectively. Then the slide was spin dried with a NimbleGen Microarray Dryer and it was immediately proceeded through the scanning of the slide to acquire data.

2.8.7. Array scanning, data extraction and normalization

The array scanning was carried out by a scanner ScanArray 4000XL (Perkin–Elmer) and associated software GenePix Pro7 (Molecular Devices, Sunnyvale, CA, USA). The wavelength at which the slide was scanned was 532 nm, corresponding to the maximum of Cy-3 absorption. Hardware was set following the manufacturer's instructions and in order to optimize the acquisition of each single array. To import the scanned image and to extract the data, it was used a

NimbleScan v2.5 software. Thanks to the oligo alignment present in the hybridization solution, the

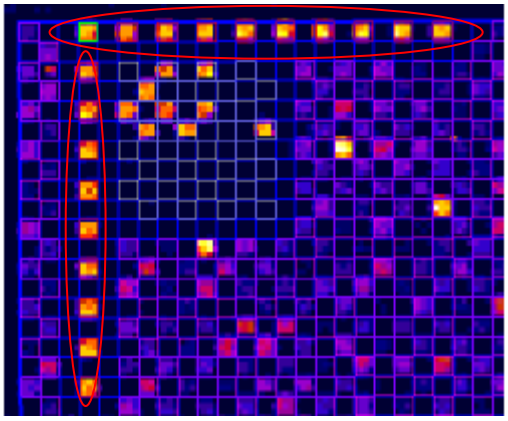


Fig. 4. Image of an array good alignment carried out by the *NimbleScan v2.5* software. The red circles represent the oligo alignment recognized by the software.

software was able to automatically positioning the grid on each single spot in the correct way (Fig. 5). A Sample Tracking Analysis confirmed the experimental integrity. The software gives back two kind of files for every probes of each sample: a Pair File, with the raw data of signal intensity for each probe; Calls File with the normalized gene expression value, deriving from the average of the intensities of the four probes for each gene.

The normalized gene expression data were finally converted in $\log_2$ value to process data.

2.8.8. Microarray data analysis.

To assess the quality of biological replicates, for each treatment at each stage was calculated the Pearson Correlation Coefficient. For variation assessment between samples due to the differential gene expression, a Principal Component Analysis (PCA SIMCA P+ (Umetrics, Umea, Sweden) was performed on all the data from the three stages analyzed. In order to identify genes modulated during berry development and the differentially expressed genes among the three treatments, a multiclass comparison of Significance Analysis of Microarrays (SAM) was performed using the TMeV software (http://www.tm4.org/mev). SAM analysis was carried out with a false discovery rate of 2%; the SAM output was further restricted to genes with fold change (FC) greater than 2 and with a threshold intensity value of 1000. in at least one of the two comparisons. This threshold value was obtained using negative controls present on the chip.

To test for significant differences between treatments in the representation of differentially expressed genes within each functional category a Pearson's chi squared test was used.

3.0 RESULTS AND DISCUSSION

3.1 Agro-meterological data and physiological and vegetative measurements

3.1.1. Agro-meteorological data

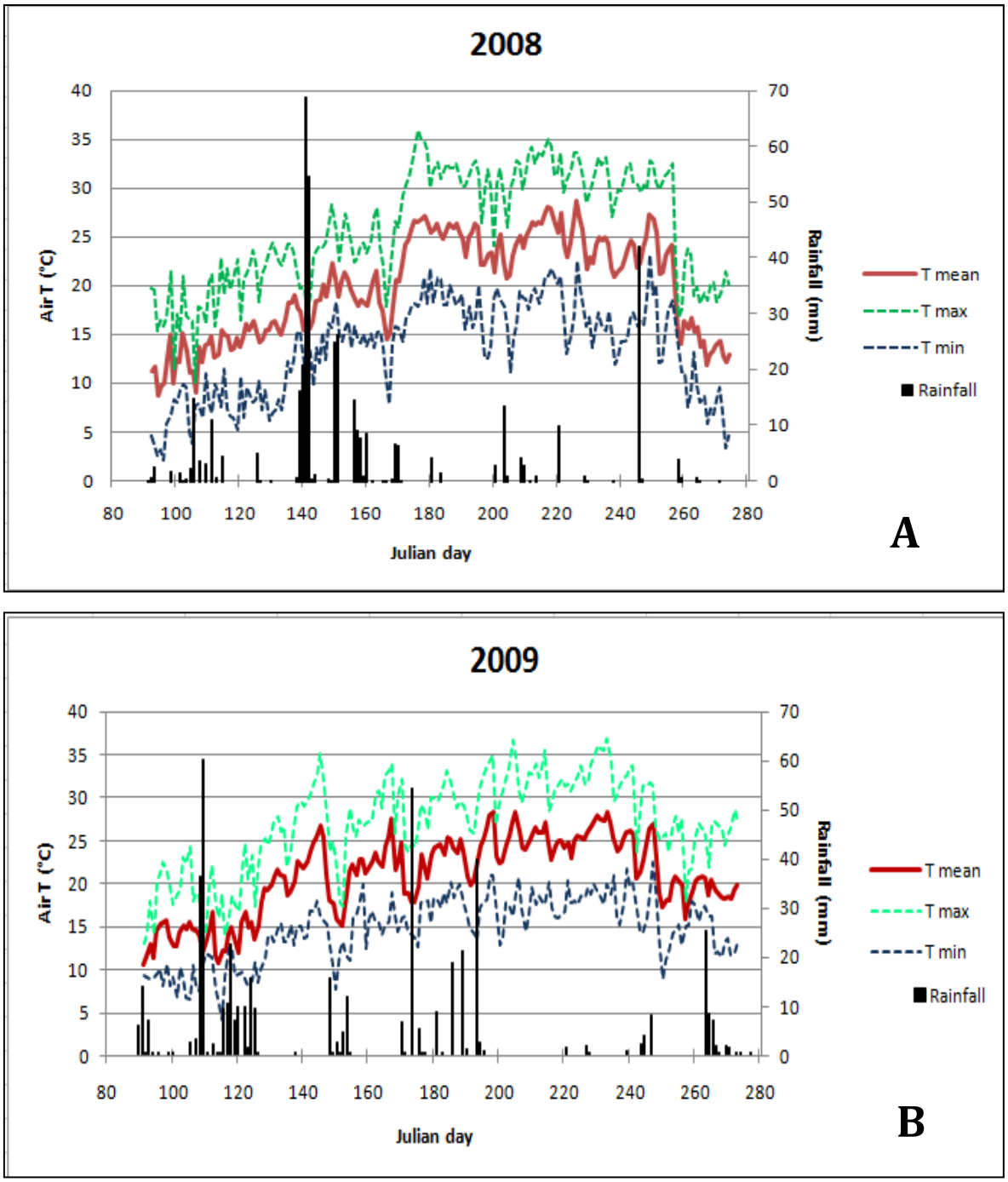


Fig. 1. Daily values (from April through September) of mean, maximum and minimum air temperature registered at the experimental site. Blue squares indicate rainfall (mm). A. Data collected in 2008. B. Data collected in 2009.

Total rainfall from April through September was comparable in 2008 and 2009 seasons (320 mm and 317.4 mm respectively). Mean temperatures registered in the same period were instead higher in 2009 (20.9 °C) compared to 2008 (19.8°C; Fig. 1). For what concern monthly values,

major differences were found at the first stages of the vegetative season. In particular, the first period of 2009 was characterized by lower rainfalls and elevated maximum temperatures (in May were reached values of 35°C). In 2009, was also registered a general tendency to present higher mean temperatures compared to the same period of 2008, which reflected on total GDD calculated from April through September (1758°C in 2008 and 2006.1°C in 2009). The late stages of berry ripening in 2009 were also characterized by higher minimum temperatures, which in September exceeded the corresponding values recorded in 2008, of 6°C (Table 1).

	2008					2009				
	Rainfall (mm)	GDD (°C)	T max (°C)	T min (°C)	T mean (°C)	Rainfall (mm)	GDD (°C)	T max (°C)	T min (°C)	T mean (°C)
April	12.8	48.2	22.9	2.1	12.8	124.4	108.4	24.2	4.3	13.6
May	191.0	224.8	28.3	6.2	17.2	38.0	304.0	35.1	7.8	19.8
June	52.4	346.4	35.9	7.8	21.5	57.4	356.4	34.0	11.1	21.9
July	24.4	446.1	34.2	11.2	24.4	54.6	451.7	36.6	12.9	24.6
August	10.2	455.3	35.1	11.9	24.7	13.2	474.5	36.8	14.6	25.3
September	42.0	247.2	32.9	3.3	18.2	29.8	311.1	31.7	9.0	20.0

Table 1. Monthly rainfall (mm), growing degree days (base 10°C, GDD), and maximum, minimum and mean temperature (T max, T min and T mean), at Cadriano experimental site in 2008 and 2009.

3.1.2 Effects of defoliation treatments on leaf area

At the beginning of the season, either in 2008 and 2009, the vine of all the treatments did not show any significant differences in total leaf area (Table 2). In both years, at pre-bloom defoliation was removed about the 70% of total leaf area per vine. In 2008 and 2009 shoot trimming was performed respectively in JD 192 and JD 160 and the leaf area removed was very similar among the treatments ranging from 1.2 to 1.5 m^2/vine in 2008 and 0.72 to 0.78 m^2/vine in 2009. In 2008, the laterals contribution in removed leaves at shoot trimming was about 30% both in C and ED vine; in 2009 laterals contribution was 15% for C and ED vine. The differences between the two years are due to the postpone moment in which shoot trimming was performed in 2008 (JD 192) compared to 2009 (JD 160). Both in 2008 and 2009 in LD vines the 40% of total leaf area was removed. In 2008 harvest, all the treatments showed a comparable total leaf area/vine. For ED vines this was due mainly to a compensatory regrowth of laterals, whose leaf area was significantly higher in ED vines compared to the other treatments. The same effect, caused by pre-bloom defoliation, was previously seen for potted vines of Sangiovese (Poni *et al*., 2006) and for Barbera (Poni *et al*., 2008). At 2009 harvest, ED and LD vines showed a lower total leaf area compared to C and CT plants, due to, in the case of ED vines, to a missed capacity of lateral to develop, as it was instead happened in

2008. This was probably caused by the higher temperature and lower rainfalls characterizing the period following the defoliation treatment in 2009, which is a key moment in triggering the recovery.

	Total leaf area / vine (m^2) immediately before pre-bloom defoliation	Removed leaf area / vine (m^2) at pre-bloom defoliation	Leaf area left/ vine (m^2) after pre-bloom defoliation (*)	Total leaf area / vine (m^2) immediately before véraison defoliation	Removed leaf area / vine (m^2) at véraison defoliation	Final main leaf area / vine (m^2)	Final laterals leaf area / vine (m^2)	Total final leaf area / vine (m^2)
2008								
C	1.70	0	1.70	4.19	0	2.52 **a**	1.32 **b**	3.84
ED	1.69	1.16	0.53	3.30	0	1.84 **b**	1.42 **a**	3.26
LD	1.75	0	1.75	4.60	1.88	1.72 **b**	1.12 **b**	2.84
CT	1.59	0	1.59	3.47	0	2.23 **ab**	1.20 **b**	3.43
2009								
C	1.77	0	1.77	5.85 **a**	0	3.26 **a**	2.51	5.77 **a**
ED	1.70	1.25	0.45	3.60 **b**	0	1.94 **b**	1.62	3.56 **b**
LD	1.64	0	1.64	5.91 **a**	2.28	2.81 **a**	0.89	3.69 **b**
CT	1.51	0	1.51	6.50 **a**	0	4.01 **a**	2.41	6.42 **a**

Table 2. Influence of pre-bloom and véraison defoliation on removed, composition and total leaf area per vine in 2008 and 2009. Data about removed leaf area per vine at shoot trimming were also reported. Means within columns followed by different letters differ significantly at $p<0.05$ by the Tukey-Kramer test. (*)In both years trimming was conducted when the shoots overcame the third wire of the system (1.1 m from the ground).

3.1.3 Monitoring of berry skin temperature in clusters of C and ED vines.

During 2008 and 2009, berry skin temperature of C and ED clusters was monitored from stage of pre-bunch closure to harvest. Among this period, three weeks in three different phases of berry development and ripening, were chosen to verify the presence and the entity of the differences in C and ED berry skin temperature. For 2008 and 2009 the weeks considered corresponding to the same stage in berry ripening.

In Figure 2, data of mean berry skin temperature of ED and C clusters registered continuously during a week at the same stage of berry development (pre-bunch closure) are represented for 2008 and 2009 (JD 182-188 in 2008 and JD 179-184 in 2009). In 2008 and 2009, during the week considered, berry skin temperature showed a comparable trend.

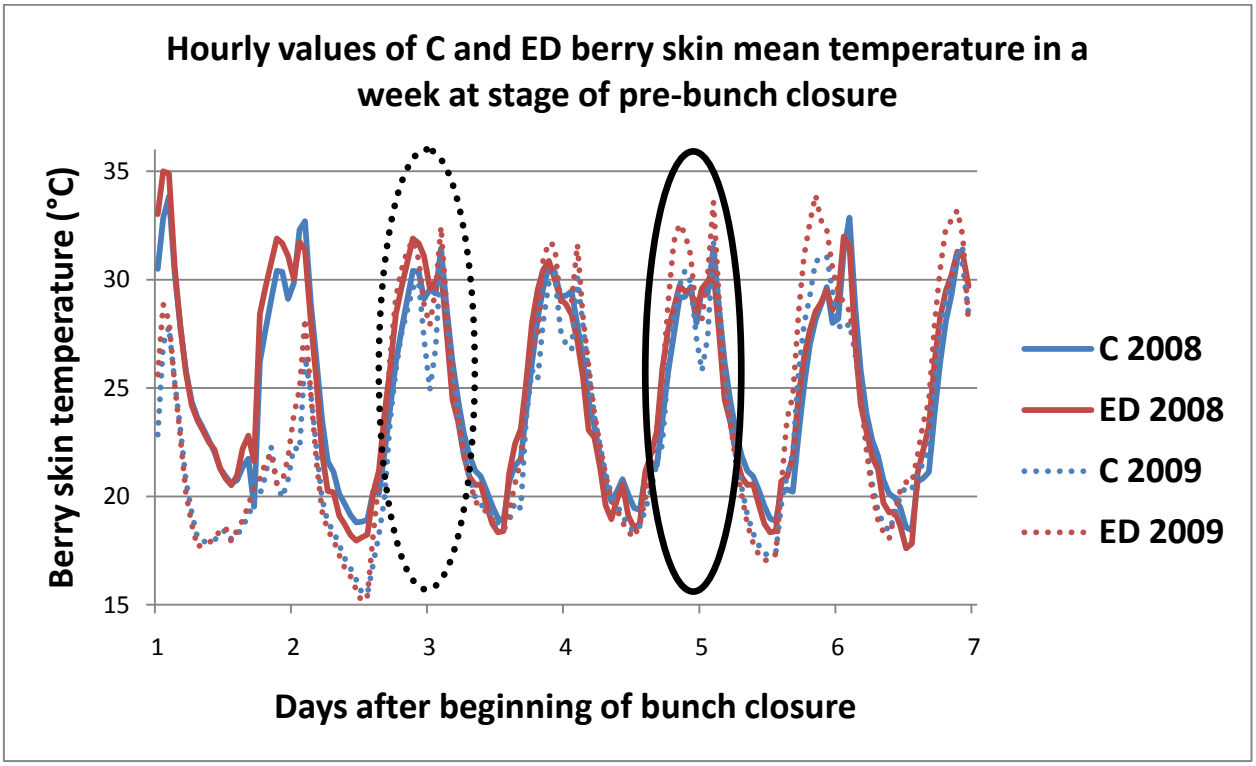


Fig. 2. Hourly values of C and ED berry skin mean temperature (°C) in a week at stage of pre-bunch closure. With black circles are signaled the days chosen for subsequent hourly statistical analyses (continuous line for 2008; dotted line for 2009).

In this period, for both years, ED mean berry skin temperature was always higher compared to C berry skin temperature, even if in 2009 bigger differences between the two treatments were registered. To better understand how this differences distributed all along the day, hourly values of C and ED berry skin temperature in a typical day of the period (JD 186 in 2008 and JD 180 in 2009), were compared (Fig.3). For both years differences between C and ED berry skin temperature were recorded from 8.00 a.m. to 8.00 p.m.. The highest differences were present at 11.00 a.m., with a ΔT(ED-C) of about 4°C. The maximum temperature of berry skin of ED vines (32.65 °C in 2008 and 32.44°C in 2009) and C vines (31.23 in 2008 and 31.11 in 2009) was reached at 19.00 p.m.. This

coincided with the time at which air maximum temperature (32.1°C) was reached in 2008, while in 2009 air maximum temperature (29.9°C) was reached 3 hours before.

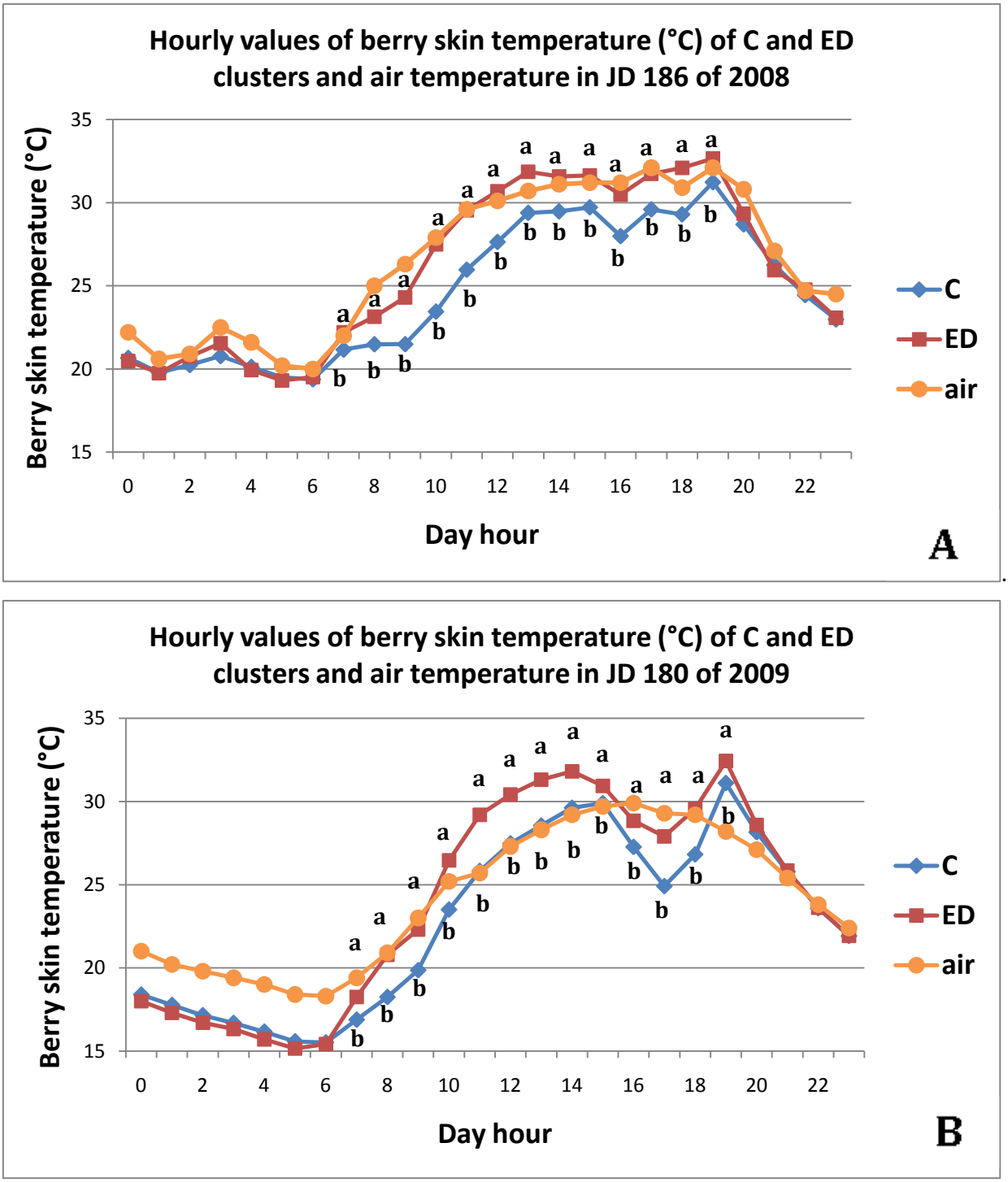


Fig. 3. Hourly values of mean berry skin temperature (°C) of C and ED in two comparable days at stage of pre-bunch closure. A. Data collected in 2008. B. Data collected in 2009. For both years also hourly values of air temperature are reported. Superscript letters indicate significant differences by the Tukey-Kramer test between the two treatments at $p<0.05$.

In Fig.4 are represented data of mean berry skin temperature of ED and C clusters recorded in a week at véraison (JD 210-216 for both 2008 and 2009). As expected, ED mean berry skin temperature was always higher compared to C mean berry skin temperature, and in 2009 in ED berry reached often values over 35°C.

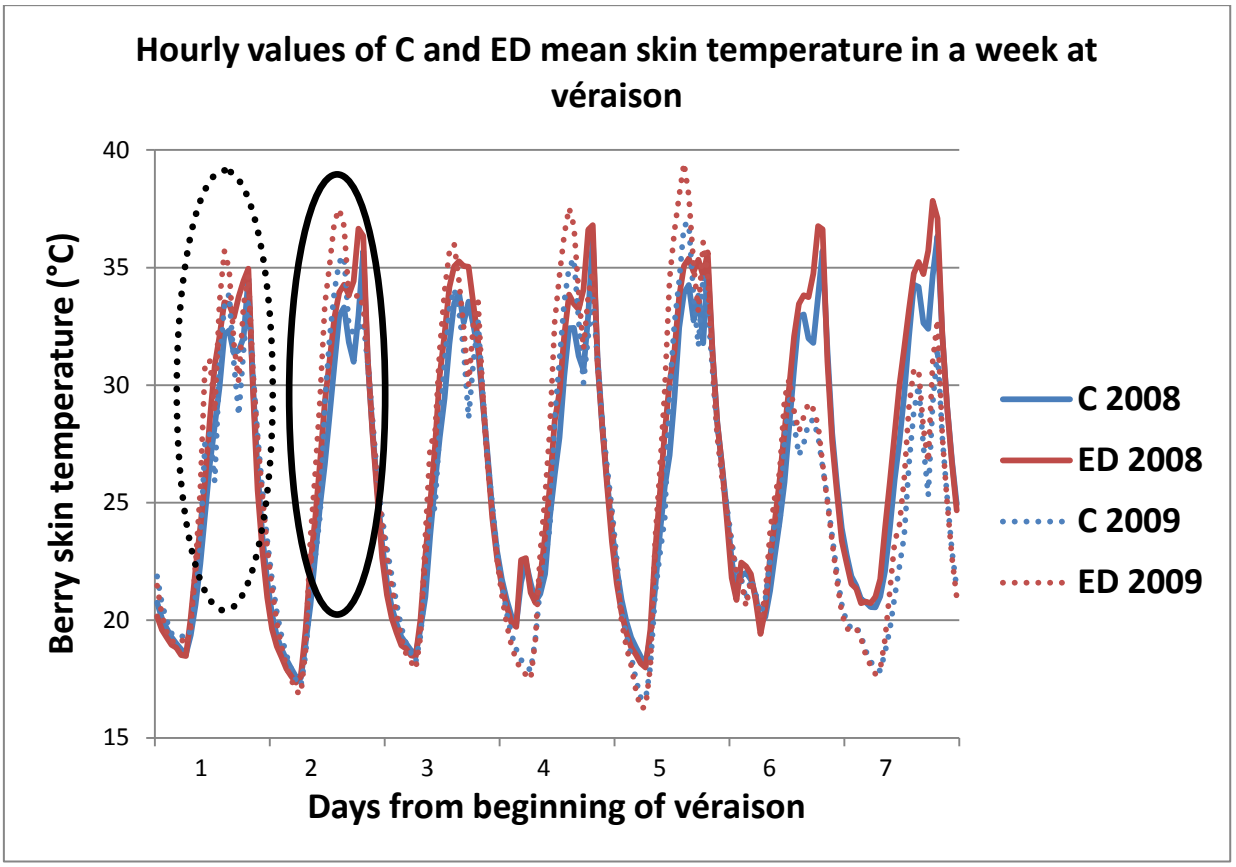


Fig. 4. Hourly values of C and ED berry skin mean temperature (°C) in a week at beginning of véraison. Black circles represent the days chosen for subsequent hourly statistical analyses (continuous line for 2008; dotted line for 2009).

The hourly trend of two comparable days chosen in 2008 and 2009 was quite different among the two years (Fig.5). In fact, in 2008, significant differences were present only early in the morning, at midday and in the afternoon. The highest differences between C and ED berry skin temperature (ΔT(ED-C)=3.7°C) were recorded at 6.00 p.m.. In 2009, ED berry skin temperature was instead higher compared to C berry skin temperature for all the morning until the early afternoon. From 2.00 p.m., in fact, no other significant differences were found. ΔT(ED-C) was maximum at 10.00 a.m. reaching a value of 3.3°C. The different behavior observed could be due to the difference observed in the night minimum temperatures, which in 2009 were higher compared to the same data collected in 2008 (as confirmed in the day considered, minimum in 2008 was 17.8°C, in 2009 19.5°C).

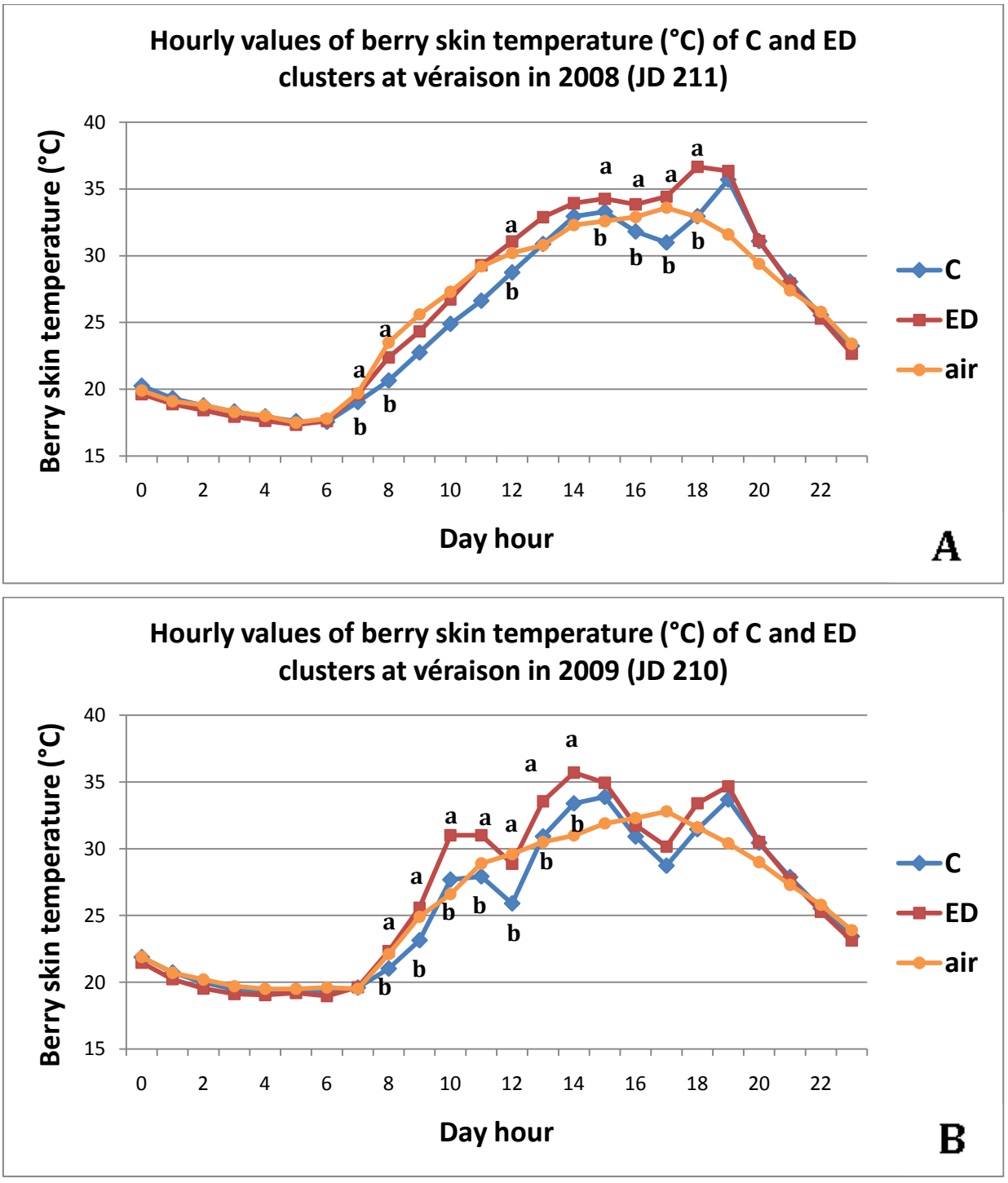


Fig. 5. Hourly values of mean berry skin temperature (°C) of C and ED in two comparable days at véraison. A. Data collected in 2008. B. Data collected in 2009. For both years also hourly values of air temperature are reported. Superscript letters indicate significant differences by the Tukey-Kramer test between the two treatments at $p<0.05$.

The differences between C and ED berry skin temperature were maintained until harvest. The trend in berry skin temperature of a week about 10 days before harvest is represented in Fig.6 for 2008 and 2009. The period closer to 2009 harvest was characterized by higher temperature, both in the day and in the night. The hourly trend in two comparable days in this period is represented in Fig.7. In 2008, ED berry skin temperature was significant different from C berry skin

temperature at 9.00 a.m., from 12.00 to 2.00 p.m. and in the late afternoon (from 4.00 to 8.00 p.m.), in a comparable way to what previously seen for a day in this year at véraison. The highest differences between the two treatments was reached at 5.00 p.m.. (ΔT(ED-C)=2.6 °C). Maximum temperature was instead reached at 6.00 p.m. Also in 2009 the course of temperature in the day near harvest considered was similar to the one previously seen at véraison. Significant differences between C and ED berry skin temperature were in fact found only from the morning to late midday (in particular, from 9.00 a.m. to 1.00 p.m.). The higher ΔT (ED-C)=4.4°C was recorded at 11.00 a.m. and the maximum around 2.00 p.m..

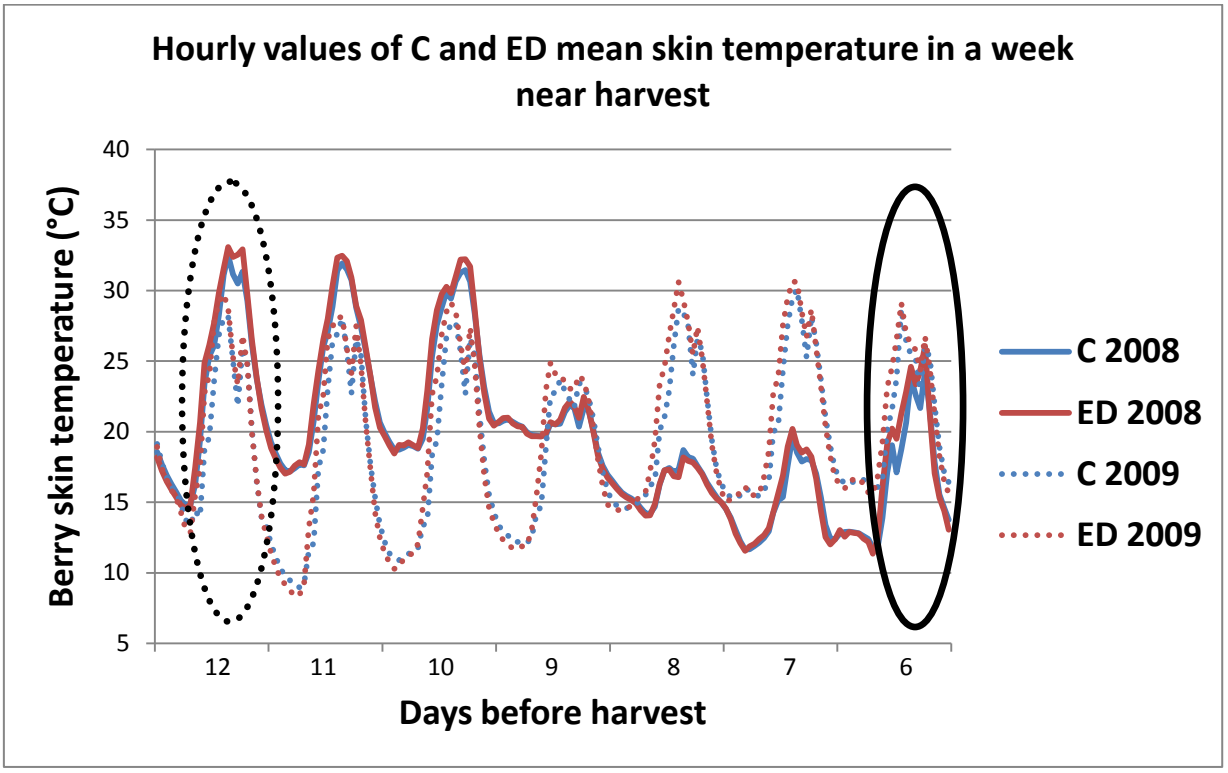


Fig. 6. Hourly values of C and ED berry skin mean temperature (°C) in a week about 10 days before harvest. Black circles represent the days chosen for subsequent hourly statistical analyses (continuous line for 2008; dotted line for 2009).

As previously seen, the difference among 2008 and 2009 in the hourly trend of berry skin temperature in the 2 days considered, could be attributed to the lower value of the minimum air temperature, which in 2008 was 11.2°C while in 2009 12.2°C. This caused an increase in the temperature of the early hours in the morning and so in 2009 at 8.00 a.m. air temperature was about 3.0°C higher compared to the same data of 2008. As a result, the phase displacement in hourly air temperature trend was reached in 2008 at 5.00 p.m., while in 2009 this was reached 3 hours before.

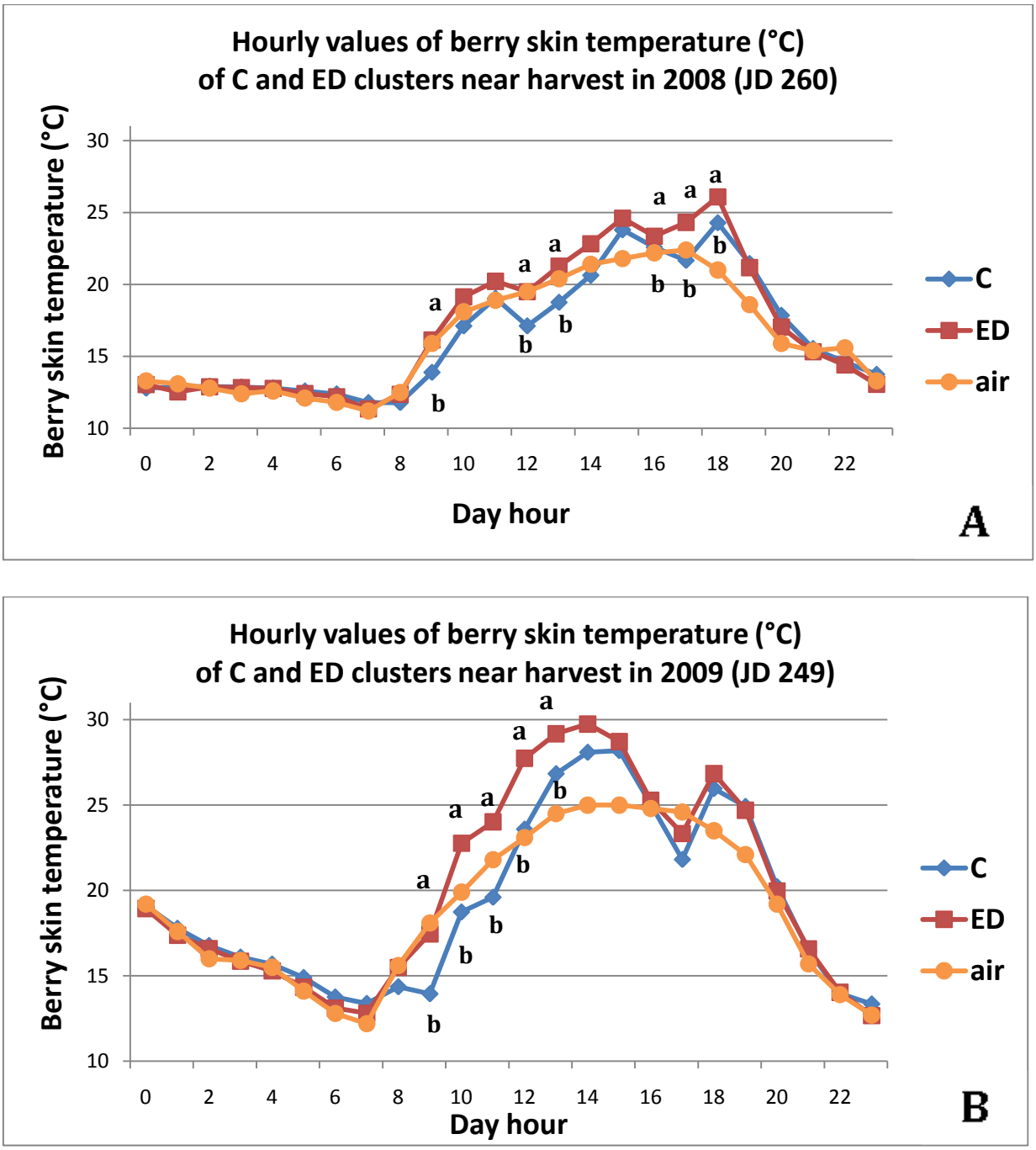


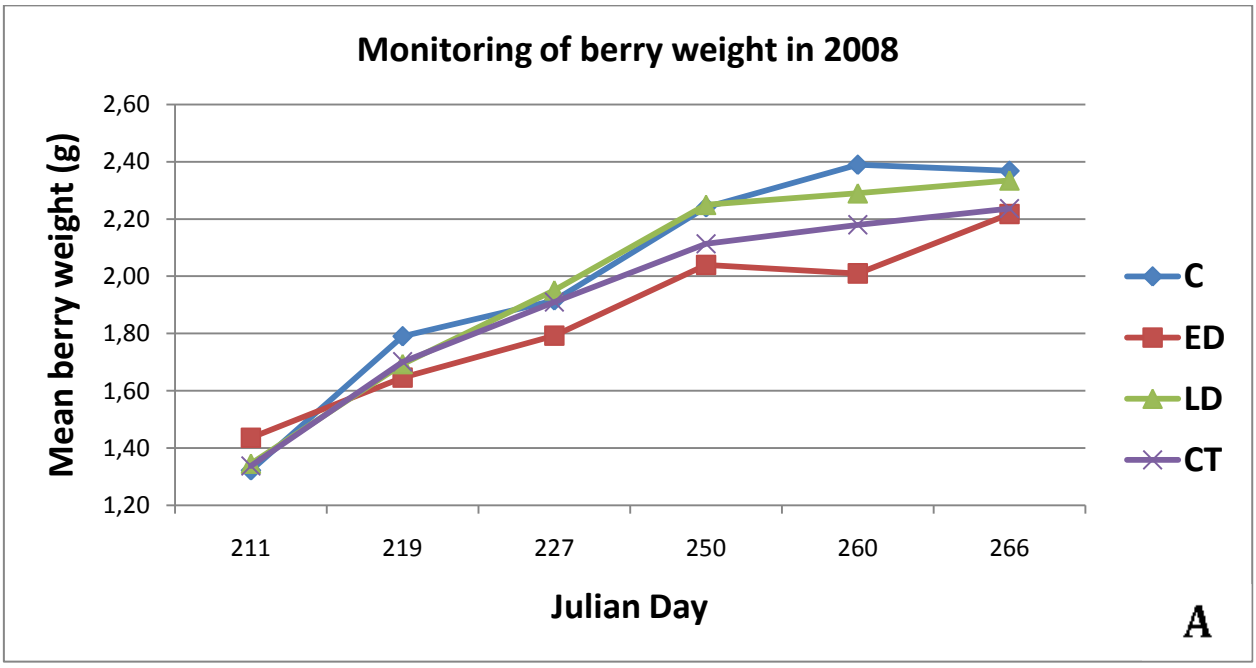
Fig. 7. Hourly values of mean berry skin temperature (°C) of C and ED in two comparable days at véraison. A. Data collected in 2008. B. Data collected in 2009. For both years also hourly values of air temperature are reported. Superscript letters indicate significant differences by the Tukey-Kramertest between the two treatments at p<0.05.

3.2 Monitor of berry ripening and yields components at harvest

3.2.1 Monitor of berry ripening

To understand the course in berry ripening and at which level the treatments could influence berry composition, berry ripening was monitored towards measurements of berry weight, °Brix, pH and TA at different intervals from the beginning of véraison to harvest.

In Fig. 8 are represented mean berry weight data collected for the 4 treatments in 2008 and 2009.



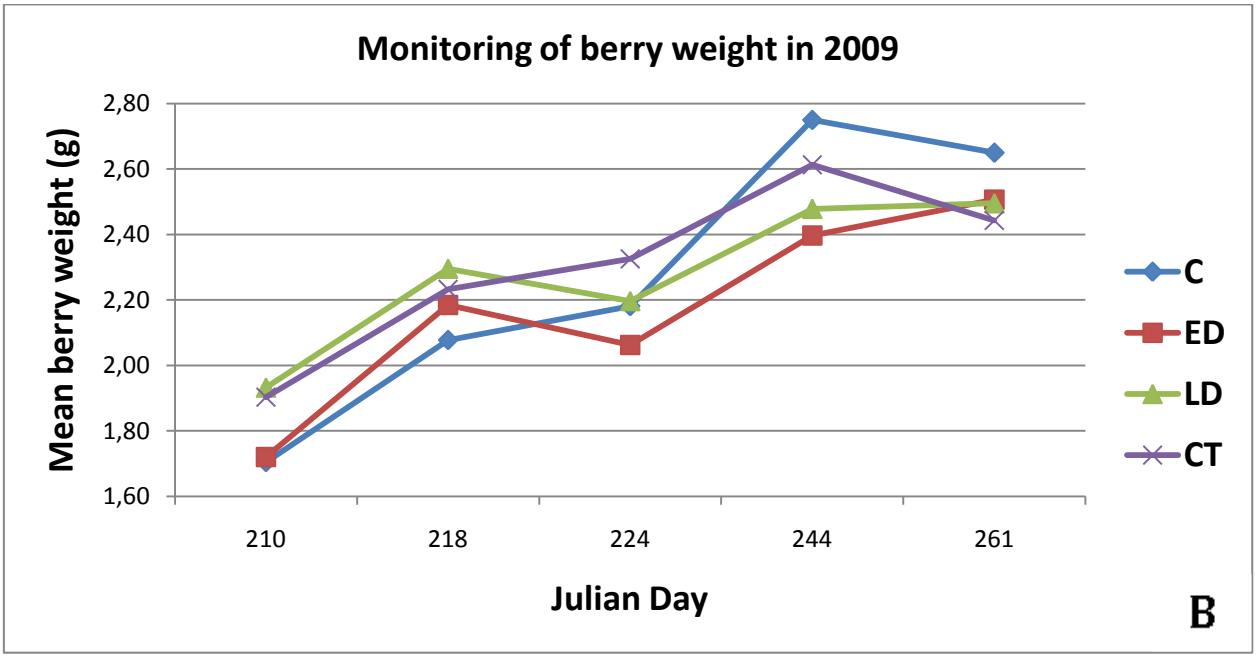
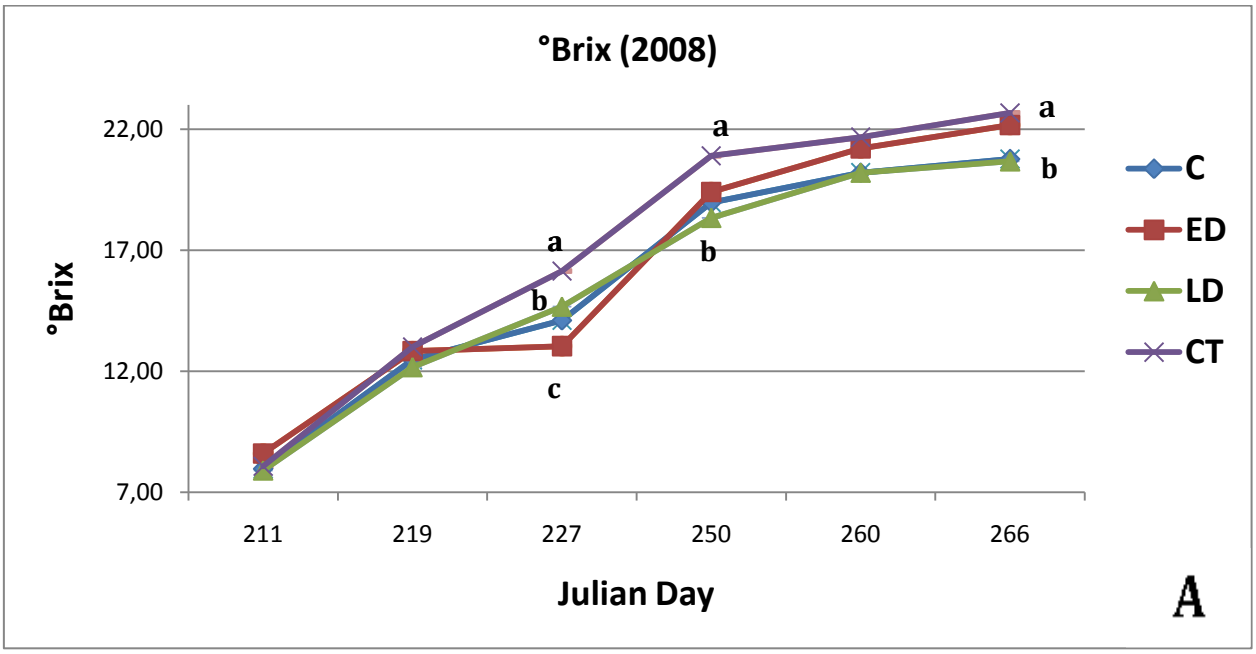


Fig. 8. Monitoring of mean berry weight at different intervals from the beginning of véraison to harvest. A. Data collected in 2008. B. Data collected in 2009.

Despite the general tendency of ED berries to present a lower mean berry weight for all the season, as in 2008, or for a large part of the season, as in 2009, the four thesis did nott show any significant difference in this parameter at harvest.



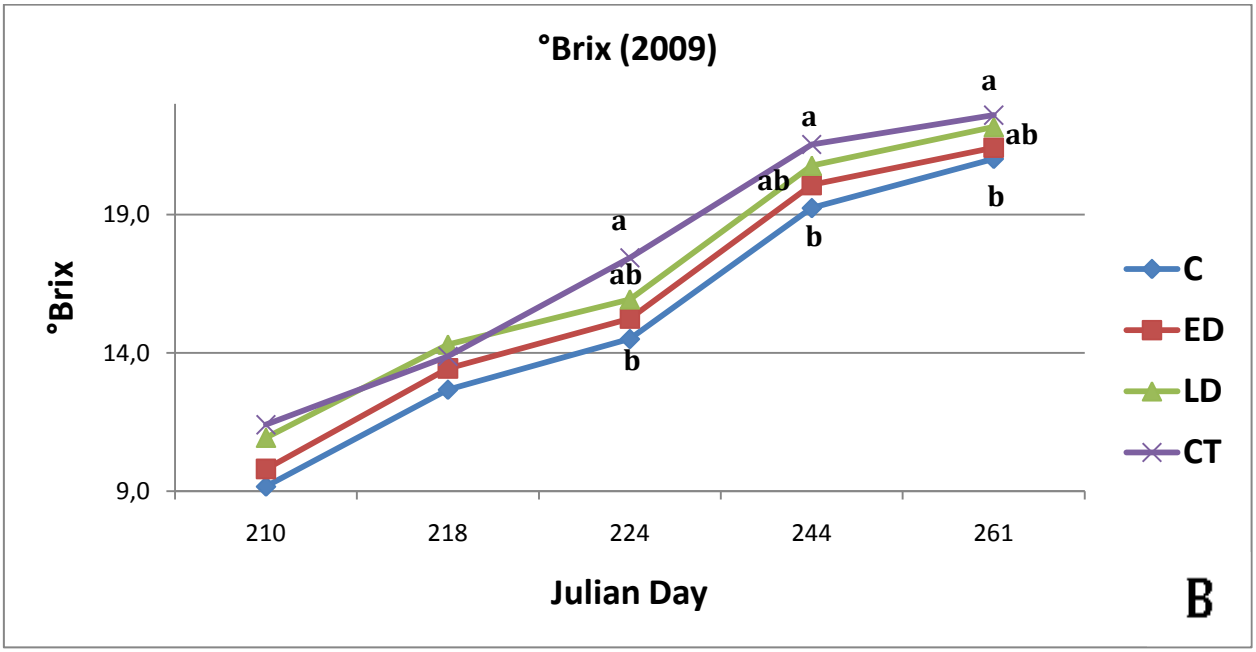


Fig. 9. Monitoring of soluble sugars(°Brix) accumulation at different intervals from the beginning of véraison to harvest. A. Data collected in 2008. B. Data collected in 2009. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05.

As expected, at the beginning of véraison all the treatments showed comparable values for all the parameters considered in both years. No differences were present until the end of véraison (JD 227), when CT berries showed an acceleration in accumulation of soluble solids (Fig.9); this implied the highest value of °Brix for this treatment at harvest in both years. The effect of pre-bloom

defoliation on sugars content seems to be less rapid and only in 2008 ED berries showed higher °Brix values compared to C at harvest. LD treatment had no influence on sugars accumulation, as demonstrated by the °Brix value associated at this treatment, which did not show differences from C values in both years considered.

	pH				TA (g/L)			
				2008				
JD	**C**	**ED**	**LD**	**CT**	**C**	**ED**	**LD**	**CT**
211	2.7	2.7	2.7	2.7	33.6	32.6	35.8	36.5
219	2.9	2.9	2.9	2.9	20.6	18.2	18.6	18.7
227	2.95**b**	2.95**b**	3.01**a**	3.04**a**	16.4**a**	16.3**a**	15.3**a**	13.9**b**
250	3.3	3.3	3.4	3.4	8.5	8.0	8.1	7.8
260	3.3	3.4	3.4	3.4	7.3	6.8	6.7	6.6
266	3.38**b**	3.45**ab**	3.45**ab**	3.5**a**	7.6**a**	6.8**b**	6.7**b**	6.9**b**
				2009				
JD	**C**	**ED**	**LD**	**CT**	**C**	**ED**	**LD**	**CT**
210	2.8	2.8	2.8	2.8	28.18	26.17	25.59	23.77
218	2.9	3.0	3.0	3.0	18.64	13.90	12.82	12.62
224	3.0	3.1	3.1	3.1	16.64	14.56	14.17	12.99
244	3.3	3.4	3.4	3.4	8.47	7.79	7.17	7.63
261	3.43**b**	3.45**ab**	3.52**a**	3.52**a**	6.94**a**	6.60**a**	6.20**b**	6.30**b**

Table 3. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning on pH and TA of Sangiovese grapevine in 2008 and 2009. Means within rows followed by different letters differ significantly at $p<0.05$ by the Tukey-Kramer test.

The faster rate of sugar accumulation in CT vines resulted in higher must pH and lower TA values (Table 3). Only in 2009, must pH of LD thesis showed a value comparable to those observed for CT, but this was most likely due to the very low TA values, characterizing this year. We may suppose that pre-bloom defoliation affected TA for a combined effect of the high sugars content associated to the higher berry temperature. It has been demonstrated in fact a general inverse relationship between temperature and TA (Spayd et al., 2002), caused mainly by the catabolism of malic acid induced by high temperature. This effect could explain also the differences observed in TA in the LD treatment, even if we did not measure malic acid.

In Table 4 are represented the vegetative-productive measurements effectuated at harvest in 2008 and 2009. As expected after the thinning treatment applied at véraison, CT vines presented at harvest a number of cluster per vine of about the half compared to the other treatments. As a result, the CT vines presented the lowest yield/vine and this was due to the reduction in cluster number since mean cluster weight didn't show any significant differences compared to C in both years

(Table 4). The strong reduction in yield in CT vines affected the leaf area/yield ratio, which in 2009 reaches even value more than 2 (Table 4).

	Clusters number/vine	Yield/vine (kg)	Cluster weight (g)	Berry weight (g)	Bunch rot/cluster (%)	Sunburn damage/cluster (%)	Leaf area/yield (m^2/kg)
				2008			
C	16.56 **a**	6.33 **a**	385.79 **a**	2.37	4.98	0.36 **b**	0.63 **a**
ED	15.89 **a**	4.78 **ab**	300.25 **b**	2.27	2.11	1.24 **b**	0.68 **a**
LD	16.11 **a**	5.55 **ab**	341.13 **a**	2.34	2.29	6.45 **a**	0.55 **a**
CT	8.33 **b**	2.91 **b**	353.28 **a**	2.24	3.22	2.00 **b**	1.17 **b**
				2009			
C	16.00 **a**	7.08 **a**	439.56	2.65	15.01	1.22 **b**	0.82 **b**
ED	16.22 **a**	5.66 **a**	352.30	2.51	3.58	6.15 **ab**	0.66 **b**
LD	16.00 **a**	5.88 **a**	364.89	2.50	11.87	13.12 **a**	0.69 **b**
CT	7.56 **b**	2.66 **b**	342.90	2.44	7.17	3.98 **b**	2.67 **a**

Table 4. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning on yield components and bunch characteristics of Sangiovese grapevine in 2008 and 2009. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test.

This justifies the effect of the treatment on the increase in soluble sugars. Towards pre bloom defoliation, it was not possible to reduce yield at the same level obtained with cluster thinning and so yield/vine was not statistically different from C, even if ED vine productivity was reduced of about the 20% in both years. In 2008, this reduction was probably linked to a decrease in fruit set percentage as it is demonstrated by the lower mean ED cluster weight, associated with any statistical difference in mean berry weight. In 2009, the tendency represented by the reduction in cluster weight due to a lower fruit set percentage was confirmed, but no statistical differences were found compared to C. The effect of pre-bloom defoliation in the reduction in fruit set was previously seen in pot Sangiovese vines (Poni et al., 2006), in Sangiovese trained to spur pruned cordon (Filippetti et al., 2009) or to COMBI training system (Intrieri et al., 2008), but also in other varieties (Poni et al., 2009). The ED value of leaf area/crop weight ratio was comparable to C in both years. In 2008 this was due to the total leaf area recovery, towards higher lateral contributions, and to the yield reduction. The increase in °Brix in 2008 for ED berries could so be attributed to the younger canopy composition, caused by the higher contribute of laterals in total leaf area (Table 2). In 2009, instead, there was not recovery in ED vine leaf area by neither main nor laterals leaves probably linked to the dryer climatic condition observed in May. As a consequence, ED berries did not show significant differences in °Brix compared to C. The véraison defoliation did not affect either yield/vine and cluster weight. Despite the leaf removal, the leaf/area weight ratio value was not significant different from C in both years. The sudden exposure to full light of LD

clusters at véraison implied their susceptibility to sunburn damages, as demonstrated by the higher percentage of sunburn berries in both years. Regards to bunch rot percentage for cluster, statistical analyses did not highlight any difference between treatments.

3.2.2 Evaluation of cluster morphology

In Table 5 are represented clusters morphology data collected in 2008 and 2009. ED clusters displayed a tendency towards lower compactness, either measured by OIV rating or by cluster weight/length ratio. This was probably due to the lower number of ED berries for cluster, which is a compatible result with the previously discussed reduction in berry set percentage associated with pre-bloom defoliation. While berry skin weight was not affected by any of the treatments, the full cluster exposure to light in the earlier stages of berry development could probably be responsible for other modifications in ED berries morphology, as demonstrated by the higher values of ED skin thickness compared to the other treatments, measured in 2008.

	Cluster compactness		Total berries/cluster	Berry skin weight/berry (g)	Berry skin thickness (µm)
	OIV rating	Cluster weight/length (g/cm)			
			2008		
C	7.47	0.015	237.44 **a**	0.324	232.3 **b**
ED	6.75	0.013	150.50 **b**	0.281	255.6 **a**
LD	7.83	0.015	187.11 **a**	0.305	223.8 **b**
CT	7.16	0.015	199.11 **a**	0.365	225.3 **b**
			2009		
C	7.72	0.015	261.33 **a**	0.323	-
ED	6.71	0.014	154.17 **b**	0.314	-
LD	7.13	0.014	210.22 **a**	0.345	-
CT	7.48	0.014	216.06 **a**	0.339	-

Table 5. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning on cluster morphology of Sangiovese grapevine in 2008 and 2009. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test.

As expected, the frequency distribution of berry weight in berry size categories was represented by a Gaussian curve (Fig.11). The most representative Sangiovese berry weight class in our trial was the class 4 (berry weight between 1.8 and 2.4 g) for all the treatments. Among the four thesis, ED clusters presented the highest uniformity in berry weight. In fact, more than 35% in 2008 and 50% in 2009 of total ED berries had a berry weight ranged from 1.8 to 2.4 g. Including class 3 and 4, it is possible to affirm that over the 70% of ED berries had a weight ranged from 1.2 to 2.4 g. Late defoliation and cluster thinning did not affect the distribution of berry weight and so LD, CT and C

showed a higher variability in berries size (only 50% in 2008 and 40% in 2009 of total berries presented a weight ranged from 1.2 and 2.4 g).

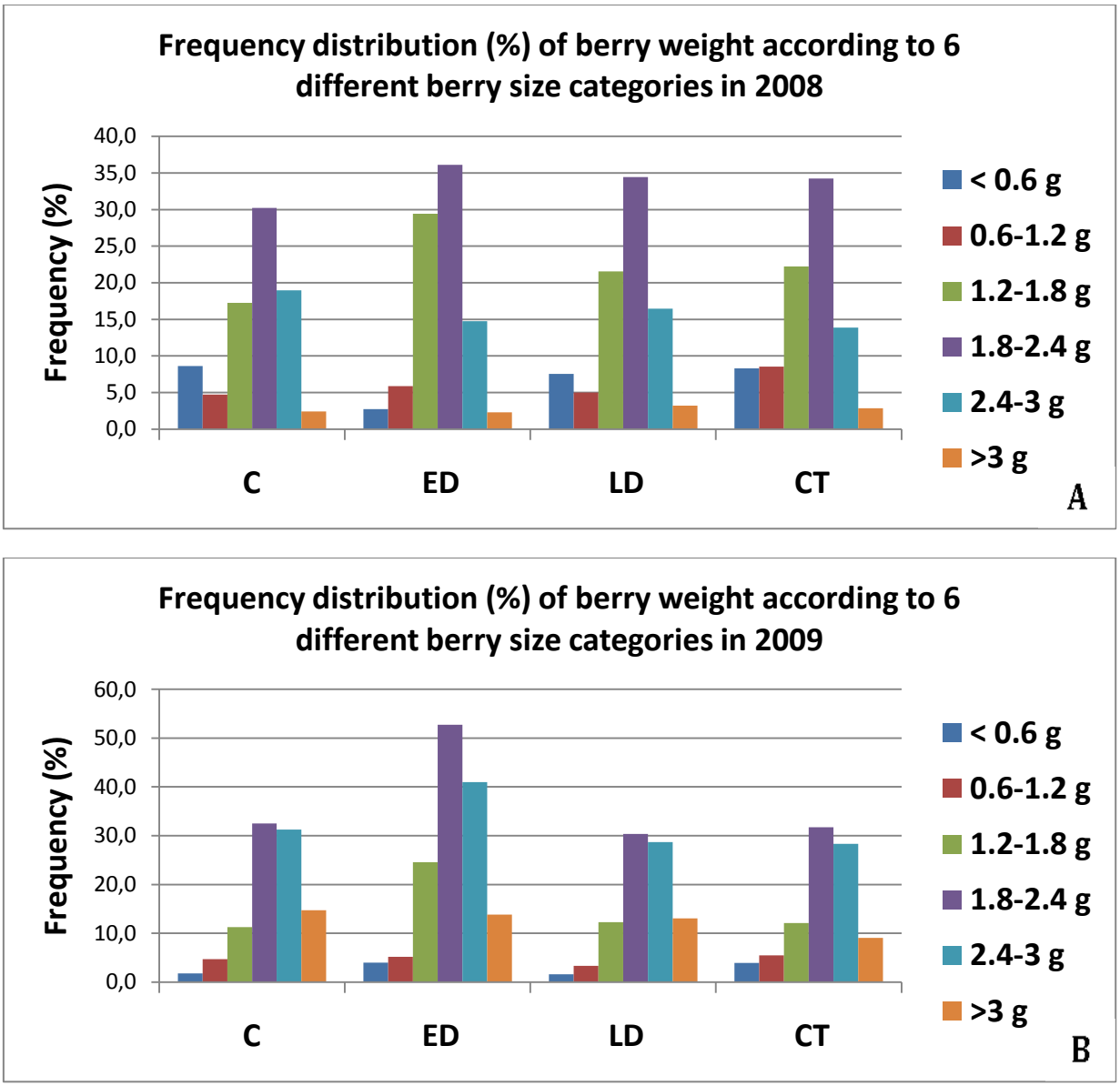


Fig. 11. Effect of pre-bloom defoliation, véraison defoliation and cluster thinning on frequency distribution (%) of berry weight for 6 different berry size categories, as indicated in the graphs. A. Data collected in 2008. B. Data collected in 2009.

In Table 6 are represented mean values of berry soluble solids for 2008 and 2009, according to the 6 berry size categories previously considered for berry weight. A pair wise comparison of °Brix data variance of all categories showed that in both years ED was firmly uniform in terms of sugars concentration, while C and other treatments showed a higher variability. This could be probably due to the more variable distribution of C, CT and LT berry size in different dimensional class, as previously seen.

	Berry size							
	< 0.6g	**0.6-1.2g**	**1.2-1.8g**	**1.8-2.4g**	**2.4-3.0g**	**> 3.0g**	**N**	**Variance of data**
				°Brix 2008				
C	20.54	19.12	21.16	20.64	20.39	19.35	570	3.69 **b**
ED	-	23.50	23.14	22.30	21.20	-	323	4.01 **b**
LD	19.54	20.45	20.87	20.77	20.47	19.05	450	5.76 **a**
CT	22.49	23.05	21.79	22.01	22.18	23.20	465	3.66 **b**
				°Brix 2009				
C	-	20.2	19.5	20.5	20.8	20.8	765	10.40 **a**
ED	22.9	20.0	23.2	22.7	22.2	21.1	602	4.71 **c**
LD	24.2	21.9	22.2	22.2	21.9	22.0	780	3.71 **d**
CT	23.7	22.1	22.0	23.1	23.0	23.0	689	6.35 **b**

Table 6. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning for different berry size categories as specified in the table in 2008 and 2009 on °Brix variance of data (N=number of data analyzed). Means within columns followed by different letters differ significantly at p<0.05 by the Student-Newman-Keuls test.

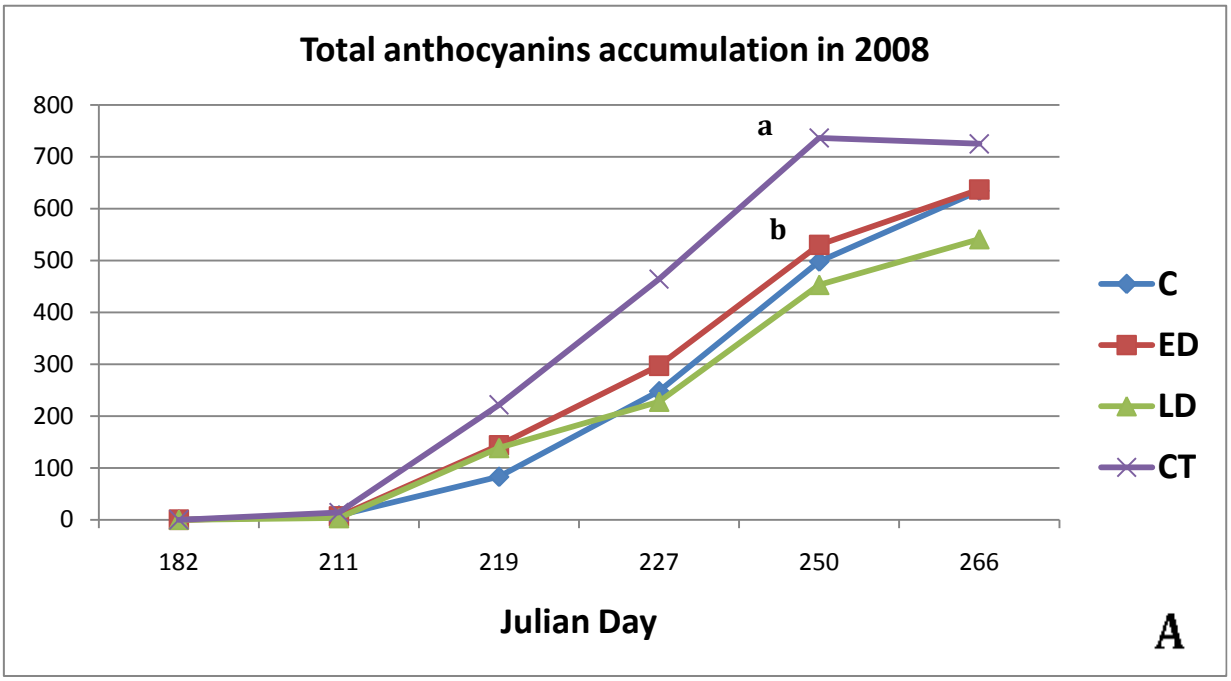
3.3 Flavonoids compounds analyses

3.3.1 Analyses of anthocyanins amount and composition

As expected, the accumulation of anthocyanins began in 2008 and in 2009 at véraison for all treatments (Fig. 12). Since no differences were found in berry weight between the treatments, all data are expressed as mg/kg berry. At the beginning of véraison C, ED, LD and CT showed comparable values of total anthocyanins. Immediately after cluster thinning, CT berries started to accumulate anthocyanins more rapidly respect to the other thesis, and, at harvest, CT presented the highest content of total anthocyanins. In 2009 this difference was significant and it should be possible to suppose a direct correlation between the alteration in source-sink balance (confirmed by the highest value of CT leaf area/crop weight ratio) and the increase in total anthocyanin amount. The defoliation, both at pre-bloom or at véraison, did not affect in a so noticeable way the rate of anthocyanin accumulation and, for both years, ED and LD showed at harvest anthocyanin concentration similar to C. The result observed in LD berries suggests that the cluster microclimate changes from véraison, represented by an increase in cluster light exposure and in cluster temperature, have no effect in enhancing the biosynthesis of anthocyanins.

Véraison defoliation was the treatment, which more than the others influenced anthocyanin composition and its action affected in particular the 3'-substituted branch of the anthocyanin pathway (Table 7). In particular, of the two 3'-substituted anthocyanins (cyanidin- and peonidin-3-O glucoside), only cyanidin 3-O-glucoside percentage was affected in LD berries, suggesting a key role of the enzyme F3'H respect to all the other enzymes of the biosynthesis in this kind of response. At harvest, cyanidin 3-0-glucoside represented over the 40% of total anthocyanins in LD berries both in 2008 and 2009. The increase in cyanidin 3-O-glucoside was always accompanied by a significant decrease in malvidin 3-O-glucoside, which at harvest represented only the 15% of total LD anthocyanins. Changes in anthocyanin profile were noticed also in CT, where peonidin-3-O

glucoside increased from 15% to 20%. This increase was accompanied by a little increase in



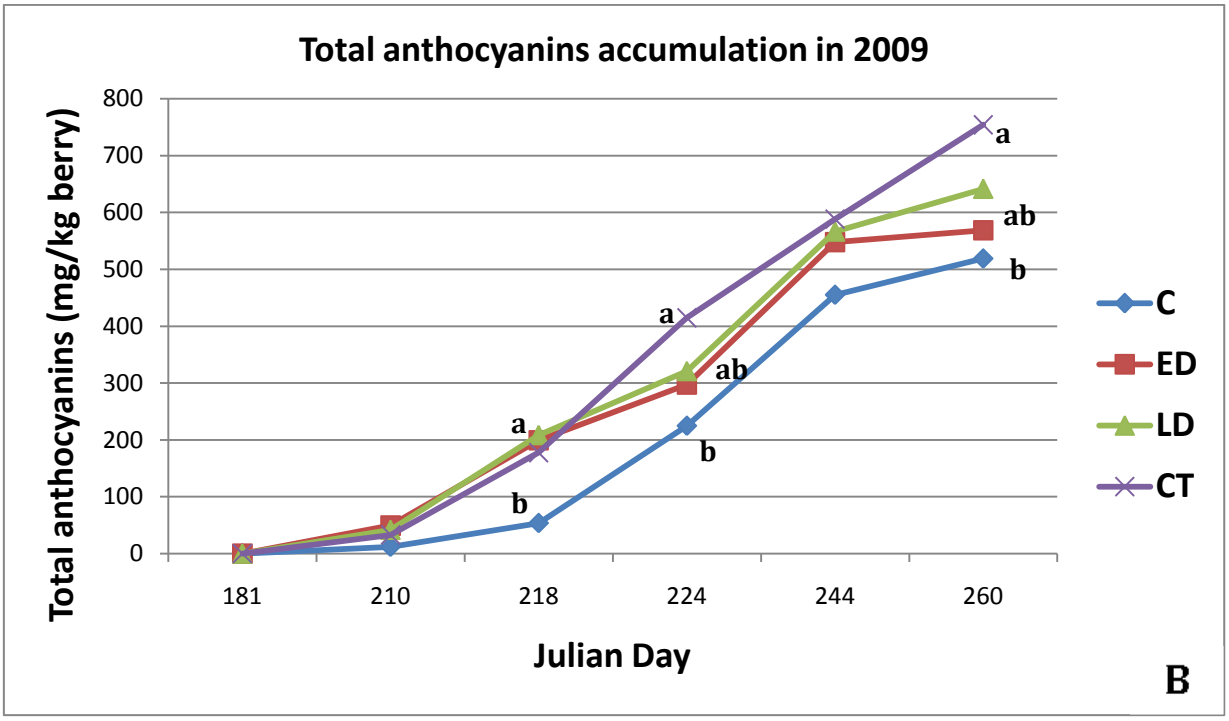


Fig. 12. Accumulation of anthocyanins in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest. A. Data collected in 2008. B. Data collected in 2009. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at $p<0.05$.

	Total anthocyanins (mg/kg berry)	Delphinidin-3-O-glucoside (%)	Cyanidin-3-O-glucoside (%)	Petunidin-3-O-glucoside (%)	Peonidin-3-O-glucoside (%)	Malvidin-3-O-glucoside (%)
			2008			
C	634.54	14.88	28.02 **b**	14.58	13.48 **b**	29.04 **a**
ED	637.02	15.29	32.70 **ab**	14.02	14.29 **b**	23.71 **ab**
LD	541.02	15.20	42.88 **a**	12.04	13.14 **b**	16.75 **b**
CT	725.00	13.21	30.61 **ab**	13.32	17.33 **a**	25.53 **ab**
			2009			
C	518.94 **b**	12.99 **ab**	31.55 **b**	12.30 **ab**	17.20 **b**	25.96 **a**
ED	568.66 **ab**	14.66 **a**	33.68 **ab**	13.21 **a**	15.30 **b**	23.14 **ab**
LD	641.60 **ab**	12.53 **ab**	47.17 **a**	10.31 **b**	14.94 **b**	15.05 **b**
CT	754.31 **a**	11.31 **b**	38.42 **ab**	10.50 **ab**	20.60 **a**	19.17 **ab**

Table 7. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning on anthocyanin amount and composition at harvest in 2008 and 2009. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test.

cyanidin-3-O glucoside together with a decrease in malvidin 3-O-glucoside (both resulting not statistically different). The noticeable changes in ED anthocyanin composition were the increases in delphinidin 3-O-glucoside and petunidin 3-O-glucoside observed in 2009. In both years, as previously seen for CT, it was found an increase in cyanidin-3-O glucoside and a decrease in malvidin 3-O-glucoside without significant differences from a statistical point of view. As a consequence of the described differences in the composition of the single anthocyanins, the four thesis showed a different profile in 3'-substituted/3'5'-substituted and in hydroxylated/methylated anthocyanins (Fig.11). The effect due to the increase in cyanidin 3-O-glucoside percentage, a 3'-substituted and hydroxylated anthocyanin, implies both in 2008 and in 2009 the highest percentage of these two classes of anthocyanins in LD berries, suggesting a role for the increase in cluster light exposure and temperature from véraison in modifying this profile. The significant variation in peonidin 3-O-glucoside (a 3'-substituted and methylated anthocyanin), observed in CT berries, did not affect the final composition neither of 3'-substituted nor methylated anthocyanins and so CT presented an intermediate profile, which was no different from the profile of any other thesis. The behavior of ED berries in terms of anthocyanin profile was instead different among the two years and so, while in 2008 ED berries showed a profile similar to CT and to the other treatments, in 2009 it presented a composition in 3'-substituted and hydroxylated anthocyanin

equal to C and CT, but different from LD berries. This result was unexpected, if it is considered that the changes in cluster microclimate caused by the defoliation treatments were equal for ED and LD berries, except the time at which defoliation occurred. In Fig. 13 is represented the 3'5'-substituted/3'-substituted ratio of C, LD and ED berries from the beginning of véraison to harvest. Differentially from C and LD berries, ED berries presented an initial higher value of 3'5'-substituted/3'-substituted ratio and, even if the trend of this ratio had the same course for both the defoliation treatments, ED presented always higher values compared to LD berries. This could be explained by the differences observed at harvest in the amount of the precursor of the 3'-substituted anthocyanins, cyanidin 3-O-glucoside, and of the 3'5'-substituted anthocyanins, delphinidin 3-O-glucoside. At the beginning of the season the ratio delphinidin 3-O glucoside/cyanidin 3-O glucoside is higher for ED (0.66) compared to C and LD berries (0.51 and 0.44, respectively), and this justify also the increase observed in ED berries in petunidin 3-O glucoside, which directly derives from delphinidin 3-O glucoside. Regards to the hydroxylated/methylated ratio, comparable values were found at the beginning of véraison among C, ED and LD berries (Fig. 15) and this could be explained by the fact that over the 70% of total hydroxylated anthocyanins are represented by cyanidin 3-O-glucoside and only 30% depend on delphinidin 3-O-glucoside. Probably, in the elapsing time between the 2009 pre-bloom defoliation and the beginning of véraison, the degradation of anthocyanin precursor was activated, cancelling any possible positive effects caused by pre bloom defoliation in terms of anthocyanins accumulation. This could explain the lower amount of 3'-substituted anthocyanins, which, since their lower substitution degree are more sensible to degradation.

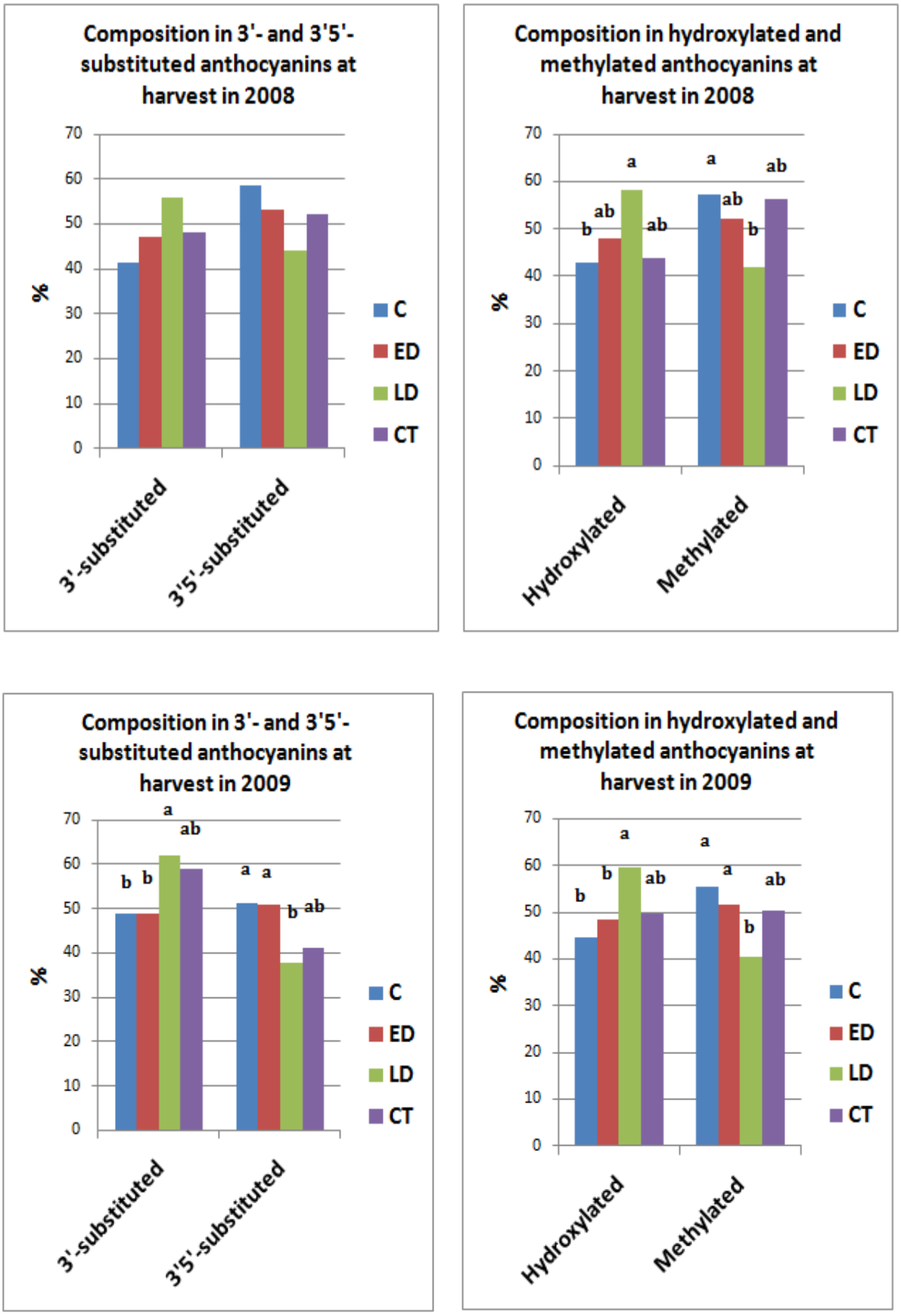


Fig. 13. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning on anthocyanin composition at harvest in 2008 and 2009. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at $p<0.05$.

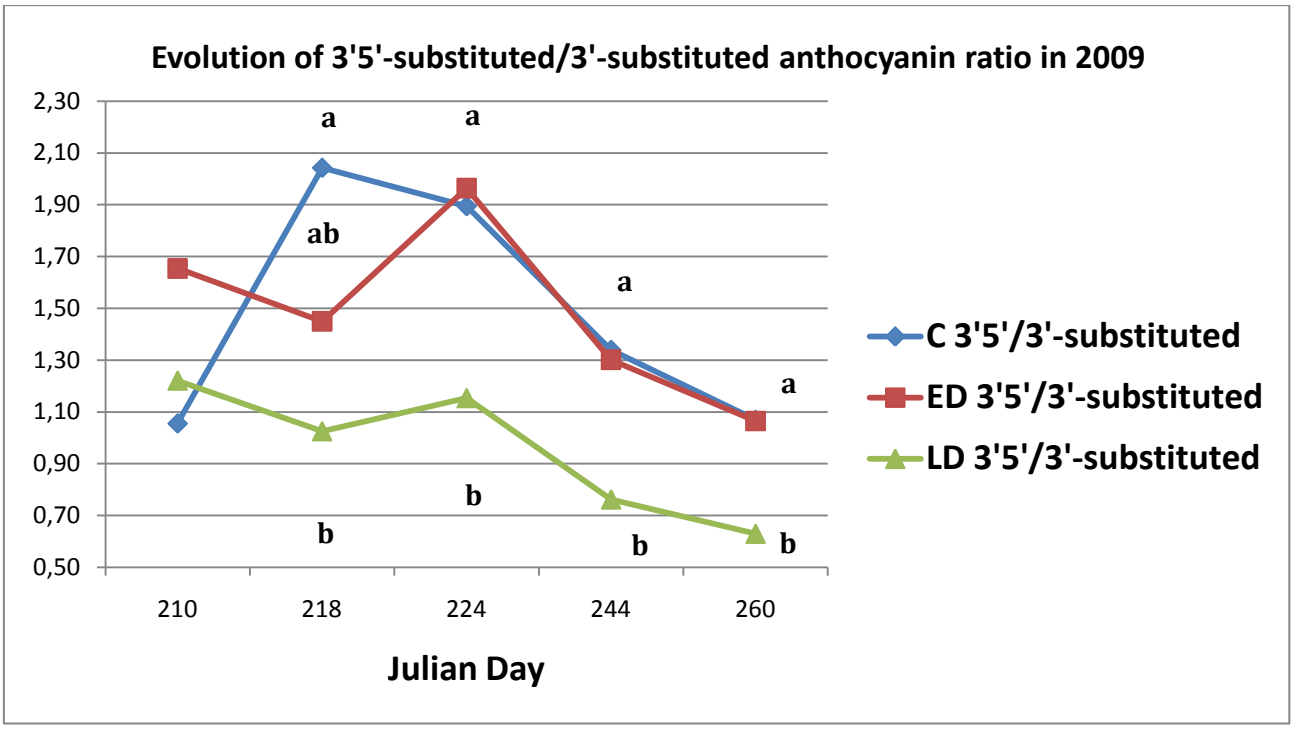


Fig. 14. Effect of the defoliation treatments on the evolution of 3'5'-substituted/3'-substituted anthocyanin ratio in 2009. Bars represent ± standard error. Superscript letters indicate significant differences by the Tukey-Kramer test between the three treatments at p<0.05.

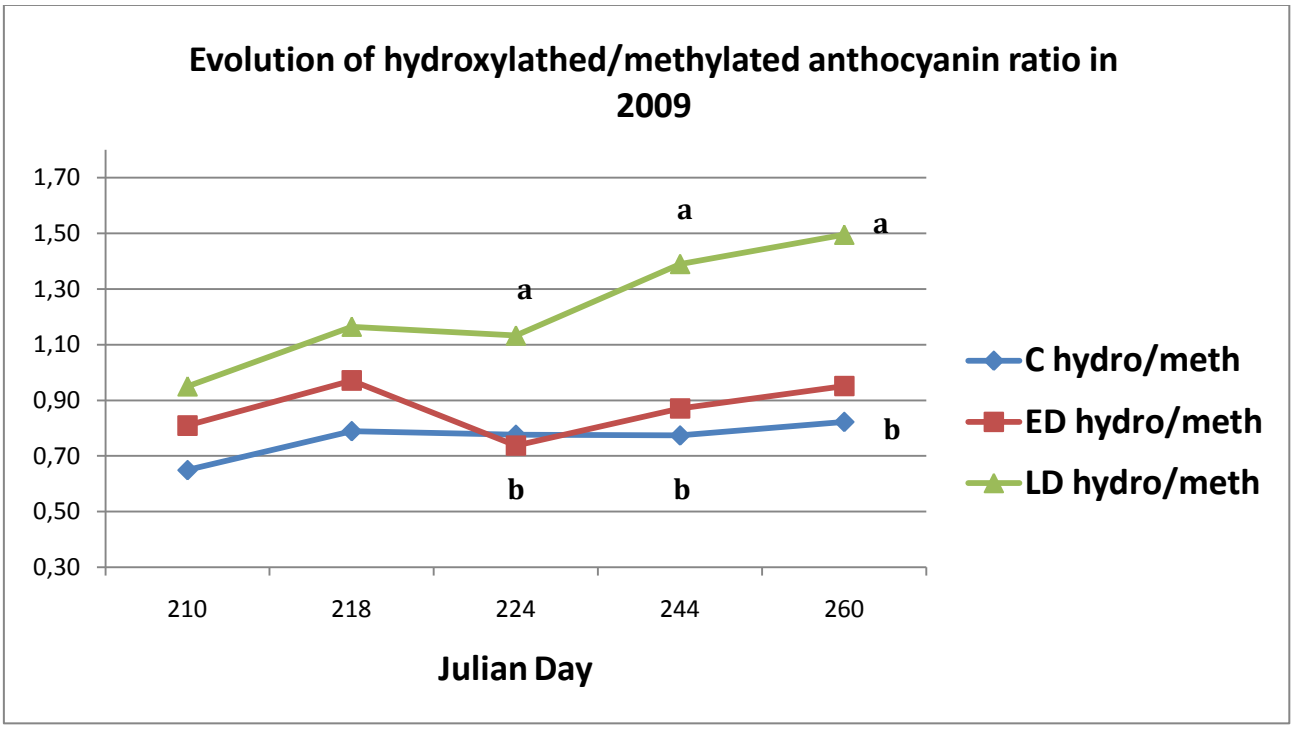


Fig. 15. Effect of the defoliation treatments on the evolution of hydroxylated/methylated anthocyanin ratio in 2009. Bars represent ± standard error. Superscript letters indicate significant differences by the Tukey-Kramer test between the three treatments at p<0.05.

3.3.2 Analyses of flavonols amount and composition in 2008 season

Final amount of flavonols is very low compared to total anthocyanins, even if their accumulation begins earlier in berry development (Fig. 16). Both defoliation treatments had a strong effect on flavonols biosynthesis. At the stage of pre-bunch closure, ED berries presented just an higher and significant different flavonols content compared to all the other treatments. Immediately after the véraison defoliation in LD berries was detected a peak in flavonol accumulations, which rapidly reached comparable values to those observed in the same stage for ED berries. Higher levels in flavonols accumulation were maintained for both ED and LD berries from the defoliation treatments until harvest, when ED and LD berries showed a total flavonol amount two-fold greater compared to that detected in C and CT berries. This suggested that high temperature are not linked with the regulation of flavonols biosynthesis, which is on the contrary stimulated by the higher cluster exposure, at whatever time it occurred. Cluster thinning did not affect flavonols biosynthesis and, during all the season, CT berries showed an overlapping trend to C in flavonols accumulation. Regards to flavonols composition, defoliation treatments implied an increase in quercetin and kaempferol percentage. Quercetin derived from the 3'-substituted branch of the flavonoids pathway, being the product of dihydroquercetin. Since dihydroquercetin is also a precursor of cyanidin, this results seems to be very comprehensible for LD berries, where it has been

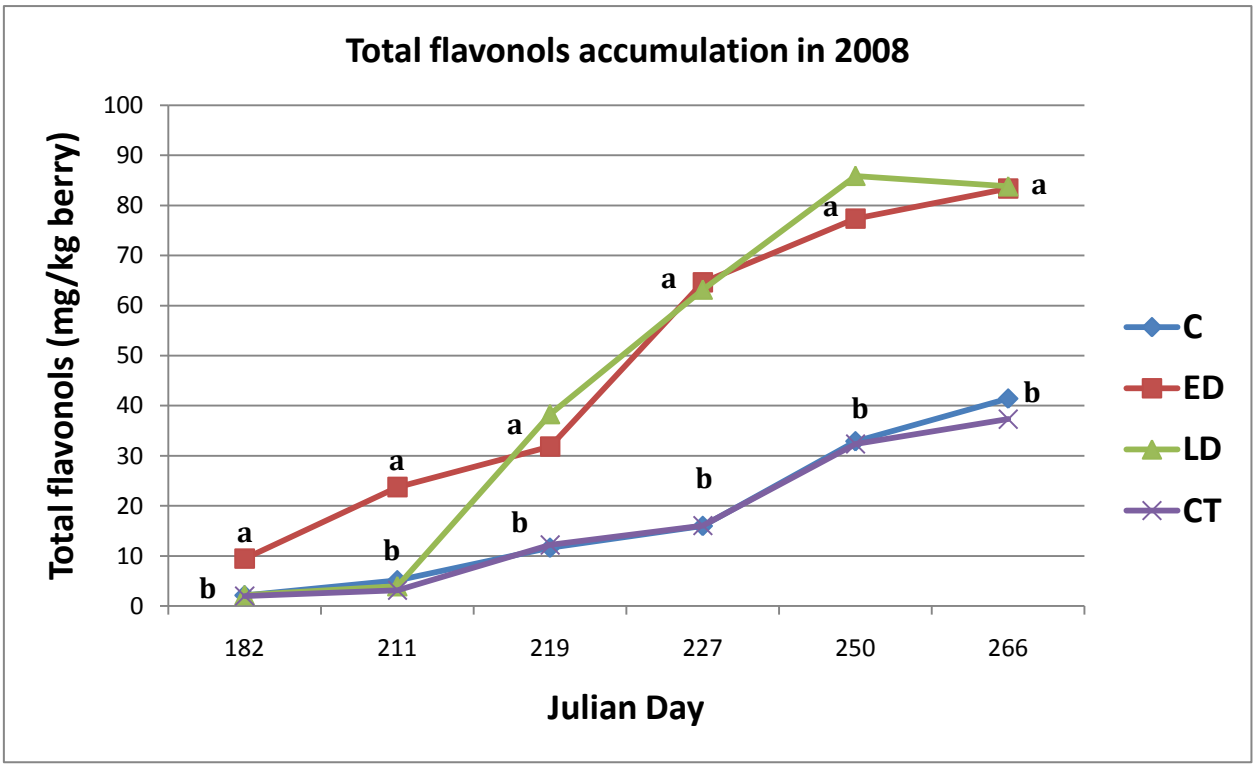


Fig. 16. Accumulation of flavonols in berry skin of C, ED, LD and CT Sangiovese vines in 2008 from pre-bunch closure stage to harvest. Bars represent ± standard error. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05.

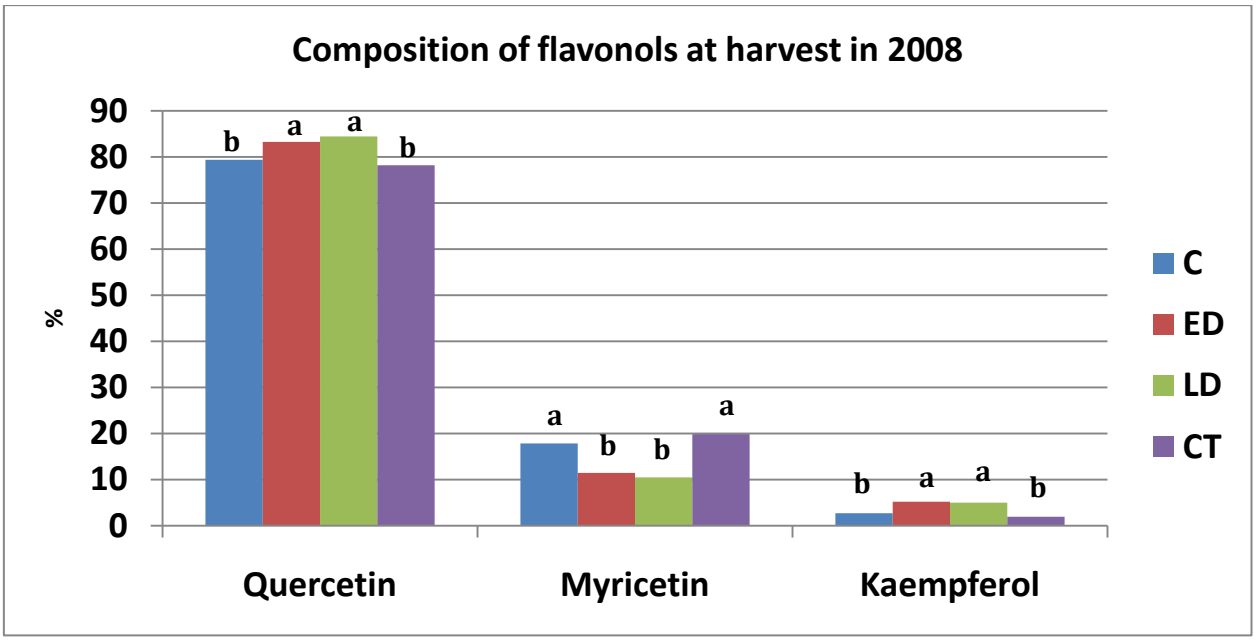


Fig. 17. Effects of pre-bloom defoliation, véraison defoliation and cluster thinning on flavonols composition at harvest in 2008 and 2009. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05.

demonstrated that the cluster microclimate changes deriving from véraison defoliation, activated this part of the flavonoids pathway in a privileged manner compared to the pathway that leads to

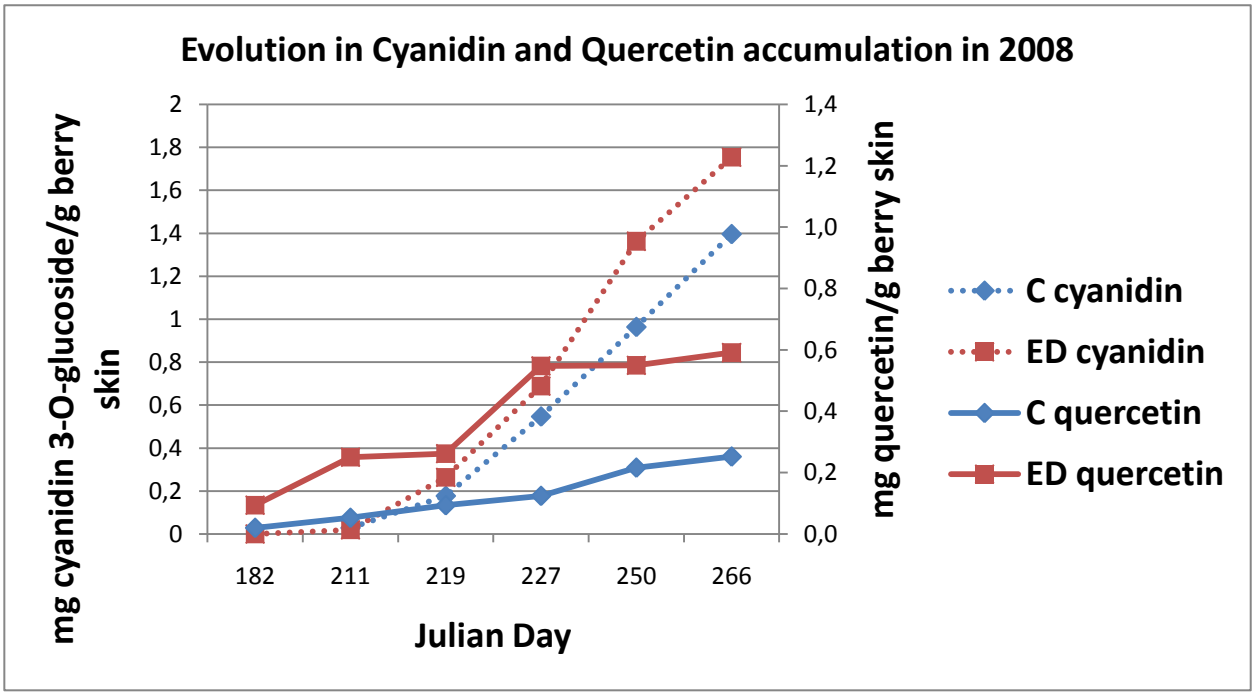


Fig. 18. Evolution of quercetin and cyanidin 3-O glucoside accumulation in C and ED berries in 2008 season from the stage of pre-bunch closure to harvest.

the biosynthesis of 3'5'-substituted anthocyanins. Usually, in fact, the key enzyme in biosynthesis of flavonols, FLS, shows a really low activity at the beginning of véraison and its activation in response

to véraison defoliation occurs in a moment in which UFGT is yet active and also anthocyanins could be synthesized. In the case of ED berries seems instead that the early increase in cluster exposure could have no effect on the cyanidin increase, as cyanidin 3-O glucoside biosynthesis begins, however, after véraison. So, when the biosynthesis of anthocyanin begins, probably could be established in ED berries a sort of competition between quercetin and cyanidin biosynthesis (Fig. 17). This could be demonstrated comparing the different trends in accumulation of cyanidin 3-0-glucoside and quercetin among C and ED berries (Fig.18). While in C berries the two trends seems to be independent one each other, in ED berries at higher level of cyanidin 3.0 glucoside accumulation corresponded a contemporary no increase in quercetin content.

3.4 RESULTS and DISCUSSION of TRANSCRIPTIONAL ANALYSES

3.4.1 Extraction and quality evaluation of total RNA

On the basis of biochemical results microarray analysis was performed on three selected stages of berry ripening: beginning of véraison (stage II), end of véraison (stage IV) and harvest (stage VI) for each treatment. Three biological replicates were analyzed for each sample, for a total of 36 hybridization reactions. Total RNA was extracted as described in material and methods section and for each sample the RNA integrity was assessed. In Figure 19 we can observe an example of Bioanalyzer results of three samples, carried through the successive transcriptional analyses.

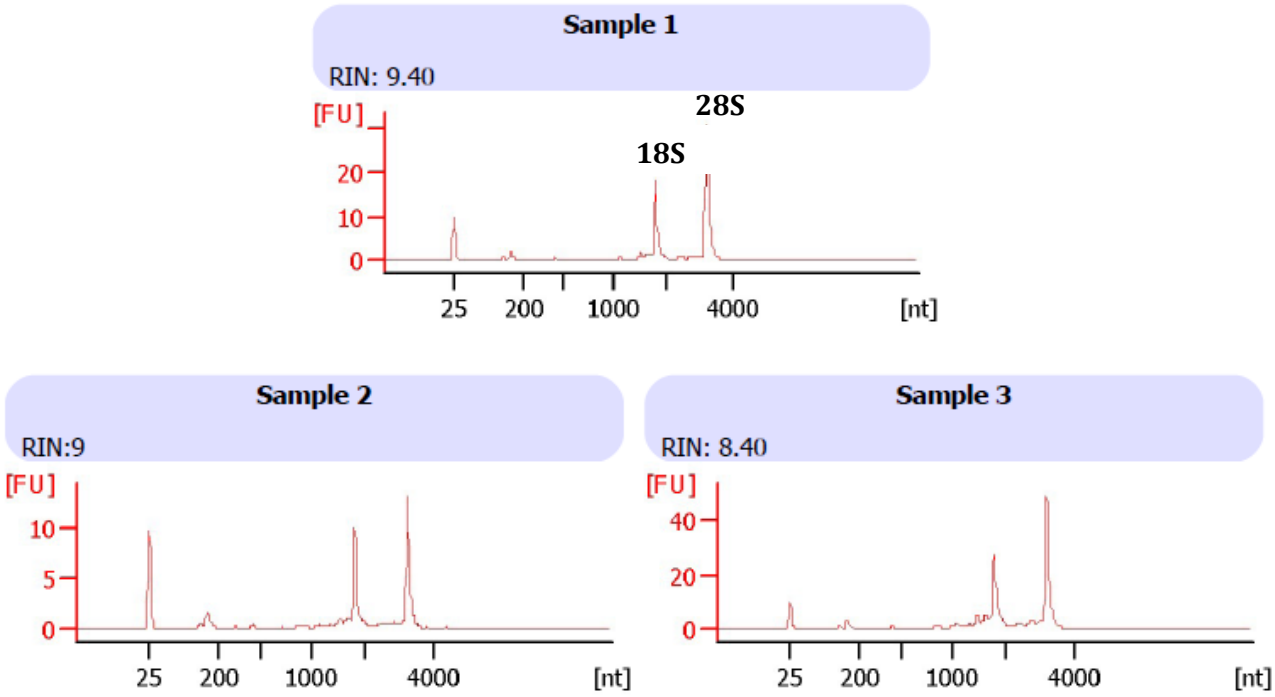


Fig. 19. Evaluation of RNA integrity of three successively analyzed samples by BioAnalyzer instrument. RNA samples showing RIN (RNA Integrity Index) higher than 6.5 could be considered of good integrity. This could be further confirmed by the electhropherogram, in which could be easily distinguished 18S and 28S rRNA peaks.

3.4.2 NimbleGen chip hybridization

RNA of good quality was carried out through labelling and hybridization steps, as described in material and methods. The hybridizations were performed on commercially available Grape Nimblegen chip, design 090407_Vitus_MD_exp_HX12, (Roche Nimblegen Company) containing 135000 probe, representing 29550 predicted genes. Figure 20 shows an example of chip scannerized image of 12 samples. Each biological replicate was tested through the Pearson's statistic to determine the robustness of sampling. All samples showed Pearson's coefficients ranging from 0.91 to 0.98 that confirmed the good reliability of biological replicates.

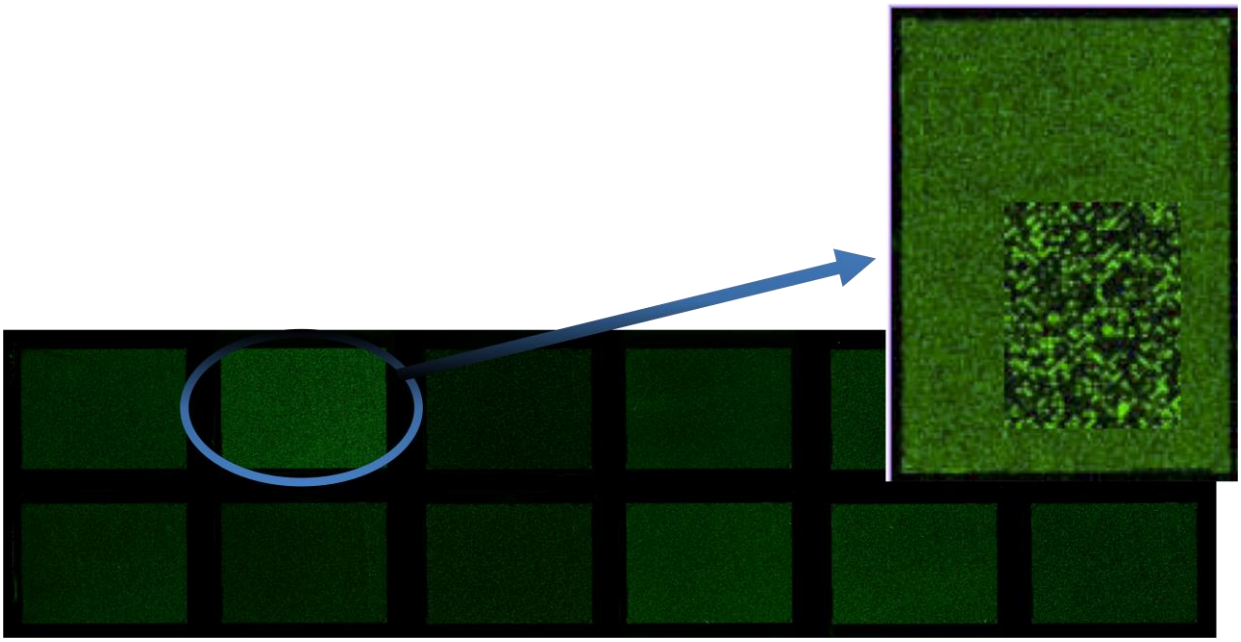


Fig. 10. NimbleGen array used to carry out microarray analysis in the experiment.

3.4.3. Principal Component Analysis

Principal Component Analysis (PCA), was used to define associations at different developmental stages between treatments within the global transcriptomic data. In Fig. 21A is represented the result of PCA analysis on data collected for the three C, CT, LD and ED biological replicates at beginning of véraison. PC1 and PC2 explained only the 48% of the overall variance and so at this stage it was not possible to clearly separate the different treatments on the basis of their transcriptomic patterns. This suggests that the transcription patterns of berries collected at beginning of véraison were similar in four treatments. This result is easy to understand in the case of CT and LD berries, in which the treatments were applied at the same time of the microarray analysis sampling. In the case of ED berries, where early defoliation occurred early in the season (8 weeks before beginning of véraison) we may suppose to find some effects on global transcriptome, but, probably due to the high sampling variability, no variation was detected at expression level at beginning of véraison.

At end of véraison the situation was quite different and PCA analyses was able to explain around the 65% of the overall variance on the basis of global transcriptome (Fig.21 B). On the second component was so possible to distinguish all the treatments from C and on the first one CT from both ED and LD transcription patterns. It is very interesting to note that ED transcription pattern exactly overlaps to LD one.

At harvest time the two principal components explained the 52% of the overall variance of transcription profiles, but this does not allow to clearly differentiate the different treatments (Fig.21 C).

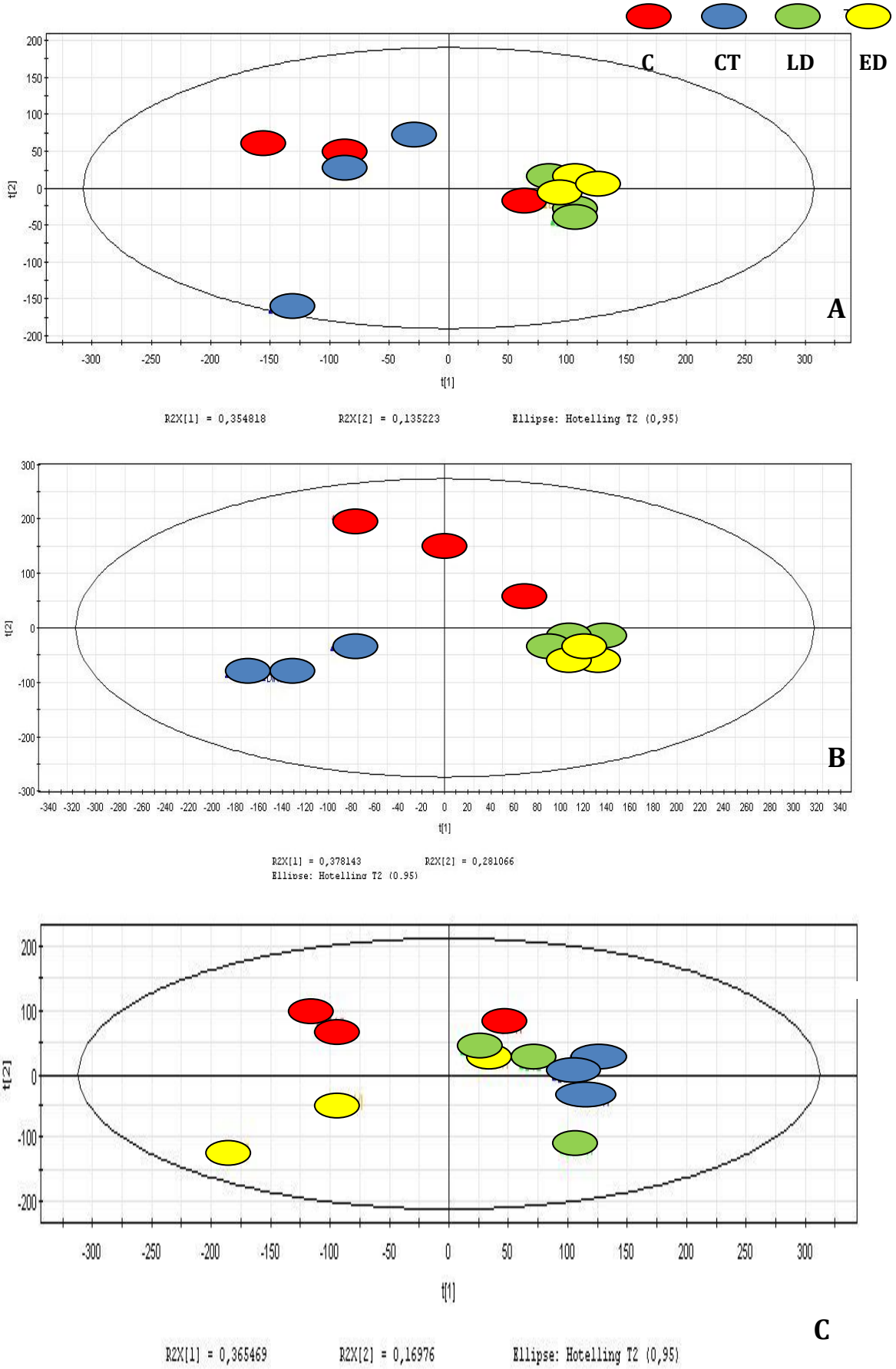


Fig. 21. Principal component analysis (PCA) of the 36 expression datasets performed at beginning of véraison (A), end of véraison (B) and at harvest (C). The three C biological replicates are respectively labeled in red for C, blue for CT, green for LD and yellow for ED.

3.4.4. Two-class unpaired comparison analyses

In Table 8 is reported the number of differential expressed genes between C and each treatment for the three ripening stages analyzed. Significance Analyses of Microarray (SAM) was carried out on genes set whose intensity value was higher than the negative control threshold. A False Discovery Rate of 2% was then chosen to identify only the effective differences; this means that with a 98% probability the differential expressed genes could be considered as really linked only to a treatment effect. Up-regulated genes with a Fold Change (FC) higher than 2 and down-regulated genes with a FC lower than -2 were finally considered as really differentially expressed compared to C.

End of véraison was the stage during berry ripening in which differences in gene expression were detected between all the treatments and C. This data confirm what previously seen at end of véraison with PCA analysis, in which was possible to separate defoliation treatments and CT from C. End of véraison could so be considered the crucial stage in berry ripening, for determine final vine response to the applied treatments. No differences were in fact obtained with SAM analysis

	Total number of differential expressed genes	Number of up regulated genes	Number of down regulated genes	Differential expressed genes on total gene analyzed (%)
Unpaired comparison	**Beginning of véraison**			
C-ED	0	0	0	0
C-LD	0	0	0	0
C-CT	0	0	0	0
	End of véraison			
C-ED	1777	1302	474	6.01
C-LD	1476	1168	308	4.99
C-CT	4070	1438	2632	13.78
	Harvest			
C-ED	5	1	4	0.02
C-LD	0	0	0	0
C-CT	490	234	260	1.65

Table 8. Percentage of different expressed genes at beginning of véraison, end of véraison and at harvest, which resulted after two-class unpaired comparison analyses between C and each treatment. Only genes with FC higher than 2 or lower than -2 were considered as differentially expressed.

for any of the treatments at beginning of véraison, while at harvest only transcription profile of CT berries differentiated from C. From this preliminary data it is possible to affirm that the source-sink balance modifications which occurs in CT vines at véraison could be considered the major cause of berry grape transcriptional modifications (13.78% of the entire transcriptome is modified by

cluster thinning) compare to ED and LD (6.01% and 4.99% of transcriptome modification respectively). These modifications, occurred at end of véraison, extend with a lower effect until harvest only in CT vines (1.65% of all genes differentially expressed, Table 8). Cluster light and temperature increase induced by early and late defoliation resulted respectively in 0.02% and 0% of difference in transcriptional profiles at harvest.

It is interesting to note that while CT treatment rose mainly in a down-regulation of the entire transcriptome, ED and LD caused instead an up-regulation of differentially expressed genes.

3.4.5. Annotation of the differentially expressed genes

The chip design was carried out on the basis of the 12X draft of *Vitis Vinifera* genome. Gene prediction and annotation were performed by the CRIBI group at University of Padova, which produced a prediction of 29971 genes (Version V1). V1 annotation was then performed by different approaches as Blastp analyses against Plant Uniprot association with Gene Ontology (GO, www.geneontology.org) and Pfam analyses (match of the protein sequence with a large collections of protein families; http://pfam.sanger.ac.uk/). Furthermore, at University of Verona each coding sequence was translated into protein and a blast analyses against all deposited proteins in UniProt database (http://www.uniprot.org/) was carried out.

Transcripts were finally grouped into 19 GO plant slim functional categories (Table 9), which describe the main process occurring in berry during the whole ripening period, from the beginning of véraison to full ripening.

FUNCTIONAL CATEGORY	GO ID	GO description
Biological process	GO:0008150	Any process specifically pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms. A process is a collection of molecular events with a defined beginning and end.
Biosynthetic process	GO:0009058	The chemical reactions and pathways resulting in the formation of substances; typically the energy-requiring part of metabolism in which simpler substances are transformed into more complex ones.
Carbohydrate metabolic process	GO:0005975	The chemical reactions and pathways involving carbohydrates, any of a group of organic compounds based of the general formula Cx(H2O)y. Includes the formation of carbohydrate derivatives by the addition of a carbohydrate residue to another molecule.
Catabolic process	GO:0009056	The chemical reactions and pathways resulting in the breakdown of substances, including the breakdown of carbon compounds with the liberation of energy for use by the cell or organism.
Cell death	GO:0008219	A biological process that results in permanent cessation of all vital functions of a cell.

FUNCTIONAL CATEGORY	GO ID	GO description
Cellular amino acid and derivative metabolic process	GO:0006519	The chemical reactions and pathways involving amino acids, organic acids containing one or more amino substituents, and compounds derived from amino acids, as carried out by individual cells.
Cellular homeostasis	GO:0019725	Any process involved in the maintenance of an internal equilibrium at the level of the cell. Any process involved in the maintenance of an internal equilibrium at the level of the cell.
Cellular process	GO:0009987	Any process that is carried out at the cellular level, but not necessarily restricted to a single cell.
Generation of precursor metabolites and energy	GO:0006091	The chemical reactions and pathways resulting in the formation of precursor metabolites, substances from which energy is derived, and any process involved in the liberation of energy from these substances.
Lipid metabolic process	GO:0006629	The chemical reactions and pathways involving lipids, compounds soluble in an organic solvent but not, or sparingly, in an aqueous solvent.
Metabolic process	GO:0008152	The chemical reactions and pathways, including anabolism and catabolism, by which living organisms transform chemical substances. Metabolic processes typically transform small molecules, but also include macromolecular processes such as DNA repair and replication, and protein synthesis and degradation.
Nucleobase, nucleoside, nucleotide, nucleic acid metabolic process	GO:0006139	Any cellular metabolic process involving nucleobases, nucleosides, nucleotides and nucleic acids.
Photosynthesis	GO:0015979	The synthesis by organisms of organic chemical compounds, especially carbohydrates, from carbon dioxide (CO2) using energy obtained from light rather than from the oxidation of chemical compounds.
Protein modification process	GO:0006464	The covalent alteration of one or more amino acids occurring in proteins, peptides and nascent polypeptides (co-translational, post-translational modifications). Includes the modification of charged tRNAs that are destined to occur in a protein (pre-translation modification).
Response to stimulus	GO:0050896	A change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus.
Signal transduction	GO:0007165	The cascade of processes by which a signal interacts with a receptor, causing a change in the level or activity of a second messenger or other downstream target, and ultimately effecting a change in the functioning of the cell.

FUNCTIONAL CATEGORY	GO ID	GO description
Transcription	GO:0006350	The cellular synthesis of either RNA on a template of DNA or DNA on a template of RNA.
Translation	GO:0006412	The cellular metabolic process by which a protein is formed, using the sequence of a mature mRNA molecule to specify the sequence of amino acids in a polypeptide chain. Translation is mediated by the ribosome, and begins with the formation of a ternary complex between aminoacylated initiator methionine tRNA, GTP, and initiation factor 2, which subsequently associates with the small subunit of the ribosome and an mRNA. Translation ends with the release of a polypeptide chain from the ribosome.
Transport	GO:0006810	The directed movement of substances (such as macromolecules, small molecules, ions) into, out of, within or between cells, or within a multicellular organism by means of some external agent such as a transporter or pore.

Table 9. Description of the 19 GO functional categories in which differentially expressed genes were grouped as reported in www.geneontology.org.

3.4.6 Differentially expressed genes at end of véraison

Functional annotation could be assigned approximately to 45% of modulated transcripts at end of véraison on total 29550 probes present on NimbleGen chip. This is a good results if it is considered that one recent publication on grape microarray analyses based on Affimetrix *Vitis* GeneChip®, which contains approximately 14500 unigenes, reported the annotation of 64% of transcripts (Deluc *et al.*, 2007).

Functional annotation of differential expressed genes at end of véraison was performed (Fig. 22). It has to be noticed that the relative frequency (%) of each functional category has been calculated as sum of the number of up- and down-regulated genes on total differentially expressed gene. As a consequence, at this level is only considered the global gene expression modulation. For all the treatments "metabolic process" and "transport" were the functional categories that had the largest number of transcripts. This is consistent with the developmental aspects of the end of véraison stage, in which are required high levels of metabolic activity.

Three comparisons (ED against LD; CT against ED; CT against LD) were performed to test for significant differences among treatments in relative gene frequency (%) of differentially expressed genes compared to C within each functional category.

From ED-LD comparison, resulted that ED berries were characterized by the highest number of modulated genes belonging to "protein modification process". This was the only statistically different category, which distinguished ED from LD, suggesting an higher level in global reorganization of proteomic pattern for ED.

CT showed an higher percentage of statistically different expressed genes belonging to "transcription" compared to both LD and ED.

"Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process" was instead the functional category, in which was present an higher number of differential expressed CT genes compared to LD. LD distinguished from CT for an elevated number of genes belonging to the functional categories of "translation", "response to stimulus" and "cell death".

Compared to CT, ED was instead characterized by the highest number of genes belonging to the "generation of precursor metabolites and energy" functional category, which is linked to high energy request processes.

It is very difficult at this level to associate the relative frequency of each functional category to one treatment rather than another. However, it is surely possible to affirm that source-sink modifications and cluster light and temperature increase are able to induce at end of véraison a global transcriptome modification, which is accountable of vine response for some physiological and biochemical aspects.

Following, on the basis of physiological and biochemical results, it will be treated in detail the modulation of genes belonging to the categories of interest for my research. The core set of differentially expressed genes was manually checked and integrated with bibliographic data (Deluc *et al*., 2007). In particular, will be reported the transcriptional profile of modulated genes belonging to:

a) carbohydrate metabolic process and transport functional categories;
b) response to stimulus functional category;
c) flavonoid biosynthesis, selected within metabolic process, transcription and transport functional categories.

For each category differentially expressed genes common to the three treatments and modification of specific gene expression for single treatment will also be described.

Functional categories distribution of differential expressed genes at end of véraison

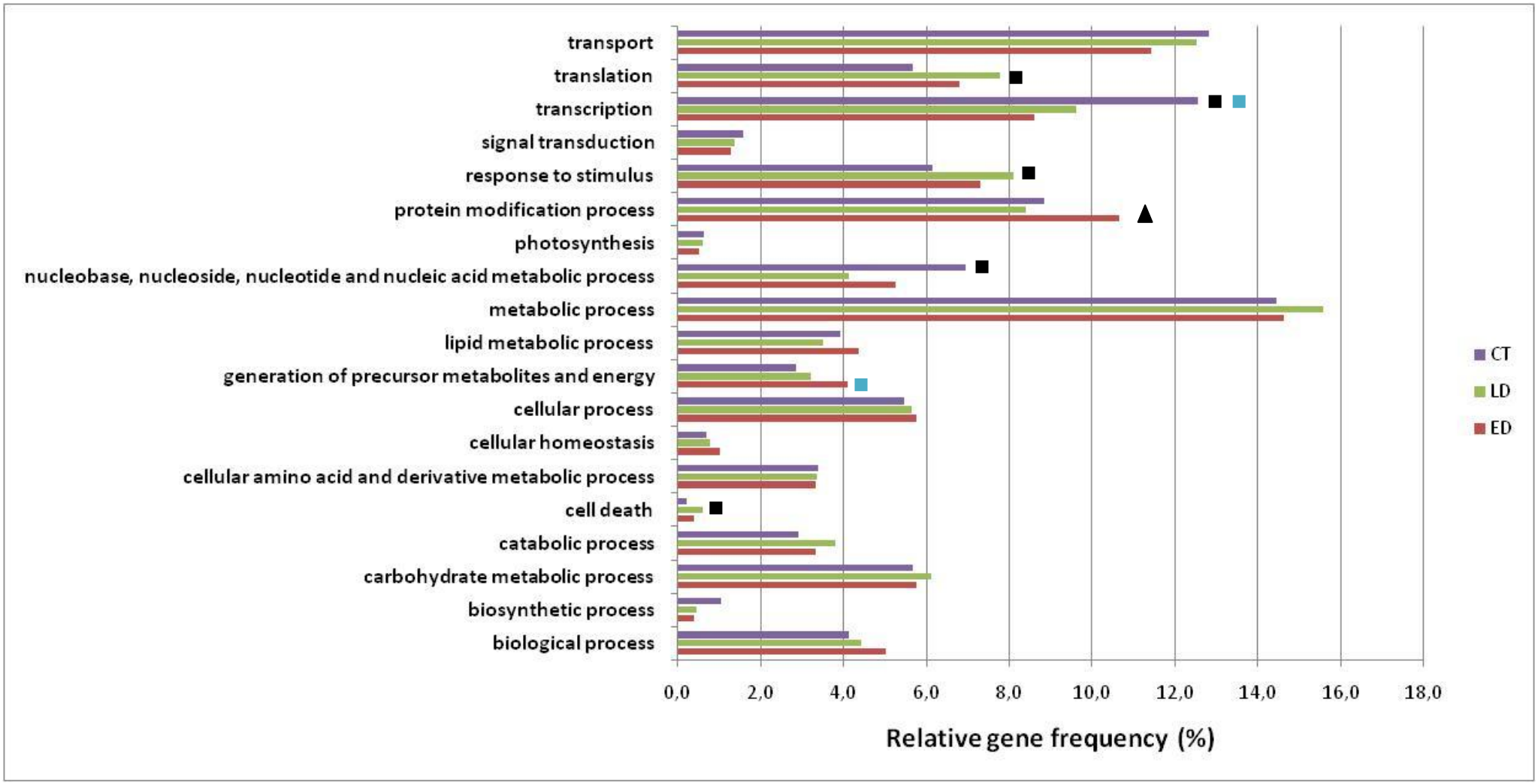


Fig. 22. Functional analyses of differential expressed transcripts compared to C at end of véraison. Statistically significant differences between CT against LD are indicated with black squares. Statistically significant differences between CT against ED are indicated with blue squares. Statistically significant differences between ED against LD are indicated with black triangles.

3.4.7. Differentially expressed genes belonging to "carbohydrate metabolic process" functional category at end of véraison

Despite no significant difference in the percentage of all modulated genes belonging to "carbohydrate metabolic process" was detected between ED, LD and CT berries the situation is quite different when up- and down-regulated genes are considered separately. In Fig. 23 is represented the relative gene frequency (%) of up- and down-regulated genes belonging to this functional category, kept separately. It has to be noticed that those genes involved in cell wall biogenesis or in protein glycosilation, even if belonging to this functional group, have been discarded and not considered at this level, because not directly involved in this study. The modulation of genes induced by cluster thinning caused at end of véraison mainly a down-regulation of the transcripts involved in the carbohydrate metabolism. About 75% of total differentially expressed genes belonging to this category were in fact repressed in CT berries compared to C. In the case of ED no down-regulated genes were detected, while in LD only one gene (corresponding to 6% of LD total differentially expressed genes) showed this trend.

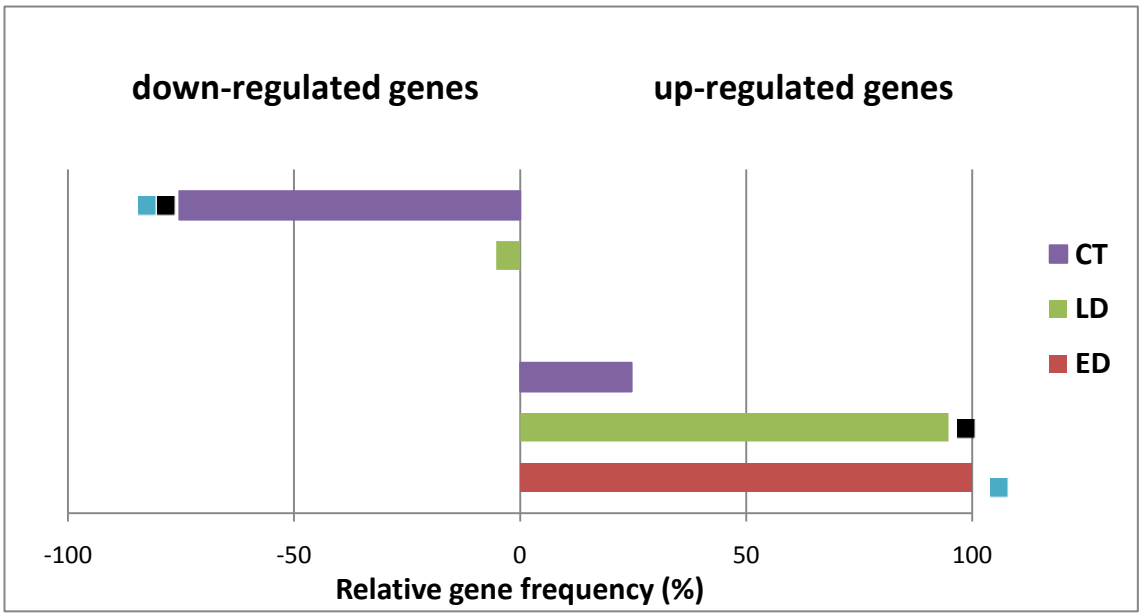


Fig. 23. Distribution of up- and down-regulated transcripts of "carbohydrate metabolic process" functional category at end of véraison. Statistically significant differences between CT against LD are indicated with black squares. Statistically significant differences between CT against ED are indicated with blue squares. No statistically significant differences were found in ED against LD comparison.

In Table 10 are listed all modulated "carbohydrate metabolic genes" for the three treatments. Among the up-regulated genes shared by the three treatments (Table 10A), genes involved in malate degradation, starch metabolism, carbohydrate catabolism (glycolysis) and sucrose metabolism and transport were detected. Two different isoforms of citosolic malate dehydrogenase (JgVv5.319 and JgVv5.318), were up-regulated in the three treatments. These enzymes are responsible of the degradation of malic acid to oxaloacetate and their activation well correlated with the rapid decline observed in total acidity in CT, ED and LD berries.

The up-regulation of one β-amylase gene (JgVv51.1) and a maltose excess protein (JgVv39.37) was also detected in all treatments. Both this enzymes take part in starch degradation that occur in berry chloroplast during ripening. Starch metabolism is poorly understood in grape berry, but could be very important in regulate the sugars cellular homeostasis. Sucrose is the form in which carbohydrates produced by leaves are transported towards bunches. The expression of a gene encoding for a sucrose synthase (JGVv5.73), which cleaves the sucrose to UDP-glucose and fructose increase in all treatments. The up-regulation of sucrose phosphatase (JGVv32.68), which catalyzes the final step of sucrose synthesis, was also detected. While until véraison the sugars imported into the berry are metabolized and no storage occurred, following véraison sugars could be accumulated in the hexose form in the vacuole. The transcripts abundance of a monosaccharide-sensing protein (JGVv38.81), which directs the transport from the citosol to different organelles, increased in ED, CT and LD berries. During the first phases of berry development the glucose obtained from sucrose cleavage is mainly used to yield ATP as an energy source. Sugars catabolism occurred in the cell via the glycolysis pathway. Some sequences encoding for key enzymes of glycolytic process are up-regulated in all the treatments (JGVv2.157 and JGVv19.365). Transcripts abundance of the UDP-glucose 4-epimerase (JGVv38.81), which catalyzes the conversion of UDP-glucose in UDP-galactose, was also detected in ED, CT and LD berries. Sometimes, after phosphorylation, also galactose can be used as substrate for glycolysis. UDP sugars can also act as prosthetic groups for the glycosilation, for instance, of proteins and phenolic compounds (flavonoids).

The pair-wise comparison of differential expressed genes at end of véraison resulted in a lack of up-regulated genes common to ED and CT and to CT and LD. Instead, ED-LD comparison(Table 10B) verifies the up-regulation of 6 genes belonging to the carbohydrate metabolism at end of véraison both in ED and LD treatments. Among these, the most involved was the gene encoded for RuBisCO (JGVv19.89), whose abundance increased 23 and 17 fold respectively in ED and LD berries, compared to C. RuBisCO takes part in the photosynthetic process, in particular in the Calvin cycle, which is light dependent and bring to glucose synthesis. A different isoform of a sucrose synthase (JGVv16.27) for sucrose cleavage and an α-amylase (JGVv63.39), which participates in starch degradation were also detected specifically in Ed and LD. The other three up-regulated genes takes directly (JGVv16.27) or indirectly (JGVv19.89) part to the glycolytic process or to the Krebs cycle (JGVv127.59).

Many genes have been found instead to be up-regulated specifically in one treatment. Eight specific up-regulated genes characterized CT treatment (Table 10D). The most representative was a transcript of a granule-bound starch synthase (JGVv22.151), that contribute to sugars storage through starch biosynthesis and whose transcripts were 60 fold more abundant compared to C. Furthermore, only CT berries showed an additional up-regulation of a NADP-dependent malic enzyme (JGVv8.9, FC=19), which it has been shown to be usually down-regulated at this stage, but

up-regulated in late ripening (Deluc *et al.*, 2007). Few other specific CT genes are involved in glycolysis or in sucrose metabolism.

Six genes were up-regulated specifically in ED berries (Table 10E). The pyruvate dehydrogenase complex (JGVv74.13), which contributes to transforming pyruvate into acetyl-CoA by a process called pyruvate decarboxylation, is the most induced. Two important enzymes (JGVv0.5 and JGVv61.144) involved in glycolytic process and a sugar transporter (JGVv54.23) are furthermore exclusively up-regulated in ED berries. In particular, the enzyme hexokinase is one of the most regulated in the glycolytic process, because it is considered a sugar sensor in higher plants. It is, in fact, activated only when its product (fructose-1,6-bisphosphate) concentration is low and so when the primary necessity for the cell is to produce energy towards carbohydrate catabolism. The hypothetical condition of energy request which may characterize ED berries at end of véraison stage, could be confirmed by the further up-regulation of the genes trehalase (JGVv25.201) and 6-phosphogluconate dehydrogenase (JGVv10.78). The products of these genes contribute in fact in cellular carbohydrate supply, through the conversion of trehalose to glucose and the synthesis of 5-carbon sugars via the pentose phosphate pathway. Several products of the pentose phosphate pathway can be in fact redirect in the glycolytic process or used for the primary metabolism, as nucleic acid or amino acid production.

LD berries showed to be the most similar to C, compared to CT and ED, in terms of carbohydrate metabolic process gene. One gene which encoded for a protein probably involved in glycolysis (JGVv2.677) and one gene involved in starch degradation (JGVv108.144) are the only two genes specifically up-regulated in LD berries (Table 10F).

As previously described, the main effect on carbohydrate metabolism genes observed in CT berries carried on the down-regulation of several genes belonging to this functional category. Among these only a β-amilase gene (JGVv25.197) was shared between CT and LD (Table 10C).

Many other genes involved in starch synthesis or degradation are strongly and specifically down regulated in CT berries compared to C (Table 10F), as one 4-alpha-glucanotransferase (JGVv31.137, FC=-10), or different α- and β-amilase isoforms. Several transcripts involved in glycolysis are also specifically down regulated in CT berries (Table 10D). Among these, it is interesting to note that the transcripts abundance of the 6-phosphofructo-2-kinase (JGVv4.148) is 6 fold lower in CT berries compared to C. The main role of 6-phosphofructo-2-kinase enzyme, is to coordinate the carbohydrate production and catabolism. Its product, fructose-2,6-bisphosphate, is in fact a key regulator of glycolysis (and gluconeogenesis, the process in which glucose is generated from non-carbohydrate carbon substrates). High levels of fructose-2,6-bisphosphate activated glycolysis, while low levels have the opposite effect. Also genes of sucrose metabolism are down-regulated in CT berries, as an isoform of sucrose synthase (JGVv29.35, FC=-6) and an invertase (JGVv22.158), which both act on sucrose cleavage. Two isoforms of chloroplastic malate dehydrogenase genes (JGVv108.80 and JGVv0.365) have been found to be down-regulated in CT berries. Their role is

mainly photosynthetic, as they decarboxylate malate to piruvate releasing CO_2, which could be taken up by RuBisCO to synthesize glucose. The remaining down-regulated genes are involved in the pentose phosphate pathway and in sugars transport. Several genes are finally indirectly involved in carbohydrate metabolism as take part in sugars isomerisation or hexose cleavage from complex polymer.

It is very interesting to note that at end of 2008 véraison (JD 227), ED berries were the one which presented the lower soluble sugars content (Fig. 24), even if, immediately after, sugars increased and remained at high levels until harvest. Probably the stress condition, due to the early leaf removal, in which berries developed and ripened, caused a delay in the initial ripening phases. No activation of carbohydrate storage occurred at end of véraison in ED berries and carbohydrate metabolism is activated mainly in order to produce energy for cellular necessity. The up-regulation of some key genes involved in many metabolic pathway with the aim to produce energy and C skeletons for primary metabolism could sustain this hypothesis. Furthermore, it has to remember, even if this specific part will be not broadened at this level, that ED berries were also the one in which it was found the highest percentage of genes belonging to "generation of precursor metabolites and energy" functional category.

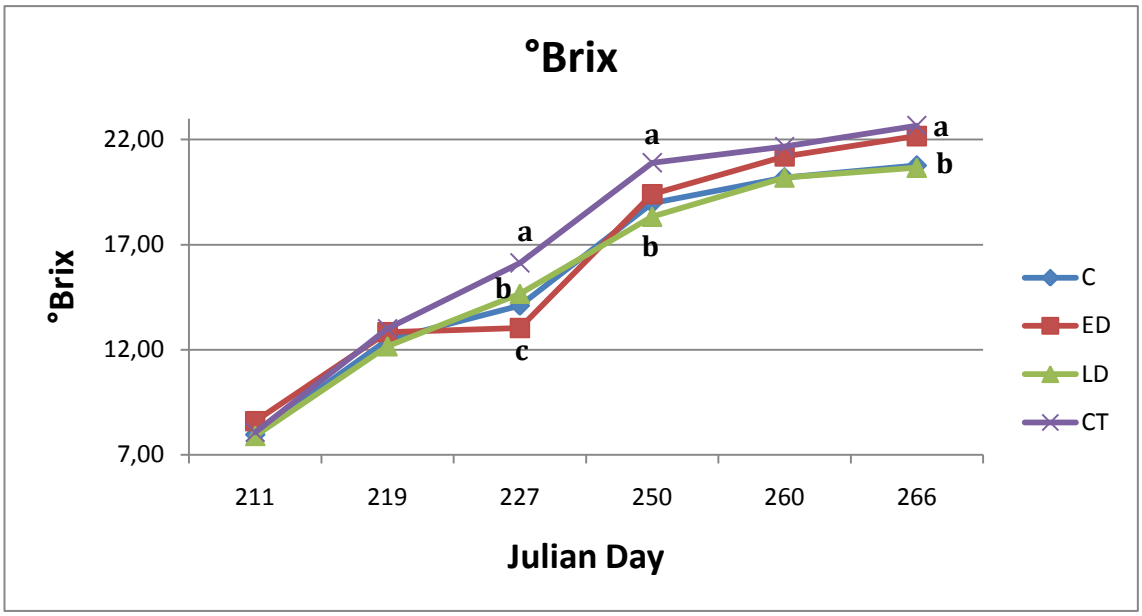


Fig. 24. Monitoring of soluble sugars(°Brix) accumulation at different intervals from the beginning of véraison to harvest. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at $p<0.05$.

Very different is, instead, the effect of cluster thinning on soluble solids accumulation (Fig. 6), which seems to occur almost contemporaneously to the cluster removal (JD211). This suggest an acceleration in carbohydrate metabolism following the treatment and this higher accumulation rate was maintained until harvest. Carbohydrate metabolism in CT berries, seems so to be at end of véraison mainly yet direct in sugars storage. Many genes involved in sugars catabolism, energy production, sucrose transport and starch degradation are in fact down-regulated in CT berries,

while genes belonging to starch biosynthesis or sucrose cleavage were more abundant compared to C.

LD berries showed a transcriptional profile in terms of carbohydrate metabolic process almost intermediate between ED and C. As expected, except the up-regulation of RuBisCo gene, probably due to the higher cluster light exposure, no strong changes occurred in this branch of cellular metabolism for LD berries. This is confirmed by the observation that LD soluble sugars accumulation did not differ from C at any stage of berry ripening.

	FOLD CHANGE					
CT-ED-LD	**CT**	**ED**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV5.319	17.28	10.34	11.90	carbohydrate metabolic process	lactate/malate dehydrogenase, NAD binding domain	Malate dehydrogenase, cytoplasmic
JGVV51.1	10.45	7.48	6.31	carbohydrate metabolic process	Glycosyl hydrolase family 14	Beta-amylase
JGVV5.318	5.48	3.22	2.94	carbohydrate metabolic process	lactate/malate dehydrogenase, NAD binding domain	Malate dehydrogenase, cytoplasmic
JGVV25.147	4.25	4.20	3.51	carbohydrate metabolic process	NAD dependent epimerase/dehydratase family	UDP-glucose 4-epimerase GEPI48
JGVV2.157	4.18	3.32	3.65	carbohydrate metabolic process	2-oxoacid dehydrogenases acyltransferase (catalytic domain)	Dihydrolipoyllysine-residue acetyltransferase component 3 of pyruvate dehydrogenase complex, mitochondrial
JGVV39.37	2.83	2.09	2.10	carbohydrate metabolic process	-	Maltose excess protein 1-like, chloroplastic
JGVV32.68	2.76	2.79	2.51	carbohydrate metabolic process	Sucrose-6F-phosphate phosphohydrolase	Sucrose-phosphatase 1
JGVV19.365	2.33	2.35	2.42	carbohydrate metabolic process	Triosephosphate isomerase	Triosephosphate isomerase, cytosolic
JGVV5.73	2.24	2.23	2.36	carbohydrate metabolic process	Glycosyl transferases group 1	Sucrose synthase 2
JGVV38.81	2.44	2.06	2.01	transport	Sugar (and other) transporter	Monosaccharide-sensing protein 2

A. Common differentially expressed gene belonging to carbohydrate metabolic process functional category in CT, ED and LD at end of véraison.

ED-LD	**ED**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV13.30	23.35	17.72	carbohydrate metabolic process	-	Ribulose bisphosphate carboxylase large chain
JGVV19.89	7.40	7.72	carbohydrate metabolic process	Phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain I	Phosphomannomutase/phosphoglucomutase
JGVV63.39	7.16	10.26	carbohydrate metabolic process	0	Alpha-amylase
JGVV127.59	3.32	3.02	carbohydrate metabolic process	ATP-grasp domain	ATP-citrate synthase
JGVV16.249	2.38	2.17	carbohydrate	Phosphofructokinase	6-phosphofructokinase 3

	FOLD CHANGE				
			metabolic process		
JGVV16.27	2.07	2.08	carbohydrate metabolic process	Glycosyl transferases group 1	Sucrose synthase

B. Common differentially expressed genes belonging to carbohydrate metabolic process functional category in ED and LD at end of véraison.

	CT	LD	GO_plant_slim	pfam_description	12X annotation
CT-LD					
JGVV25.197	-2.1	-2.02	carbohydrate metabolic process	Glycosyl hydrolase family 14	Beta-amylase

C. Common differentially expressed genes belonging to carbohydrate metabolic process functional category in CT and LD at end of véraison.

CT	CT	GO_plant_slim	pfam_description	12X annotation
JGVV22.151	60.34	-	Granule-bound starch synthase 1, chloroplastic/amyloplastic	Granule-bound starch synthase 1, chloroplastic/amyloplastic
JGVV1562.1	19.45	carbohydrate metabolic process	Sucrose synthase	Sucrose synthase 2
JGVV171.41	10.41	carbohydrate metabolic process	Glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3
JGVV59.203	6.10	carbohydrate metabolic process	Aconitase family (aconitate hydratase)	Aconitate hydratase, cytoplasmic
JGVV25.145	4.65	carbohydrate metabolic process	NAD dependent epimerase/dehydratase family	UDP-glucose 4-epimerase GEPI48
JGVV127.79	4.20	carbohydrate metabolic process	Pyruvate phosphate dikinase, PEP/pyruvate binding domain	Alpha-glucan water dikinase, chloroplastic
JGVV31.137	-10.28	carbohydrate metabolic process	4-alpha-glucanotransferase	4-alpha-glucanotransferase, chloroplastic/amyloplastic
JGVV875.1	-9.43	carbohydrate metabolic process	Phosphoglycerate mutase family	-
JGVV30.39	-7.58	carbohydrate metabolic process	Ribose 5-phosphate isomerase A (phosphoriboisomerase A)	Probable ribose-5-phosphate isomerase
JGVV29.35	-6.42	carbohydrate	Glycosyl transferases group 1	Sucrose-phosphate synthase 2

	FOLD CHANGE			
		metabolic process		
JGVV4.148	-6.27	carbohydrate metabolic process	Phosphoglycerate mutase family	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
JGVV11.607	-5.38	carbohydrate metabolic process	-	Probable 6-phosphogluconolactonase 1
JGVV78.31	-5.22	carbohydrate metabolic process	-	Galactokinase
JGVV38.349	-4.21	carbohydrate metabolic process	pfkB family carbohydrate kinase	Uncharacterized sugar kinase yeiC
JGVV32.39	-4.08	carbohydrate metabolic process	Glycosyl hydrolase family 3 N terminal domain	Lysosomal beta glucosidase
JGVV87.54	-4.03	carbohydrate metabolic process	Glycosyl hydrolase family 14	Beta-amylase
JGVV38.367	-4.02	carbohydrate metabolic process	Fructose-bisphosphate aldolase class-I	Probable fructose-bisphosphate aldolase 1, chloroplastic
JGVV7.152	-3.97	carbohydrate metabolic process	-	Probable galactose-1-phosphate uridyl transferase
JGVV7.654	-3.79	carbohydrate metabolic process	Starch binding domain	Phosphoglucan, water dikinase, chloroplastic
JGVV137.20	-3.77	carbohydrate metabolic process	-	Alpha-galactosidase
JGVV780.1	-3.33	carbohydrate metabolic process	Phosphofructokinase	6-phosphofructokinase 2
JGVV31.225	-3.15	carbohydrate metabolic process	Glycosyl hydrolases family 35	Beta-galactosidase 3
JGVV7.703	-3.14	carbohydrate metabolic process	Fructose-1-6-bisphosphatase	Fructose-1,6-bisphosphatase, chloroplastic
JGVV2.205	-3.07	carbohydrate metabolic process	Glycosyl hydrolases family 35	Beta-galactosidase 17
JGVV109.24	-2.99	carbohydrate metabolic process	Phosphoribulokinase / Uridine kinase family	Phosphoribulokinase, chloroplastic
JGVV3.153	-2.88	carbohydrate metabolic process	Glycosyltransferase family 20	Probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 7
JGVV88.73	-2.82	carbohydrate metabolic process	Pyruvate kinase, barrel domain	Pyruvate kinase isozyme A, chloroplastic

	FOLD CHANGE			
JGVV259.12	-2.79	carbohydrate metabolic process	5'-AMP-activated protein kinase, beta subunit, complex-interacting region	SNF1-related protein kinase regulatory subunit beta-2
JGVV3.262	-2.77	carbohydrate metabolic process	Starch synthase catalytic domain	-
JGVV57.83	-2.74	carbohydrate metabolic process	Glucose-6-phosphate dehydrogenase, NAD binding domain	Glucose-6-phosphate 1-dehydrogenase, chloroplastic
JGVV26.99	-2.74	carbohydrate metabolic process	Alpha amylase, catalytic domain	Alpha-amylase
JGVV23.90	-2.66	carbohydrate metabolic process	Fructose-bisphosphate aldolase class-I	Probable fructose-bisphosphate aldolase 1, chloroplastic
JGVV0.735	-2.60	carbohydrate metabolic process	Fructose-bisphosphate aldolase class-II	Uncharacterized oxidoreductase ygbJ
JGVV25.381	-2.57	carbohydrate metabolic process	NAD dependent epimerase/dehydratase family	UDP-glucose 4-epimerase
JGVV7.109	-2.51	carbohydrate metabolic process	Glycosyl hydrolases family 28	Exo-poly-alpha-D-galacturonosidase
JGVV5.506	-2.51	carbohydrate metabolic process	Glycosyltransferase family 28 N-terminal domain	Sterol 3-beta-glucosyltransferase
JGVV46.23	-2.45	carbohydrate metabolic process	Eukaryotic phosphomannomutase	Phosphomannomutase
JGVV25.256	-2.45	carbohydrate metabolic process	Glycosyl hydrolases family 17	Putative glucan endo-1,3-beta-glucosidase 11
JGVV52.42	-2.43	carbohydrate metabolic process	FGGY family of carbohydrate kinases, N-terminal domain	FGGY carbohydrate kinase domain-containing protein
JGVV22.158	-2.41	carbohydrate metabolic process	Glycosyl hydrolases family 32 N terminal	Beta-fructofuranosidase, soluble isoenzyme I
JGVV60.191	-2.36	carbohydrate metabolic process	Glucose-6-phosphate dehydrogenase, NAD binding domain	Glucose-6-phosphate 1-dehydrogenase 4, chloroplastic
JGVV108.80	-2.27	carbohydrate metabolic process	lactate/malate dehydrogenase, NAD binding domain	Malate dehydrogenase, chloroplastic
JGVV20.140	-2.26	carbohydrate metabolic process	galactosyl transferase GMA12/MNN10 family	Putative glycosyltransferase 2

	FOLD CHANGE			
JGVV17.53	-2.21	carbohydrate metabolic process	NAD dependent epimerase/dehydratase family	UDP-arabinose 4-epimerase 1
JGVV78.22	-2.18	carbohydrate metabolic process	Alpha amylase, catalytic domain	Glycogen operon protein glgX homolog
JGVV0.365	-2.17	carbohydrate metabolic process	lactate/malate dehydrogenase, NAD binding domain	Malate dehydrogenase, chloroplastic
JGVV63.232	-2.16	carbohydrate metabolic process	Glycosyl hydrolases family 17	Putative glucan endo-1,3-beta-glucosidase 7
JGVV16.273	-2.08	carbohydrate metabolic process	NAD binding domain of 6-phosphogluconate dehydrogenase	Uncharacterized oxidoreductase ykwC
JGVV12.247	-2.00	carbohydrate metabolic process	Glycosyl hydrolase family 14	Beta-amylase
JGVV63.210	-5.91	transport	Sugar (and other) transporter	Sugar-proton symporter PLT5
JGVV1.502	-5.66	transport	-	Sucrose transport protein SUC4
JGVV16.303	-2.97	transport	Sugar (and other) transporter	Sugar transport protein 14
JGVV16.303	-2.97	transport	Sugar (and other) transporter	Sugar transport protein 14
JGVV207.7	-2.81	transport	Nucleotide-sugar transporter	Probable UDP-sugar transporter protein SLC35A5
JGVV26.73	-2.17	transport	-	Sucrose transport protein SUC3

D. Differentially expressed genes belonging to carbohydrate metabolic process functional category specific for CT at end of véraison.

ED	ED	GO_plant_slim	pfam_description	12X annotation
JGVV74.13	5.21	carbohydrate metabolic process	2-oxoacid dehydrogenases acyltransferase (catalytic domain)	Dihydrolipoyllysine-residue acetyltransferase component 1 of pyruvate dehydrogenase complex, mitochondrial
JGVV10.78	2.04	carbohydrate metabolic process	6-phosphogluconate dehydrogenase, C-terminal domain	6-phosphogluconate dehydrogenase, decarboxylating
JGVV0.5	2.41	carbohydrate metabolic process	Glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
JGVV61.144	2.30	carbohydrate metabolic process	Hexokinase	Hexokinase-3
JGVV25.201	3.27	carbohydrate	Trehalase	Trehalase

	FOLD CHANGE				
		metabolic process			
JGVV54.23	2.50	transport	Sugar (and other) transporter	-	

E. Differentially expressed genes belonging to carbohydrate metabolic process functional category specific for ED at end of véraison.

LD	LD	GO_plant_slim	pfam_description	12X annotation
JGVV2.677	4.50	carbohydrate metabolic process	FGGY family of carbohydrate kinases, C-terminal domain	Probable glycerol kinase
JGVV108.144	2.1	carbohydrate metabolic process	Carbohydrate phosphorylase	Alpha-1,4 glucan phosphorylase L-1 isozyme, chloroplastic/amyloplastic

F. Differentially expressed genes belonging to carbohydrate metabolic process functional category specific for LD at end of véraison.

Table 10. Differential expressed genes belonging to "carbohydrate metabolic process" functional category as reported in www.geneontology.com. For each gene , identified by the specific SEQ_ID, fold change (FC) value, pfam description (http://pfam.sanger.ac.uk/) and 12X annotation on the basis of protein sequence homology (www.uniprot.org) are reported.

3.4.8 Genes involved in "response to stimulus" functional category

In Table 11 are listed all the genes which matched with the "response to stimulus" functional category. From the list have been deliberately excluded genes involved in response to biotic stimulus, which in the future should be object of more in deep researches, even on the basis of the effect that in particular ED treatments could have on cluster compactness and so on pathogens infections.

At this level, it will be considered only the effects that changes in source/sink modifications or in cluster exposure and temperature increase could have in terms of abiotic stress response.

Two main classes of genes seems to be the most affected by the treatments: gene involved in oxidative stress and genes involved in response to high temperature.

3.4.8.1 Genes involved in oxidative stress response

The occurrence of oxidative stress during ripening was demonstrated also for grape berry in a recent study (Pilati *et al.*, 2007), where it was shown an increase in H_2O_2 content from the véraison stage (reaching its maximum one-two weeks after) and then a decrease until harvest. Several specific enzymes take part in cellular detoxification by H_2O_2 and their modulation could drive the vine response to oxidative stress. Ascorbate and glutathione are low-molecular-weight antioxidant, which are the most important reducing substrates for H_2O_2 detoxification.

An isoform of a citosolic L-ascorbate peroxidase (JGVv14.213) have been identified to be up-regulated in all the treatments (Table 11A). This enzyme is an integral component of the glutathione-ascorbate cycle and it is activated by the plant with the aim to detoxify peroxides using ascorbate as a substrate. Glutathione is then used to regenerate ascorbate from dehydroascorbate (the oxidized form of ascorbate). Glutathione-S-transferases (GST) are not directly involved in the glutathione-ascorbate cycle. Their primary function is to catalyze the conjugation of the glutathione to a variety of hydrophobic, electrophilic and usually cytotoxic substrates. In addition to the catalytic function, GSTs serve as non enzymatic carrier proteins (ligandins) involved in the intracellular transport of different compounds among which flavonoid (as previously described in Introduction paragraph). GSTs are an ancient and diverse protein family, existing as multigene families in bacteria, fungi, animals and plants (Edwards *et al.*, 2000). Phi (ϕ) and Tau (τ) GSTs classes are plant specific and are both involved in detoxification mechanisms and ligandin activity.

Ten GSTs belonging to τ class are up-regulated in all the treatments. Among these, 3 genes (JGVv93.7, JGVv27.95 and JGVv15.214) show a strong up-regulation in CT berries, suggesting their further activation following thinning treatment. Two ϕ type GSTs are also up-regulated in all the treatments. In other transcriptional studies (Deluc *et al.*, 2007; Pilati *et al.*, 2007) neither Catalase nor Super Oxide Dismutase (SOD) transcripts, which codify for two important detoxifying enzymes, were found to be modulated during ripening. In our study, two isoforms of Super Oxide Dismutase genes (SOD, JGVv30.111 and JGVv30.115), which is known to be involved in oxidative stress response, were found to be up-regulated in all the treatments. SOD genes are more induced in

defoliation treatments, suggesting their more direct involvement in light and/or temperature increase response.

The pair-wise comparison of up-regulated genes, showed that no genes involved in oxidative stress response were shared between CT and ED and only one GTS gene (JGVv24.22) was up-regulated in both CT and LD berries (Table 11D). A different τ-type GST isoform (JGVv24.22) was instead up-regulated specifically in ED and LD treatments and it could be hypothesized its direct involvement in response to cluster microclimate changes (Table 11C).

Concerning one treatment-specific modulation of transcripts, no genes involved in oxidative stress response were found to be differentially expressed specifically in LD berries and only a specific GST (JGVv26.125) was weakly up-regulated in ED berries (Table 11F).

Several genes involved in response to oxidative stress were instead specifically differentially expressed in CT berries (Table 11G). It is very interesting to note among these, many genes have been shown to be down-regulated. Five transcripts coding for five different peroxidases, one probable monodehydroascorbate reductase, 9 GSTs (among which a DHAR, dehydro-ascorbate reduttase, type) and 2 thioredoxines showed an expression decrease compared to C. In strawberry it has been demonstrated that peroxidase activity varies during ripening, with the highest levels at stage of green fruits and a low, yet clearly detectable, specific enzymatic activity in the remaining ripening stages (Civello *et al.*, 1995). The observed trend in H_2O_2 could suggest a similar trend for grapevine, with a peak of oxidative burst after véraison and then a H_2O_2 concentration decrease. The down-regulation of the previously described genes taking part in the oxidative stress response, could be a consequence of the advance in berry ripening observed in CT berries. On the basis of this considerations, 7 GSTs genes specifically strongly up-regulated in CT berries could be considered probably more involved in ligandin process rather than in stress response. Among these GSTs transcripts it is interesting to note that one (JGVv240.3) is 160 fold more abundant compared to C, that means a strong induction following thinning treatment. Another sequence (JGVv15.206, FC=31) has been identified as VvGST5, which encoding a GST protein identified in pigmented *Vitis Vinifera* cell suspensions. VvGST5 did not show any increase in its activity under those conditions enhancing anthocyanin biosynthesis in vitro. Furthermore, it was found only weakly expressed in post-véraison berry skin of cv Shiraz (Conn *et al.*, 2008). This suggests that differences in transcriptional profiles of GSTs genes are greatly related to interspecies variation or response to different stimuli. Probably, sugars content is the main factor inducing the up-regulation of GST genes specifically expressed in CT berries.

3.4.8.2 Genes involved in response to high temperature

Plant response to oxidative stress is strictly related to high temperature stress. Many genes involved in both responses are in fact induced simultaneously in stress conditions. The accumulation of heat shock proteins (HSPs), under the control of heat stress transcription factors (HSFs), play a central role in the heat stress response (Kotak *et al.*, 2007), but also in the responses

to other several abiotic stresses. HSPs protein are molecular chaperones and are classified into five major families: the Hsp70 (DnaK) family; the chaperonins (GroEL and Hsp60); the Hsp90 family; the Hsp100 (Clp) family; and the small Hsp (sHsp) family. Chaperones are responsible for protein folding, assembly, translocation and degradation in normal cellular processes; they also function in the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions. It has to be noticed that some members of Hsp70 are constitutively expressed during development and so their activity can be not correlated to abiotic stress conditions. These enzymes are often referred to 70-kDa Heat shock cognate (Wang *et al.*, 2004).

Many genes coding for HSPs are in fact up regulated in all treatments (Table 11A). It is interesting to note that more abundant HSPs CT transcripts (e.g. PDVv5.557or JGVv198.8) usually coding for Heat shock cognate proteins. Small HSPs are involved in the first phases of stress response with the aim to bind non-native proteins, facilitating their subsequent refolding by the other classes of HSPs. Several small HSPs, common to the three treatments, are instead more induced in defoliation treatments (e.g. JGVv8.144 or JGVv19.236).

Pair-wise comparison has shown that several small HSPs are also specifically induced in defoliation treatments and are so present only in ED and LD berries (Table 11C).

Modulation of some HSP genes occurred also specifically in CT berries (Table 11E), where three genes coding for 70-kDa Heat shock cognate are up-regulated and two small HSPs were instead down-regulated.

Concerning to defoliation treatments, it is interesting to underline that, while ED berries showed the up-regulation of only one HSP70 gene and Heat shock cognate 70 kDa gene (Table 11F), LD berries were instead characterized by the up-regulation of 4 different small HSPs. This probably suggests the reaction to a close imposed heat stress for LD berries (Table 11G). ED berries seems instead at end of véraison to be more adapted to heat stress condition.

Trend in temperature, observed towards thermocouples measurements in 2008 season, suggests that during all development for ED berries and from véraison for LD one, higher temperature characterized clusters of defoliated vines compared to C and also to CT, as it is possible to hypothesize. At end of 2008 véraison, berry skin temperature of C and defoliated berries exceeded 30°C for the most part of the day and so probably heat stress in field conditions affected all the treatments and also C at this stage of berry ripening.

SEQ_ID	FOLD CHANGE					
CT-ED-LD	**CT**	**ED**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV93.7	141.97	24.44	26.11	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
PDVV5.557	68.74	13.52	11.46	response to stimulus	-	Heat shock cognate 70 kDa protein 2
JGVV27.95	40.37	6.24	8.05	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV93.21	33.63	22.04	25.90	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV77.10	21.60	13.18	13.08	response to stimulus	-	-
JGVV15.214	20.51	6.51	5.48	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV14.213	17.71	8.77	10.04	response to stimulus	-	L-ascorbate peroxidase 2, citosolic
JGVV30.111	13.74	16.07	17.12	metabolic process	Copper/zinc superoxide dismutase (SODC)	Superoxide dismutase [Cu-Zn] 2
JGVV30.115	13.30	18.84	20.28	metabolic process	Copper/zinc superoxide dismutase (SODC)	Superoxide dismutase [Cu-Zn] 2
JGVV40.35	10.65	11.06	12.34	response to stimulus	-	Glutathione S-transferase (ϕ type)
JGVV15.212	10.17	6.06	5.41	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV93.10	9.75	6.95	6.83	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV15.220	9.68	4.85	5.69	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV104.3	9.29	12.66	9.92	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (ϕ type)
JGVV8.144	9.27	12.10	13.05	response to stimulus	Hsp20/alpha crystallin family	17.9 kDa class II heat shock protein
JGVV198.8	9.25	5.75	7.96	response to stimulus	-	Heat shock 70 kDa protein, mitochondrial
JGVV19.240	6.96	8.77	10.40	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein
JGVV254.2	6.37	5.12	5.18	response to stimulus	-	Heat shock cognate 70 kDa protein 2
PDVV75.19	6.22	6.47	7.86	response to stimulus	-	Heat shock 70 kDa protein, mitochondrial
JGVV93.14	5.87	3.53	4.06	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV15.219	5.85	5.57	5.19	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV19.236	5.52	8.04	8.76	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein
JGVV98.37	5.44	2.89	2.80	response to stimulus	Hsp70 protein	Luminal-binding protein 5
JGVV43.48	4.25	2.77	2.59	response to stimulus	Hsp70 protein	Hypoxia up-regulated protein 1
JGVV15.205	4.23	3.56	3.65	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV1569.1	3.40	4.39	4.36	response to stimulus	-	Heat shock cognate protein 80
JGVV8.147	3.01	2.34	2.34	response to stimulus	Hsp20/alpha crystallin family	17.9 kDa class II heat shock protein
JGVV8.145	2.72	2.50	2.60	response to stimulus	Hsp20/alpha crystallin family	17.3 kDa class II heat shock protein
JGVV207.22	-3.82	-2.37	-2.62	response to stimulus	NPH3 family	BTB/POZ domain-containing protein At1g30440
JGVV7.157	-8.20	-3.38	-2.49	response to stimulus	DnaJ domain	Chaperone protein dnaJ

A. Common differentially expressed genes belonging to response to stimulus functional category in CT, ED and LD at end of véraison.

SEQ_ID	FOLD CHANGE				
CT-ED	CT	ED	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV172.4	-4.87	-2.04	response to stimulus	-	-
JGVV60.113	-2.83	-2.46	response to stimulus	DnaJ domain	DnaJ homolog subfamily B member 6
JGVV11.327	-2.57	-2.08	response to stimulus	Eukaryotic protein of unknown function, DUF292	-

B. Common differentially expressed genes belonging to response to stress functional category in CT and ED at end of véraison.

ED-LD	**ED**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV8.140	8.06	7.41	response to stimulus	Hsp20/alpha crystallin family	17.2 kDa class II heat shock protein
JGVV19.241	7.23	8.43	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein
JGVV19.205	6.07	6.37	response to stimulus	Hsp20/alpha crystallin family	17.6 kDa class I heat shock protein
JGVV29.78	5.22	4.94	response to stimulus	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	Glycine-rich RNA-binding protein 2, mitochondrial
JGVV119.3	5.02	5.38	response to stimulus	Protein of unknown function (DUF760)	-
JGVV19.243	4.18	4.73	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein
JGVV24.22	3.60	3.56	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV4.256	3.70	3.50	response to stimulus	Hsp20/alpha crystallin family	17.6 kDa class I heat shock protein
JGVV8.141	2.75	2.89	response to stimulus	Hsp20/alpha crystallin family	17.9 kDa class II heat shock protein
JGVV1.1087	2.70	2.57	response to stimulus	Hsp90 protein	Endoplasmin homolog
JGVV8.138	2.01	2.32	response to stimulus	Hsp20/alpha crystallin family	17.9kDa class II heat shock protein

C. Common differentially expressed gene belonging to response to stress functional category in LD and ED at end of véraison.

CT-LD	**CT**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV24.22	3.60	3.66	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)

D. Common differentially expressed genes belonging to response to stress functional category in CT and LD at end of véraison.

CT	**CT**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV240.3	161.52	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV93.15	48.78	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV19.375	41.87	response to stimulus	Hsp70 protein	Heat shock cognate 70 kDa protein
JGVV93.19	34.13	response to stimulus	Glutathione S-transferase, C-terminal domain	Probable glutathione S-transferase parA
JGVV15.206	30.95	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (**VvGST5**)
JGVV93.11	8.63	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV11.290	5.88	response to stimulus	Universal stress protein family	Universal stress protein A-like protein

SEQ_ID	FOLD CHANGE			
JGVV35.19	5.73	response to stimulus	Glutathione S-transferase, N-terminal domain	Glutathione S-transferase (ζ type)
JGVV4.263	4.64	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV5.132	2.50	response to stimulus	Universal stress protein family	Universal stress protein A-like protein
JGVV4.367	2.25	response to stimulus	Hsp70 protein	Heat shock cognate 70 kDa protein
JGVV19.350	2.06	response to stimulus	Hsp70 protein	Heat shock cognate 70 kDa protein 2
JGVV28.247	-11.14	response to stimulus	-	Small heat shock protein C2
JGVV1.424	-6.92	response to stimulus	Universal stress protein family	Universal stress protein A-like protein
JGVV46.142	-6.77	response to stimulus	Late embryogenesis abundant protein	Late embryogenesis abundant protein Lea14-A
JGVV191.5	-6.54	response to stimulus	Peroxidase	Peroxidase 17
JGVV16.476	-5.55	response to stimulus	Peroxidase	Peroxidase 25
JGVV55.61	-5.21	response to stimulus	Peroxidase	Peroxidase 43
JGVV5.17	-4.84	response to stimulus	Rubber elongation factor protein (REF)	Stress-related protein
JGVV510.1	-4.63	response to stimulus	-	-
JGVV14.71	-4.56	response to stimulus	NB-ARC domain	BES1/BZR1 homolog protein 4
JGVV134.35	-4.39	response to stimulus	Universal stress protein family	-
JGVV4.518	-4.28	response to stimulus	Thioredoxin	Protein disulfide-isomerase
JGVV20.145	-4.05	response to stimulus	CBS domain pair	5'-AMP-activated protein kinase subunit gamma-2
JGVV38.234	-3.64	response to stimulus	Peroxidase	L-ascorbate peroxidase 3, peroxisomal
JGVV20.432	-3.62	response to stimulus	-	-
JGVV66.99	-3.61	response to stimulus	Pyridine nucleotide-disulphide oxidoreductase	Probable monodehydroascorbate reductase, cytoplasmic isoform 2
JGVV8.631	-3.52	response to stimulus	Glutathione peroxidase	Phospholipid hydroperoxide glutathione peroxidase 1, chloroplastic
JGVV72.105	-3.52	response to stimulus	-	-
JGVV7.27	-3.49	response to stimulus	Pyridine nucleotide-disulphide oxidoreductase	Thioredoxin reductase
JGVV77.56	-3.26	response to stimulus	-	Calmodulin-4
JGVV65.27	-3.21	response to stimulus	Proteasome A-type and B-type	Proteasome subunit beta type-6
JGVV50.119	-3.20	response to stimulus	DnaJ domain	Translocation protein SEC63 homolog
JGVV19.319	-3.15	response to stimulus	Zinc finger, C3HC4 type (RING finger)	E3 ubiquitin-protein ligase RNF12
JGVV324.4	-3.06	response to stimulus	DnaJ domain	Chaperone protein dnaJ
JGVV11.596	-2.96	response to stimulus	Protein of unknown function (DUF1675)	UPF0737 protein AFP2
JGVV8.616	-2.80	response to stimulus	NPH3 family	Coleoptile phototropism protein 1
JGVV0.815	-2.80	response to stimulus	-	Uncharacterized protein At3g48680, mitochondrial
JGVV3.397	-2.76	response to stimulus	NPH3 family	BTB/POZ domain-containing protein At1g30440

SEQ_ID	FOLD CHANGE			
JGVV98.19	-2.76	response to stimulus	TAP42-like family	PP2A regulatory subunit TAP46
JGVV4.260	-2.70	response to stimulus	Universal stress protein family	Universal stress protein A-like protein
JGVV21.114	-2.69	response to stimulus	GRAM domain	GRAM domain-containing protein 1A
JGVV66.102	-2.67	response to stimulus	Universal stress protein family	Universal stress protein A-like protein
JGVV108.139	-2.67	response to stimulus	ATPase family associated with various cellular activities (AAA)	Chaperone protein clpB 2
JGVV50.16	-2.65	response to stimulus	ATPase family associated with various cellular activities (AAA)	Cell division protease ftsH homolog 11, chloroplastic/mitochondrial
JGVV3.245	-2.62	response to stimulus	Cold acclimation protein WCOR413	-
JGVV19.380	-2.34	response to stimulus	Hsp20/alpha crystallin family	18.1 kDa class I heat shock protein (Fragment)
JGVV7.236	-2.18	response to stimulus	DnaJ domain	Chaperone protein dnaJ
JGVV317.5	-2.19	response to stimulus	Glutathione S-transferase, N-terminal domain	Glutathione S-transferase (DHAR)
JGVV1.46	-4.27	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (ϕ type)
JGVV48.194	-3.81	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (ζ type)
JGVV0.664	-5.00	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)
JGVV49.99	-3.62	response to stimulus	Glutathione S-transferase, N-terminal domain	Glutathione S-transferase (τ type)
JGVV49.94	-2.87	response to stimulus	Glutathione S-transferase, N-terminal domain	Glutathione S-transferase (τ type)
JGVV49.96	-2.35	response to stimulus	Glutathione S-transferase, N-terminal domain	Glutathione S-transferase (τ type)
JGVV51.16	-3.22	response to stimulus	Glutathione S-transferase, N-terminal domain	Glutathione S-transferase (τ type)
JGVV5.1	-2.00	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (ϕ type)

E. Differentially expressed genes belonging to response to stimulus functional category specific to CT at end of véraison.

ED	ED	GO_plant_slim	pfam_description	12X annotation
JGVV27.97	4.16	response to stimulus	DnaJ domain	DnaJ homolog subfamily C member 14
JGVV7.837	2.45	response to stimulus	Hsp70 protein	Heat shock cognate 70 kDa protein 2
JGVV26.125	2.06	response to stimulus	Glutathione S-transferase, C-terminal domain	Glutathione S-transferase (τ type)

F. Differentially expressed genes belonging to response to stimulus functional category specific to ED at end of véraison.

LD	LD	GO_plant_slim	pfam_description	12X annotation
JGVV91.44	3.30	response to stimulus	Interferon-related developmental regulator (IFRD)	Interferon-related developmental regulator 1
JGVV19.239	4.14	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein
JGVV19.234	3.40	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein
JGVV8.148	2.61	response to stimulus	Hsp20/alpha crystallin family	17.9 kDa class II heat shock protein

SEQ_ID	FOLD CHANGE			
JGVV19.233	2.28	response to stimulus	Hsp20/alpha crystallin family	18.2 kDa class I heat shock protein

G. Differentially expressed genes belonging to response to stimulus functional category specific to LD at end of véraison.

Table 11. Differential expressed genes belonging to "response to stimulus" functional category as reported in www.geneontology.com. For each gene , identified by the specific SEQ_ID, fold change (FC) value, pfam description (http://pfam.sanger.ac.uk/) and 12X annotation on the basis of protein sequence homology (www.uniprot.org) are reported.

3.4.9 Differentially expressed genes involved in flavonoid biosynthesis

3.4.9.1. Structural genes involved in flavonoid bioynthesis

Many genes involved in flavonoid biosynthesis are strongly affected by thinning and defoliation treatments. In Table 12 are listed differentially expressed genes involved in flavonoid accumulation, divided into common to the three treatments, detected after pair-wise comparison and single treatment specific genes. According to the localization of accumulation of flavonoid pigments, the majority of the genes encoding flavonoid biosynthetic enzymes were specifically expressed in the skin or seed (Grimplet *et al.*, 2007). Flavonoid compounds are present in flesh only as traces and so differential expression of flavonoid genes could be specifically linked to flavonoid synthesis in berry skin (Boss *et al.*, 1996a).

Among genes up-regulated in all treatments compared to C (Table 12A), DFR (JGVv15.1) and LDOX (JGVv64.134), which operate in the last step of anthocyanin pathway (Table 12A), were found. In CT berries these genes presented a FC of 19.8 and 11.2 respectively, while in ED berries, the increase in DFR transcription was represented by a 7.2 FC but LDOX was only few affected (FC=2.9). LD showed similar results in terms of DFR and LDOX gene expression to ED. Flavonoids transporter belonging to ABC and MatE families were also up-regulated in all treatments (e.g. JGVv15.4, JGVv3.13, JGVv5.437). Several common up-regulated transcripts were identified as Flavonoid glucosyltransferase (e.g. JGVv55.22, JGVv2.220), but any matched exactly with the known grapevine UFGT sequence.

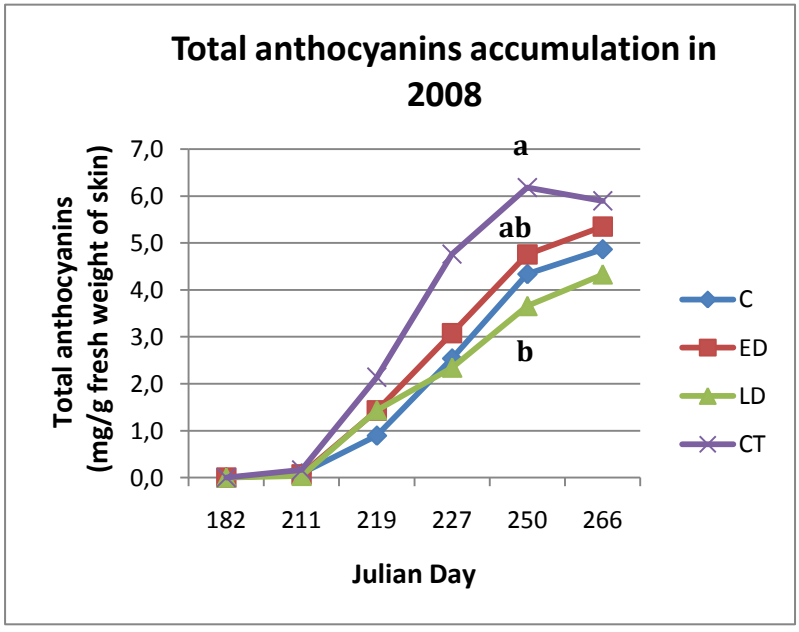


Fig. 25. Accumulation of anthocyanins in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest.

Among specific to one treatment modulated genes, only in ED berries (Table 12E), one up-regulated sequence corresponded to UFGT (JGVv52.20, FC=7.7).

Modulation of other genes involved in common step of flavonoids biosynthesis was found specifically in CT berries (Table 12D). An additional DFR isoform and several flavonoid glucosyltransferase genes were up-regulated in CT berries. Moreover, CT berries were mainly characterized at end of véraison by the up-regulation of several genes involved in flavonoids transport. Genes belonging to the early step of flavonoids biosynthesis pathway were instead down-regulated specifically in CT. Among these, 2 F3H isoforms (JGVv25.273 and JGVv1.1071) and CHS2 (JGVv67.6) were identified. Down-

regulation of specific flavonoid glucosyltransferase genes and flavonoids transporter were also found. In Fig. 25 is represented the trend in 2008 anthocyanin accumulation expressed as mg per g of fresh weight skin. At end of véraison (JD227), CT berries showed an anthocyanin content approximately two-fold greater compared to C. This result matches with the previously observed increase in the expression of the structural (DFR, LDOX) and transport genes involved in anthocyanins accumulation. This effect should be linked to the increase in source-sink ratio and so to the higher sugars berry availability (Fig. 24, Table 4, Gollop et al., 2001; Gollop et al., 2002).

ED and LD total anthocyanin amount were similar to C at end of véraison despite the differences observed in anthocyanin genes transcriptional profile at the same stage. In fact, DFR and LDOX expression levels were lower and additional transporters, characterizing CT berries, lacked in ED and LD berries.

FLS is the main genes in determining flavonols accumulation. ED and LD berries were characterized by up-regulation of FLS (JGVv1.213) gene at end of véraison (Table 12C). The same FLS gene was down-regulated in CT berries compared to C (Table 12D). ED and LD berries showed the highest flavonols concentration from defoliation treatments until harvest. Light induction of FLS genes, as previously reported (Downey *et al.*, 2004), after defoliation treatments could be considered a determining factor in increase flavonols concentration (Fig. 26). CT result is instead difficult to explain because C and CT showed no differences in flavonols concentration in any stage of berry ripening (Fig. 26).

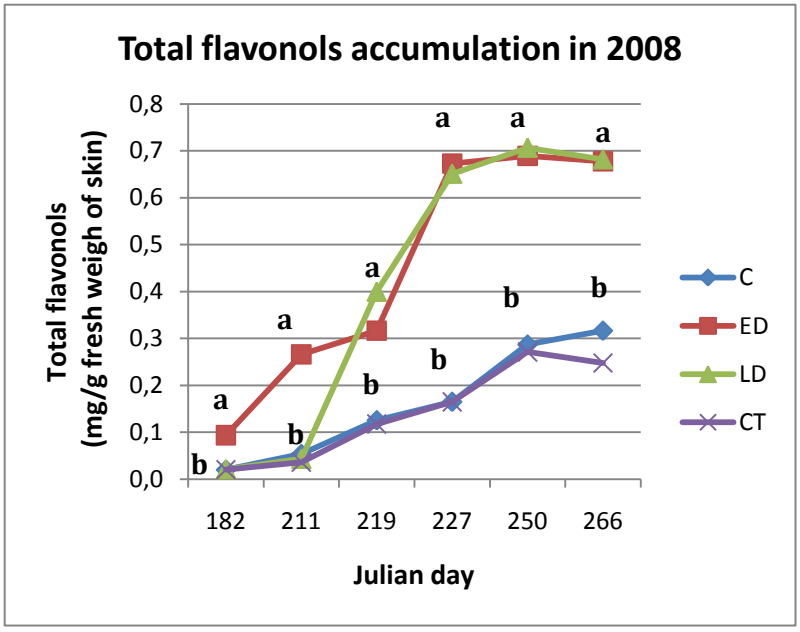


Fig. 26. Accumulation of flavonols in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest.

Proanthocyanidins analyses were not performed, but from transcriptional analyses emerged that LAR1 and LAR2, which catalyze the synthesis of catechin are down-regulated respectively in CT (Table 12D)and in CT and ED (Table 12B) berries at end of véraison.

3.4.9.2. Genes involved in flavonoids composition

According to the biosynthesis of flavonoids (Fig.6 in Introduction paragraph) we discussed separately the flavonoids composition derived from F3'H genes and the one from F3'5'H.

Two isoforms of F3'H genes were found to be up-regulated in all treatments (JGVv55.6 and JGVv52.29, Table 12A), one specifically up-regulated in LD and ED berries (Table 12C, JGVv0.280)

and an additional isoform over-expressed specifically in ED berries (Table 12E, JGVv16.82). A further isoform is found to be down-regulated only in CT berries (Table 12D, JGVv2.90). Until now, several papers reported the identification of one or two copies of F3'H genes (Bogs *et al.* 2006; Castellarin *et al.*, 2006) in grape berry skin. It is reasonable to hypothesize that additional isoforms could be modulated after thinning or defoliation treatments.

In Fig. 27 and 28 are represented the trends in 3'-substituted anthocyanin (cyanidin and peonidin3-O-glucoside) and of the flavonol quercetin accumulation respectively, which both derive from F3'H action. At end of véraison (JD227), CT berries were characterized by the highest levels of 3'-substituted anthocyanins, while ED and LD berries from the highest quercetin concentration. It is possible to affirm that both source/sink ratio modification and cluster light and temperature increase, act on the stimulation of genes belonging to this branch of flavonoid pathway. In CT berries, where no increase in cluster light exposure occurred, the up-regulation of F3'H genes caused at end of véraison an increase in 3'-substitued anthocyanins. In defoliation treatments (ED and LD), a competition mechanism between products of F3'H genes, could occur when flavonols and anthocyanins biosynthesis are simultaneously activated, which corresponded to véraison time. F3'H genes are activated very early in berry ripening (Castellarin *et al.*, 2006; Bogs *et*

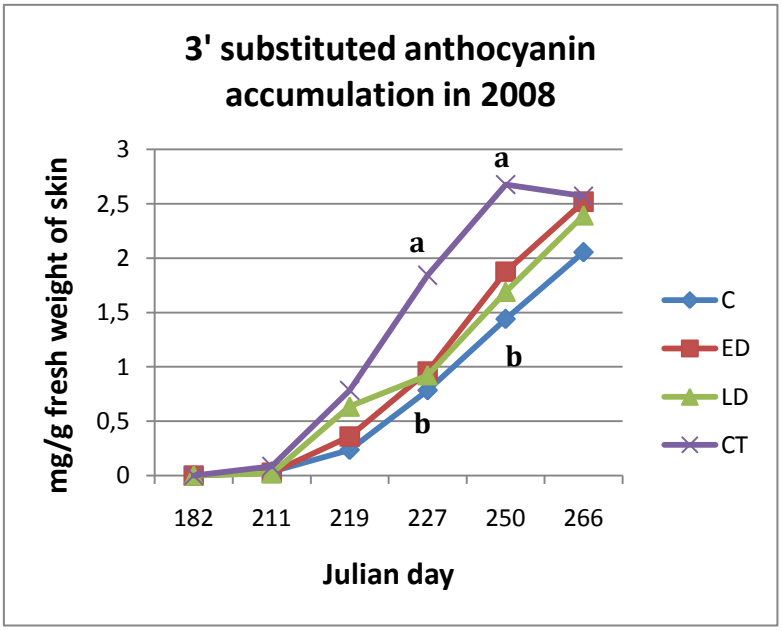


Figure 27. Accumulation of 3'-substituted anthocyanins in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05.

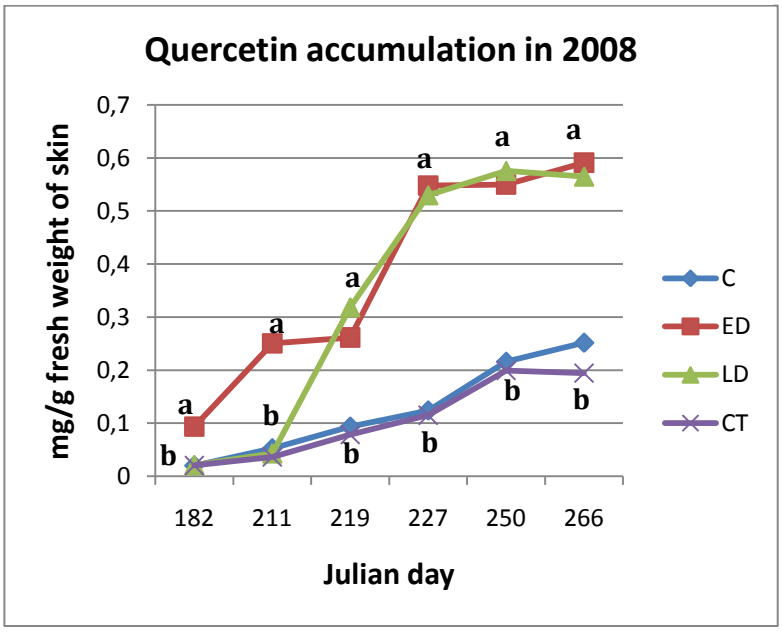


Fig. 28. Accumulation of the flavonol quercetin in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05..

al., 2006) and this is confirmed also by the presence of F3'H derivatives, as quercetin, early in the season (JD 182). In this conditions, it could be hypothesized that when a competition for F3'H products between flavonols and anthocyanins biosynthesis could be triggered, F3'H products are yet synthesized and so both the biosynthetic pathway could proceed in a usual way. Furthermore, no differences in 3'-substituted anthocyanins could be observed in 2008 between C and ED and LD.

Concerning 3'5'-substituted branch of flavonoids biosynthesis, a total number of 8 F3'5'H genes are modulated at end of véraison. Interestingly, any modulated F3'5'H gene is shared among CT, ED and LD transcripts, suggesting a more specific sensitivity at end of véraison of these gene family to the changes induced by cluster thinning or cluster microclimate modifications. Four different isoforms were induced in defoliation treatments: one in both ED and LD treatments (Table 12C, JGVv9.60), and 3 specifically in ED berries (Table 12E, JGVv9.67, JGVv9.79 and JGVv9.66). One F3'5'H transcript (JGVv9.68) is more abundant, while 3 F3'5'H genes (JGVv9.75, JGVv9.71 and JGVv9.81) are instead down-regulated in CT berries (Table 12D).

In Fig. 29 and 30 are reported trends in accumulation of 3'5'-substituted (delphinidin, petunidin and malvidin 3-O-glucoside) anthocyanins and of flavonol myricetin respectively, which both derived from F3'5'H action. At end of véraison, CT berries were characterized by the highest concentration of 3'5'-substituted anthocyanin, while highest myricetin concentration was found in ED and LD berries. At harvest LD berries presented the lower 3'5'-substituted concentration and no differences were found in myricetin concentrations among treatments. It has to be noticed that despite of the early cluster light exposure occurring after pre-flowering defoliation, myricetin accumulation began for ED berries at the same time in which it occurred in other treatments. This suggests a more dependent berry ripening stage regulation of F3'5'H genes, whose activation at the beginning of véraison seems to be independent from endogenous or exogenous berry conditions. These results suggest that following defoliation treatments, light and temperature increase could act on inhibiting the 3'5'-substituted branch of anthocyanin biosynthesis, but not via a direct effect on down-regulation of F3'5'H genes. Rather, a direct competition between FLS and DFR for F3'5'H

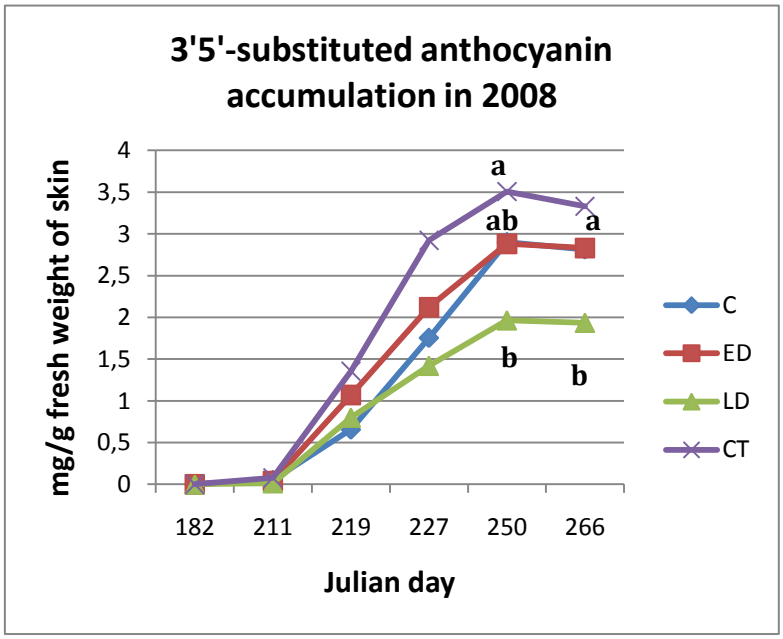


Figura 29. Accumulation of 3'5'-substituted anthocyanins in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05.

genes products could occur and this antagonism could be greater much more FLS further activation occur in proximity of F3'5'H activation.

The up-regulation of the three specific F3'5'H isoforms observed in ED and not in LD berries could bring to an higher accumulation of F3'5'H products and so they could be probably linked to the observed ED recovery in 3'5'-substituted anthocyanin accumulation.

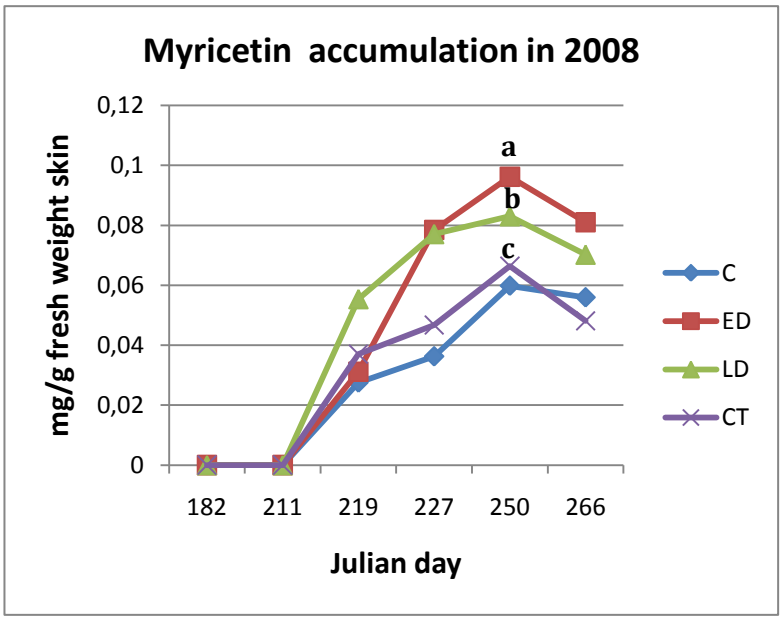


Fig. 30. Accumulation of the flavonol myricetin in berry skin of C, ED, LD and CT Sangiovese vines from pre-bunch closure stage to harvest. Superscript letters indicate significant differences by the Tukey-Kramer test between the treatments at p<0.05..

3.4.9.3. Genes involved in flavonoids transcriptional regulation

Up-regulation of VvMYBA1 (JGVv33.33) gene was detected in all treatments at end of véraison (Table 12A). Despite this, as previously described, no common up-regulation was found in characterized grapevine UFGT sequences. It is interesting to note that, exclusively in ED berries, where instead a sequence matched with UFGT was found, a weak induction of VvMYBA2 (JGVv33.31, FC=2.2) and of a bHLH transcription factor (JGVv141.54) were also detected (Table 12E). Another MYB transcription factor (JGVV6.70), that matched within an anthocyanin-related MYB subclade, was over-expressed (FC=14.4) specifically in CT berries (Table 12D).

Myb5a (JGVv7.172), which regulates early step in flavonoids biosynthesis (Deluc *et al*., 2008), was found to be down-regulated in all treatments (Table 12A). Myb5b (JGVv4.726), which seems to have a similar role to Myb5a in flavonoids biosynthesis regulation, is instead down-regulated only in CT berries (Table 12D). CT berries was also characterized by the down-regulation of two members of bHLH transcription factors, VvMYC1 (JGVv104.164) and VvMYCA1 (Table 12D, JGVv46.101). VvMYC1 is homologue to AtTT8 and PhAN1, which are necessary for anthocyanin induction in *Arabidopsis* and *Petunia Hybrida* respectively, interacting with some MYB transcription factors. It has been demonstrated that VvMYC1 displayed a minimal expression during berry

development (data not published, oral communication), starting at the véraison stage. VvMYC1 down-regulation in CT berries could confirm that, differentially from what demonstrated for homologue genes found in *Arabidopsis* and Petunia, the VvMYC1 role in grape berry is not to activate MYB transcription factors to enhance anthocyanin accumulation.

	FOLD CHANGE					
ED-LD-CT	**CT**	**ED**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV55.22	33.9	23.3	25.7	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase 6 (Fragment)
JGVV2.220	31.5	16.2	17.8	metabolic process	#N/D	Flavonoid-glucosyltransferase 6 (Fragment)
JGVV55.6	24.5	17.5	16.6	metabolic process	#N/D	Flavonoid 3'-monooxygenase (F3'H)
JGVV15.1	19.8	7.2	6.6	metabolic process	NAD dependent epimerase/dehydratase family	Dihydroflavonol reductase (DFR)
JGVV52.29	19.2	5.4	5.0	metabolic process	Cytochrome P450	Flavonoid 3'-monooxygenase (F3'H)
JGVV16.83	18.8	4.7	4.7	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase 6 (Fragment)
JGVV64.134	11.2	2.9	3.1	metabolic process	2OG-Fe(II) oxygenase superfamily	Leucoanthocyanidin dioxygenase (LDOX)
JGVV15.4	10.4	12.7	13.9	transport	ABC transporter	Multidrug resistance-associated protein 6
JGVV3.313	9.7	14.0	11.0	transport	ABC transporter	Multidrug resistance-associated protein 3
JGVV5.437	9.2	10.8	11.9	transport	MatE	Protein TRANSPARENT TESTA 12
JGVV16.84	7.7	6.9	4.6	transport	ABC transporter	Multidrug resistance-associated protein 3
JGVV34.197	5.1	8.7	5.5	transport	#N/D	Multidrug resistance-associated protein 5
JGVV15.5	4.2	9.0	10.2	transport	ABC transporter	Multidrug resistance-associated protein 5
JGVV17.46	2.6	3.1	2.6	transport	ABC transporter	Multidrug resistance-associated protein 3
JGVV33.33	10.0	7.2	6.5	transcription	Myb-like DNA-binding domain	VvMybA1
JGVV7.172	-7.28	-2.17	-2.32	transcription	Myb-like binding domain	VvMyb5a

A. Common differentially expressed genes involved in flavonoids biosynthesis and transport in CT, LD and ED at end of véraison.

	FOLD CHANGE				
ED-CT	**CT**	**ED**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV30.113	5.8	3.6	transport	ABC transporter	Multidrug resistance-associated protein 6
JGVV4.199	-3.1	-2.2	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase 6 (Fragment)
JGVV11.360	-4.0	-2.8	metabolic process	NmrA-like family	Leucoanthocyanidin reductase 1 (LAR1)

B. Common differentially expressed genes involved in flavonoids biosynthesis and transport in CT and ED at end of véraison.

	FOLD CHANGE				
ED-LD	**ED**	**LD**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV1.213	2.3	2.8	-	2OG-Fe(II) oxygenase superfamily	Flavonol synthase (FLS)
JGVV0.280	2.2	2.6	metabolic process	Cytochrome P450	Flavonoid 3'-monooxygenase (F3'H)
JGVV9.60	5.3	4.0	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase
JGVV34.196	4.6	4.2	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase 1
JGVV56.90	5.2	3.7	transport	MatE	Protein TRANSPARENT TESTA 12
JGVV62.40	-4.4	-4.3	metabolic process	#N/D	Flavonoid-glucosyltransferase
JGVV4.196	-5.1	-5.1	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Anthocyanidin 3-O-glucosyltransferase 1

C. Common differentially expressed genes involved in flavonoids biosynthesis and transport in LD and ED at end of véraison.

CT	**FOLD CHANGE**	**GO_plant_slim**	**pfam_description**	**12X annotation**
JGVV41.133	10.8	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase
JGVV107.12	8.8	metabolic process	3-beta hydroxysteroid dehydrogenase/isomerase family	Dihydroflavonol reductase (DFR)
JGVV55.18	6.1	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase 6 (Fragment)
JGVV41.124	5.1	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-O-glucosyltransferase
JGVV9.68	4.5	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase (F3'5'H)
JGVV4.196	3.8	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-glucosyltransferase 1
JGVV2.486	24.4	transport	ABC transporter	Pleiotropic drug resistance protein 1
JGVV2.487	7.1	transport	ABC transporter	Pleiotropic drug resistance protein 1
JGVV31.68	4.1	transport	MatE	Multidrug and toxin extrusion protein 1
JGVV74.52	3.7	transport	ABC transporter	Pleiotropic drug resistance protein 2
JGVV1.376	3.0	transport	MatE	Protein TRANSPARENT TESTA 12
JGVV50.169	2.7	transport	MatE	Protein TRANSPARENT TESTA 12
JGVV6.70	14.4	transcription	Myb-like DNA-binding domain	Myb transcription factor (anthocyanin-related subclade)

	FOLD CHANGE			
JGVV67.6	-2.2	cellular amino acid and derivative metabolic process	Chalcone-flavanone isomerase	Chalcone isomerase 2 (CHS2)
JGVV1.213	-3.3	cellular amino acid and derivative metabolic process	2OG-Fe(II) oxygenase superfamily	Flavonol synthase (FLS)
JGVV115.23	-4.8	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-O-glucosyltransferase
JGVV0.557	-3.4	metabolic process	NmrA-like family	Leucoanthocyanidin reductase 2 (LAR2)
JGVV41.140	-2.8	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-O-glucosyltransferase
JGVV63.5	-2.7	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid-O-beta-glucosyltransferase
JGVV2.90	-6.2	metabolic process	Cytochrome P450	Flavonoid 3'-monooxygenase (F3'H)
JGVV25.273	-3.6	metabolic process	2OG-Fe(II) oxygenase superfamily	Flavanone 3-hydroxylase (F3H)
JGVV9.75	-3.3	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase (F3'5'H)
JGVV9.71	-2.3	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase (F3'5'H)
JGVV1.1071	-2.3	metabolic process	2OG-Fe(II) oxygenase superfamily	Flavanone 3-hydroxylase (F3H)
JGVV9.81	-2.1	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase(F3'5'H)
JGVV7.373	-5.4	transport	ABC transporter	Multidrug resistance protein 1
JGVV1.500	-3.7	transport	MatE	Multidrug and toxin extrusion protein 1
JGVV100.92	-5.2	transport	ABC transporter	Non-intrinsic ABC protein 8
JGVV68.165	-4.5	transport	ABC transporter	Non-intrinsic ABC protein 14, chloroplastic
JGVV5.347	-4.4	transport	ABC transporter	Non-intrinsic ABC protein 4
JGVV0.400	-2.7	transport	ABC transporter	Probable ABC2 homolog 6
JGVV4.726	-3.7	transcription	Myb-like DNA-binding domain	Myb5b
JGVV104.164	-2.9	transcription	U1 zinc finger	VvMYC1

	FOLD CHANGE			
JGVV46.101	-3.5	transcription	-	VvMYCA1

D. Differentially expressed genes involved in flavonoids biosynthesis and transport specific to CT at end of véraison.

ED	FOLD CHANGE	GO_plant_slim	pfam_description	12X annotation
JGVV10.257	3.4	cellular amino acid and derivative metabolic process	O-methyltransferase	Caffeoyl-CoA O-methyltransferase
JGVV9.67	2.8	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase(F3'5'H)
JGVV9.79	3.1	metabolic process	Cytochrome P450	Flavonoid 3',5'-hydroxylase(F3'5'H)
JGVV52.20	7.7	metabolic process	UDP-glucoronosyl and UDP-glucosyl transferase	Flavonoid 3-O-glucosyltransferase (**UFGT**)
JGVV9.66	2.1	metabolic process	#N/D	Flavonoid 3',5'-hydroxylase(F3'5'H)
JGVV16.82	7.3	metabolic process	#N/D	Flavonoid 3'-monooxygenase (F3'H)
JGVV141.54	2.26	transcription	Helix-loop-helix DNA-binding domain	bHLH transcription factor
JGVV33.31	2.2	transcription	Myb-like DNA-binding domain	VvMybA2

E. Differentially expressed genes involved in flavonoids biosynthesis and transport specific to ED at end of véraison.

LD only	FC LD	GO_plant_slim	pfam_description	12X annotation
JGVV28.271	2.8	multidrug transport	MatE	Protein TRANSPARENT TESTA 12

F. Differentially expressed genes involved in flavonoids biosynthesis and transport specific to ED at end of véraison.

Table 12. Differential expressed genes belonging to flavonoid biosynthesis, transport and transcriptional. For each gene, identified by the specific SEQ_ID, fold change (FC) value, pfam description (http://pfam.sanger.ac.uk/) and 12X annotation on the basis of protein sequence homology (www.uniprot.org) are reported.

3.4.10 Differentially expressed genes at harvest

LD berries global transcriptome did not show any significant difference compare to C at harvest.

Only 5 differentially expressed genes compare to C were found in ED berries at harvest. The only up-regulated gene (JGVv151.33) encoding for a cupin protein and was 25 fold more abundant in ED berries compare to C. The cupin superfamily is a group of functionally different proteins, which were initially identified in higher plant only as seed storage proteins. Recently a further role in response to biotic and abiotic stress was also proposed for cupin protein (Dunwell *et al.*, 2001). Among 4 down-regulated genes only one gene (JGVv1.547), which presented a domain of protein-protein interaction, was annotated.

CT berries was the treatment which presented the most differential transcriptional pattern compared to C at harvest. Among all modulated genes 34% of the transcripts on total 29550 probes on the microarray chip were annotated with the previously described annotation method. In Fig.31 is represented the functional annotation of CT modulated genes at harvest.

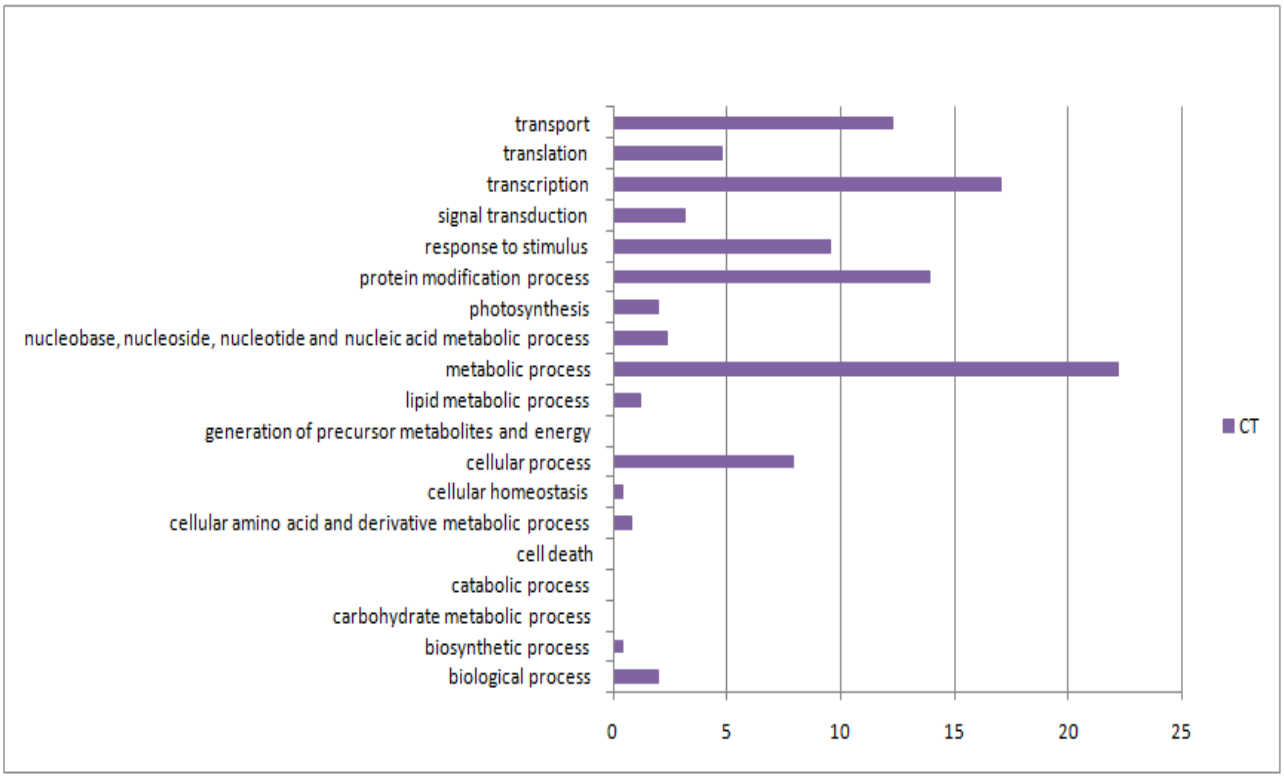


Fig. 31. Functional analyses of CT differential expressed transcripts compared to C at harvest.

Transcripts distributed only into 15 of the 19 GO functional categories in which they previously distributed at end of véraison. No genes belonging to "carbohydrate metabolic process", "catabolic process", cell death" and "generation of precursor metabolites and energy" were modulated at harvest. "Metabolic process" was the functional category with the highest gene number followed by "transcription", "protein modification process" and "transport". Among the

“response to stimulus “ functional category 11 genes were up-regulated and 14 genes were instead down regulated in CT berries. Several genes were involved in response to biotic stress.

Three isoforms of Heat shock cognate proteins (JGVv83.107, JGVv32.75 and JGVv131.20), different from those identified at end of véraison, were found to be up-regulated in CT berries at harvest. Two DNA-repair protein were instead down-regulated (JGVv7.480 and JGVv23.191).

It is interesting to note that, among gene involved in oxidative stress response, an additional SOD isoform (GSVIVT00021953001, FC=14) was found to be up-regulated in CT berries compared to C. It has been shown that after ripening decrease, SOD activity shows an increases at over-ripe stage in tomato (Jimenez *et al.*, 2002) or during senescence process in apple (Du and Bramlage, 1994). Furthermore, one GST (JGVv15.205), which was up-regulated in all treatments at end of véraison, still presented at harvest 2.60 more abundant transcripts in CT compare to C. Only a probable citosolic monodehydroascorbate reductase (JGVv66.99), which was yet down-regulated in CT berries at end of véraison, was found to be still down-regulated in CT compare to C at harvest.

Concerning flavonoids biosynthesis, only a flavonoid-glucosyltransferase gene (JGVv55,22), which was expressed in all treatments at end of véraison, was found to be up-regulated in CT berries at harvest. The down-regulation of a F3’H gene (JGVv2.90) and of a flavonoid-glucosyltransferase (JGVv115.23) observed at end of véraison specifically in CT berries was further confirmed at harvest. Besides, an additional DFR isoform (JGVv39.107) and two transporter involved in flavonoids transport (JGVv14.215 and JGVv108.42) were also down-regulated in CT berries compare to C at harvest.

EXPERIMENT 2: physiological and biochemical response of cv. Cabernet Sauvignon, Nero d'Avola and Raboso Piave (*Vitis Vinifera* L.) to véraison defoliation.

4.0 MATERIAL AND METHODS

4.1 Plant material

The trial was conducted in 2007 and 2008 on adult *Vitis Vinifera* L. Cabernet Sauvignon (grafted to SO_4), Nero d'Avola (grafted to SO_4) and Raboso Piave (grafted to K5BB) vines, in a vineyard located in Bologna, Italy (44°30'N, 11°24'E), with North–South oriented rows. Vine spacing was 0.74 m x 3.0 m and the training system was a vertical shoot positioned spur pruned cordon (12 buds per vine), with cordon height at 1.0 m above ground and a canopy height of about 1.3-1.4 m. Pest management was conducted according to local practice of Regione Emilia Romagna. Each vine in trial was uniformed per bud load and cluster number at flowering. Nine vines per treatment in three blocks were selected in a single uniform row and each vine was randomly assigned to the following treatments: a) control (C), no treatments; b) véraison defoliation (Late Defoliation, LD), hand defoliation of six basal leaves at véraison. In the defoliation treatments, any laterals growing in the 6 basal node shoot zone were also removed. Defoliation treatments and harvest were carried out differentially, according to the trend in berry ripening of each year and cultivar (Table 1).

cultivar	Julian Day in 2007		Julian Day in 2008	
	Véraison defoliation	Harvest	Véraison defoliation	Harvest
Cabernet Sauvignon	199	253	226	276
Nero d'Avola	199	256	226	276
Raboso Piave	207	267	226	287

Table 1. Julian Day in which véraison defoliation treatment and harvest were carried out in 2007 and 2008 for Cabernet Sauvignon, Nero d'Avola and Raboso Piave.

4.2 Measurements of berry temperature

Infrared temperature were collected in the field using a hand held infrared thermometer (Raynger ST, Raytek, Santa Cruz, CA, USA). In one day in 2007 and two days in 2008, temperature data were collected in three moments of the day: in the morning (9.00-9.30 a.m.), when the sun position is at its Zenith (incident radiation orthogonal to the horizon layer, 1.30-2.00 p.m.) and in late afternoon (5.30-6.00 p.m.). For each variety, one cluster per vine on the east-side and one cluster per vine on the west side were chosen to carry out temperature measurements in defoliated and control vine. For each cluster, the percentage of cluster exposure was visual estimated and temperature data were collected for both the exposed and

shaded side of the cluster in the external and internal side of the row. The overall cluster temperature was then calculated as pondered average with the formula:

$$T=T_{exp(ext)} * \%exp_{(ext)} + T_{shaded\ (ext)} * \%\ shaded_{(ext)} + T_{exp(int)} * \%exp_{(int)} + T_{shaded(int)} * \%\ shaded_{(int)}$$

where:

- $T_{exp(ext)}$ / $T_{exp(int)}$ = temperature of the exposure part of each tagged cluster respectively in the external or internal side of the row;
- $\%\ exp_{(ext)}$ / $\%exp_{(int)}$ = cluster light exposure percentage respectively in the external or internal side of the row;
- $T_{shaded\ (ext)}$ / $T_{shaded(int)}$ = temperature of the shaded part of each tagged cluster respectively in the external or internal side of the row;
- $\%\ shaded_{(ext)}$ / $\%\ shaded_{(int)}$ = cluster shading percentage respectively in the external or internal side of the row.

Each time and day in which temperature data were collected, Photosyntethically Active Radiation (PAR) was measured with a single channel light sensor (Skye Instruments, UK) in the exposed and shaded side of the canopy.

4.3 Berry sampling

At 2007 and 2008 harvest berry sampling was effectuated kept each sample separately per vine. For each vine, samples of 30 berries were taken, immediately crushed and used to perform analyses of total soluble solids concentration (°Brix), pH and titratable acidity (TA).

For HPLC analyses of skin anthocyanins (both in 2007 and 2008) and flavonols (only in 2008) 20 berries for vine were stored in a -20°C freezer until analysis was carried out.

4.4 Analyses at harvest

The analyses of total soluble solids concentration (°Brix), pH and titratable acidity (TA), were carried out as previously reported for the experiment 1 (Paragraph 2.4). All clusters on each vine in trial were counted and weighed and a visual evaluation of sunburn and Botrytis damage (% for each cluster) was also carried out.

4.5 HPLC analyses of anthocyanins and flavonols

The berries sampled for HPLC analyses of anthocyanins and flavonols were treated as previously described in the experiment 1 (Paragraph 2.6). The HPLC separation was carried out with the same column and equipment reported for the experiment 1, according to Mattivi *et al*. (2006) with suitable modifications.

4.6 Statistical analyses

Concerning yield, vegetative and berry composition (°Brix, pH, acidity, anthocyanins) data, combined analyses over years after Gomez and Gomez (1984) was carried out using the mixed

General Linear Model (GLM) procedure of the SAS statistical package (SAS Institute, Cary, North Carolina, USA). Year was considered as a random variable, and the error term for the defoliation treatments was the year by treatment interaction mean square. Mean separation between defoliation levels was performed with the Tukey-Kramer test. The year by treatment interaction was tested over the pooled error and considered only if significant.

Flavonols data, recorded only in 2008, were analyzed by mixed GLM procedure of the SAS statistical package procedure, considering only one year data.

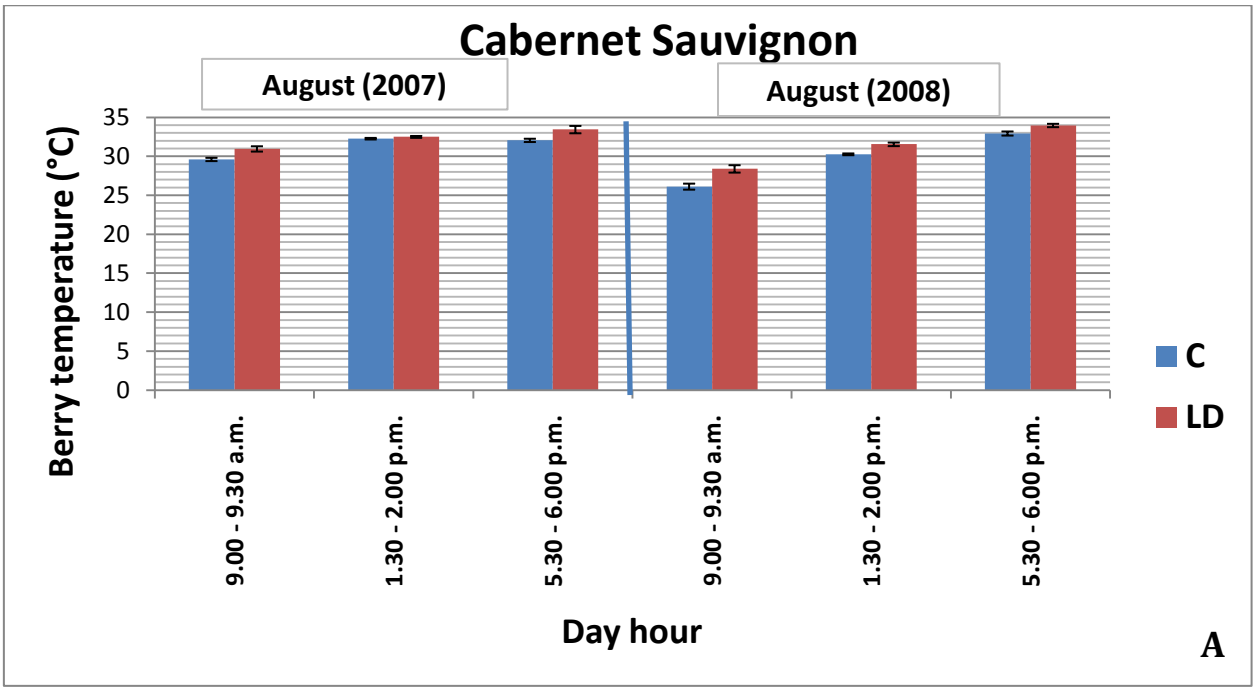
Visual ratings of sunburn damage and Botrytis damage percentage, such as cluster exposure and anthocyanins percentage data, were subjected to square root transformation prior to analysis.

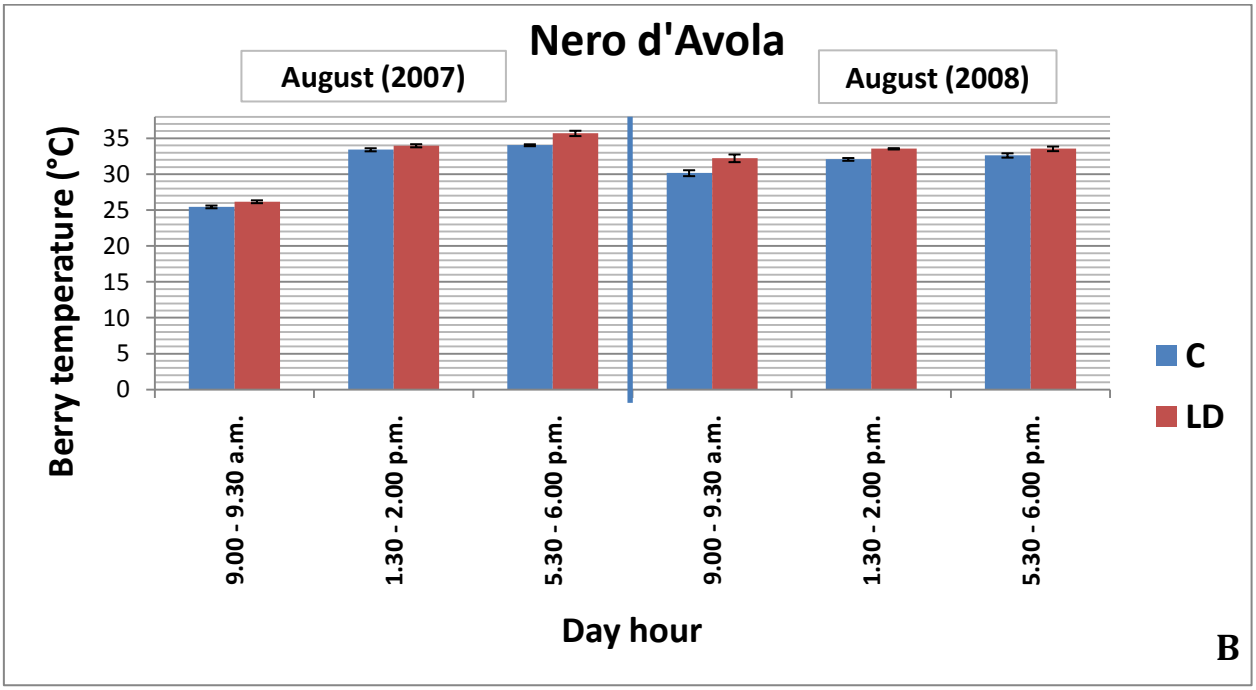
5.0 RESULTS AND DISCUSSION

5.1 Physiological and vegetative-productive measurements at harvest

5.1.1 Effects of véraison defoliation on bunches temperature and exposure

In Fig.1 are represented berry temperature data collected in a representative day chosen during ripening of August 2007 and 2008.





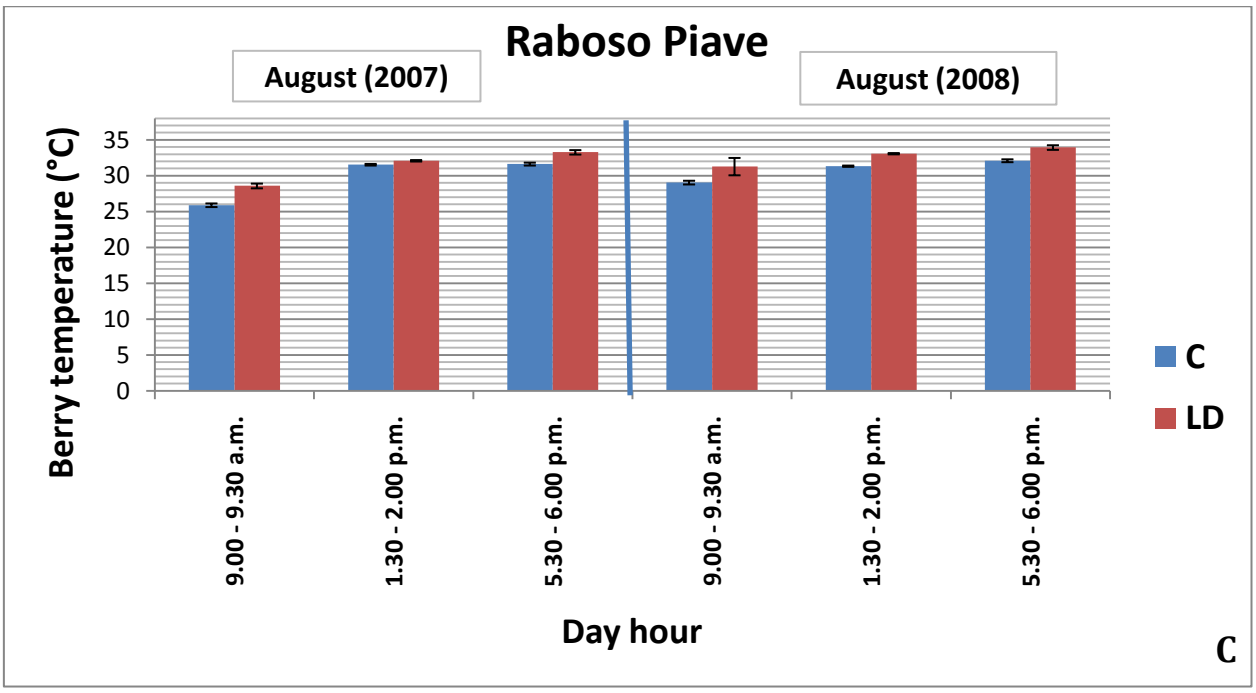


Fig. 1. IR measurements of berry temperature in two representative days chosen in August 2007 and 2008. A. Data collected for Cabernet Sauvignon; B. Data collected for Nero d'Avola; C. Data collected for Raboso Piave.

Berry temperature of véraison defoliated vines (Late Defoliated, LD) was, as expected, higher compared to control vines (C). The effect of late defoliation in increasing berries temperature seems to be cultivar-independent, as all the varieties in trial showed the same behavior. The highest differences between berry temperature of LD and C berries, were recorded in the morning and in the afternoon. In the central hour of the day no differences were instead found between the treatments. The daily measurements carried out through IR thermometer confirmed what previously seen for Sangiovese, in which thermocouples data were collected. The lower entity of differences between C and LD berry temperature data collected with the IR thermometer, compared with the data measured in Sangiovese with thermocouple, could be attributed to the estimation of whole bunch temperature by a pondered average, which also accounted of the percentage of shading in each bunch.

		Cluster exposure (%)		
cultivar	**treatment**	**9.00-9.30 a.m.**	**1.30-2.00 p.m.**	**5.30-6.00 p.m.**
Cabernet Sauvignon	**C**	7.18 **b**	0.06	8.58 **b**
	LD	38.06 **a**	5.57	30.37 **a**
Nero d'Avola	**C**	7.13 **b**	0.30	2.50 **b**
	LD	33.38 **a**	0.97	35.93 **a**
Raboso Piave	**C**	2.82 **b**	0.19	3.40 **b**
	LD	36.34 **a**	1.04	34.23 **a**

Table 1. Average 2007-2008 cluster exposure data collected for Cabernet Sauvignon, Nero d'Avola and Raboso Piave at about 9.00 a.m., 1.30 p.m. and 5.30 p.m. Means within columns followed by different letters differ significantly at $p<0.05$ by the Tukey-Kramer test.

The percentage of cluster exposure, registered in the same day and at the same time of collecting temperature data, is shown in table 1, in which average data collected in 2007 and 2008 are reported. The differences in cluster exposure directly correlates with the berry temperature differences measured both in the morning and in the afternoon. When no differences were pointed out (as at 1.30-2.00 p.m.), no differences were also found in cluster exposure.

5.1.2 Vegetative-productive measurements at harvest

The results concerning the vegetative-productive measurements at harvest previously obtained for Sangiovese in the Experiment 1, have been confirmed also for Cabernet Sauvignon, Nero d'Avola and Raboso Piave. In Table 2 are showed the average of 2007 and 2008 data. Véraison defoliation, causing the elimination of basal leaves already senescent and with low photosynthetic efficiency, did not affect the source-sink balance.

Cultivar	Treatment	Clusters number/vine	Yield/vine (kg)	Cluster weight (g)	Berry weight (g)	Sunburn damage/cluster (%)	Ravaz index
Cabernet Sauvignon	**C**	20.22	2.01	100.11	1.30	0	1.83
	LD	19.44	2.17	111.14	1.31	0	2.47
Nero d'Avola	**C**	15.83	2.62	161.52	2.04	0	3.19
	LD	15.28	2.93	190.31	2.10	0.88	3.39
Raboso Piave	**C**	8.11	2.71	336.71	1.93	0	5.77
	LD	7.72	2.66	347.17	1.82	2.83	5.70

Table 2. Effects of véraison defoliation on yield components of Cabernet Sauvignon, Nero d'Avola and Raboso Piave grapevine. Average of 2007 and 2008 data. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test.

Cultivar	Treatment	°Brix	pH	TA (g/L)
Cabernet Sauvignon	**C**	23.39	3.57	6.86
	LD	23.24	3.65	5.92
Nero d'Avola	**C**	22.19	3.21	10.87
	LD	21.61	3.22	11.15
Raboso Piave	**C**	22.86	3.33	7.81
	LD	22.84	3.37	7.27

Table 3. Effects of véraison defoliation on berry composition of Cabernet Sauvignon, Nero d'Avola and Raboso Piave grapevine at harvest. Average of 2007 and 2008 data. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test

Comparable values of Ravaz index between treatments were recorded. As a consequence, no differences were found in any of the considered vegetative-productive parameter for any of the cultivar in trial. In the condition of the trial, the increase in cluster exposure and temperature associated with véraison defoliation did not affect the entity of sunburn damage.

As expected, the changes in cluster microclimate induced by the treatment were not linked to soluble sugars content and pH (Table 3). Despite the increased temperature of LD berries (and differentially from what observed in Sangiovese) no significant differences were found in titrable acidity, even if LD berries of Cabernet Sauvignon and Nero d'Avola showed a tendency to lower acidity compared to C.

5.2 Flavonoids compounds analyses

5.2.1 Analyses of anthocyanin amount and composition at harvest

Total anthocyanin amount and composition at harvest is showed as average of 2007 and 2008 data in Table 4. Differentially from what previously seen for cultivar Sangiovese, in Cabernet Sauvignon, Nero d'Avola and Raboso Piave also acetate and para-coumarate forms of the five main anthocyanins are present. As expected, the increase in cluster light and exposure after véraison defoliation did not affect final anthocyanin content in any of the variety in trial. Among the three varieties Raboso Piave was the one which contained the highest anthocyanin content.

cultivar	treatment	Total anthocyanins (mg/kg)	Del-3-O-Glc	Cy-3-O-Glc	Pet-3-O-Glc	Peo-3-O-Glc	Mal-3-O-Glc	Total acetil-Glc	Total coumaroyl-Glc
Cabernet	**C**	1170.4	120.4	13.5	79.4	51.9	470.0	313.0	101.7
Sauvignon	**LD**	1220.5	133.5	17.0	93.3	64.9	504.1	293.5	114.1
Nero	**C**	1118.0	101.4	8.6 **b**	98.2	40.9	540.1	151.9	177.0
d'Avola	**LD**	1053.0	115.2	14.5 **a**	106.5	50.9	479.0	135.5	151.5
Raboso	**C**	2001.1	180.0	314.2	181.5	552.0	490.4	154.3	128.8
Piave	**LD**	1925.2	173.9	335.1	171.4	545.2	440.4	142.5	116.7

Table 4. Effects of véraison defoliation on anthocyanin amount and composition in Cabernet Sauvignon, Nero d'Avola and Raboso Piave, expressed as mg/kg berry. Average of 2007 and 2008 data. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test.

Regard to composition, it has to be noticed that the three varieties are characterized by different anthocyanic profiles. Cabernet Sauvignon and Nero d'Avola are in fact showed very low amount of 3'-substitued anthocyanin (cyanidin and peonidin 3-O-glucoside), which in complex represent in these varieties about the 8% of total anthocyanins. Raboso Piave anthocyanic profile distinguishes from the other two varieties for the high 3'-substituted anthocyanin

content, which correspond to about 50% of total anthocyanin. In addition, compared to the other varieties, Cabernet Sauvignon contains higher amount of acetate anthocyanins (25% of total anthocyanins), which in Nero d'Avola and Raboso Piave represent respectively only the 12% and 7% of total anthocyanins. Despite this genetic differentiation in terms of anthocyanin composition, in both Cabernet Sauvignon and Raboso Piave no significant differences were found after the defoliation treatment in anthocyanic profile. A significant variation in cyanidin 3-O-glucoside concentration, which was almost 2-fold greater in LD than in C berries was detected in Nero d'Avola. It is interesting to note that a tendency in cyanidin 3-O glucoside increase can be observed also in Cabernet Sauvignon and Raboso Piave and it could be considered as mainly responsible of the significant differences observed in 3'-substituted and hydroxylated anthocyanins final percentages (Fig.2). All the varieties showed an increase in LD berries for both 3'-substituted and hydroxylated anthocyanins. This increase was significantly different in the case of 3'-substitued for Nero d'Avola and Cabernet Sauvignon and in the case of hydroxylated anthocyanins for Nero d'Avola and Raboso Piave. This confirms what previously seen for Sangiovese about the high increase in 3'-substituted anthocyanin percentage following the increase in cluster light and temperature. As expected, also a difference in hydroxylated anthocyanin was found, except in the case of Cabernet Sauvignon.

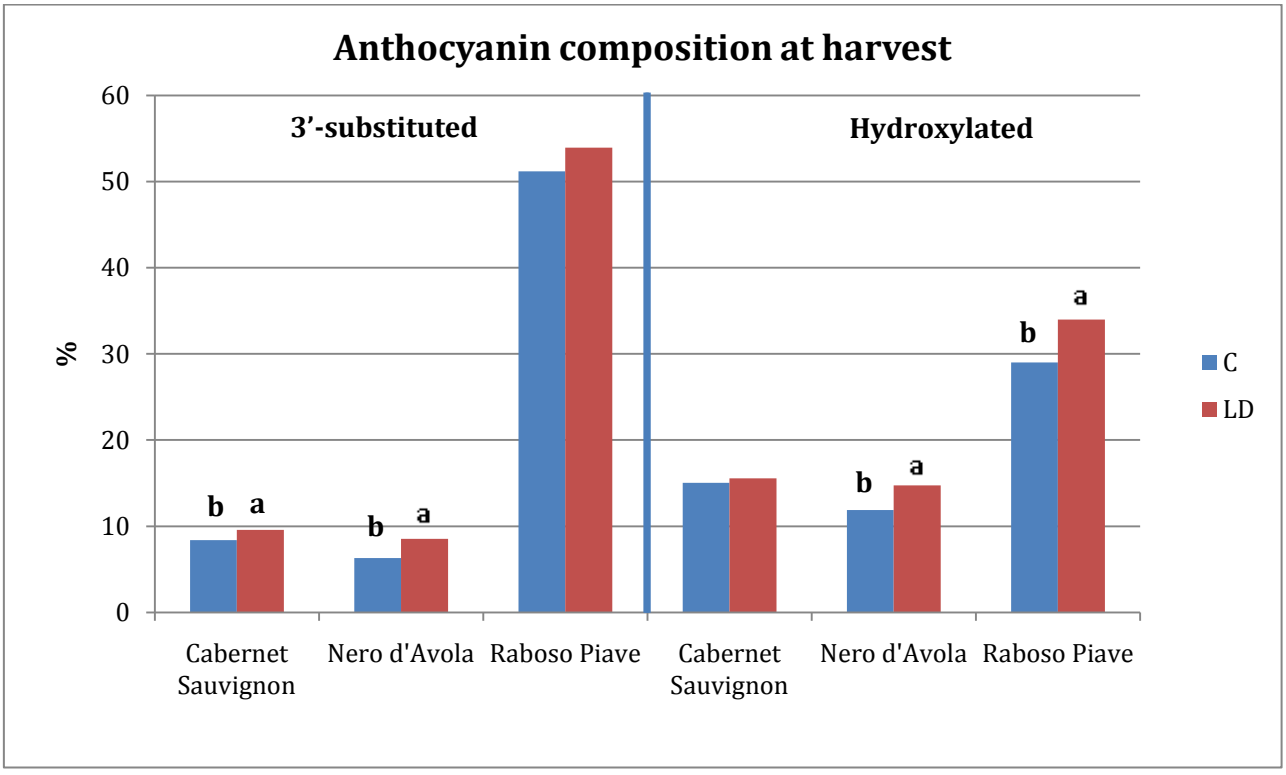


Fig. 2. Effects of véraison defoliation on 3'-substituted and hydroxylated anthocyanin percentage in Cabernet Sauvignon, Nero d'Avola and Raboso Piave. Average of 2007 and 2008 data. Means within columns followed by different letters differ significantly at p<0.05 by the Tukey-Kramer test.

5.2.2 Analyses of flavonols amount and composition at 2008 harvest

The final amount of flavonols increased significantly in defoliated berries in all varieties in trial, confirming the lacking of the cluster temperature increase effect in this pathway. The differences observed in composition, seems to be, instead, cultivar dependent and in particular it seems to exist a strict correlation between anthocyanin and flavonol composition. In those cultivar characterized by low amount of 3'substitued anthocyanin as Cabernet Sauvignon or Nero d'Avola, it was observed an increase in all flavonols classes, which does not affect the final flavonol percentage composition. In Raboso Piave, which has a composition similar to Sangiovese, characterized by higher amount of 3'-substituted anthocyanin, the defoliation treatment induces a further activation of this branch of flavonoid pathway resulting in a five-fold increase in quercetin in defoliated berries. This reflects in the final quercetin percentage, which in C berries was around 40% and in LD berries reaches the 75%.

cultivar	treatment	Total flavonols (mg/kg)	Kaempferol (mg/kg)	Myricetin (mg/kg)	Quercetin (mg/kg)
Cabernet Sauvignon	**C**	**22.70**	0.59 **b**	11.47 **b**	10.64 **b**
	LD	**51.51**	2.59 **a**	23.57 **a**	25.36 **a**
Nero d'Avola	**C**	**39.51**	0.00 **b**	21.94 **b**	16.35
	LD	**70.71**	2.98 **a**	36.22 **a**	30.32
Raboso Piave	**C**	**24.13**	1.23 **b**	13.94	10.19 **b**
	LD	**69.62**	4.16 **a**	15.87	50.78 **a**

Table 5. Effects of véraison defoliation on flavonols amount and composition in Cabernet Sauvignon, Nero d'Avola and Raboso Piave, expressed as mg/kg berry. Average of 2007 and 2008 data. Means within columns followed by different letters differ significantly at $p<0.05$ by the Tukey-Kramer test.

While anthocyanin amount seems to be not affected neither by cultivar nor véraison defoliation treatments, anthocyanin composition is very sensitive to both this factor.

As expected, light induction plays a key role in determining flavonol accumulation independently by the cultivar. Also at this level, the response in terms of composition is strictly linked to cultivar.

It has been demonstrated the existence of at least two different isoforms of F3'H, several isoforms of F3'5'H and two isoforms of FLS (Castellarin *et al*., 2006; Bogs *et al*., 2006; Downey *et al.,* 2003). The combination of these different gene isoforms could be considered as responsible of the different response cultivar-dependent in terms of anthocyanins and flavonols.

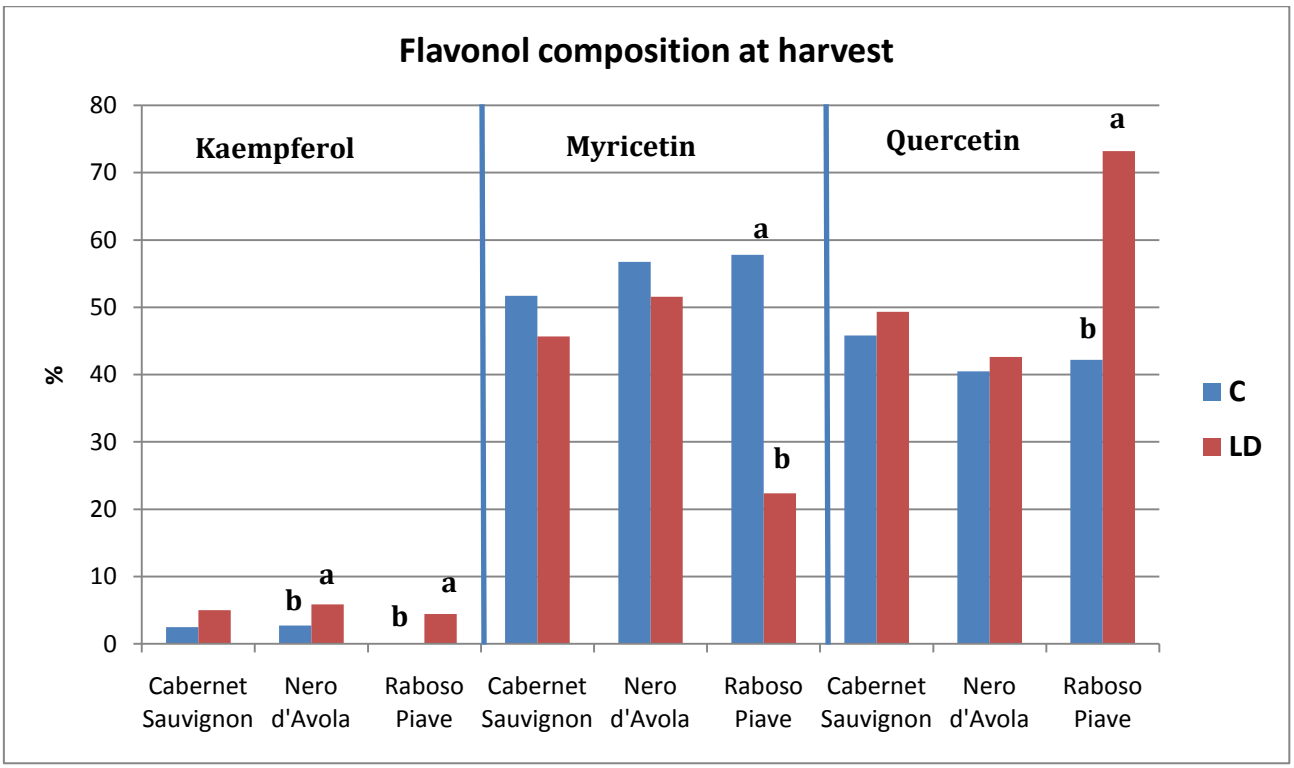


Fig. 3 Effects of véraison defoliation on kaempferol, myricetin and quercetin percentage in Cabernet Sauvignon, Nero d'Avola and Raboso Piave. Average of 2007 and 2008 data. Means within columns followed by different letters differ significantly at $p<0.05$ by the Tukey-Kramer test.

6.0 CONCLUSION

Interesting results were obtained through an integrated agronomic, biochemical and molecular approach, employed in order to understand the mechanisms involved in berry composition of Sangiovese vines submitted to modification of source/sink ratio and/or microclimatic changes.

Among the management techniques applied in the present research (cluster thinning, pre-bloom defoliation and véraison defoliation), cluster thinning (CT) was the treatment which causes the most strong changes in yield and berry composition. The highest yield reduction observed in CT vines (removal of 50% of total clusters per vine at véraison), implied the highest leaf area/crop weight ratio, which caused an acceleration in berry ripening rate and the highest values of soluble sugars at harvest in both years compared to the other treatments and C.

Pre bloom defoliation (ED) has shown in recent works to be a powerful tool in inducing berry set and yield reduction, while achieving improved must composition on white and black varieties (Poni et al., 2006; Intrieri et al., 2008). In present work, despite the lower berries per cluster, only a slight effect was found in berry and cluster weight reduction in ED respect the other thesis following pre-bloom defoliation. Soluble sugars concentration resulted increased at harvest in ED compared to C only in 2008, despite in both years no differences were found in leaf area/crop weight ratio. The different behavior observed between ED and C in must composition at 2008 harvest associated with no differences in leaf area/crop weight ratio, was probably due to the combined effect of photosynthetic compensation (Poni et al., 2008) and lateral re-growth. In 2009, the higher temperature compared to 2008, which characterized the period following pre-bloom defoliation, probably impeded this kind of response to leaf removal. This confirmed that canopy photosynthetic efficiency is a crucial issue to assess source/sink balance in the vine, rather than total canopy surface.

Among the expected effects following ED in modifying cluster morphology, only a slight reduction in ED cluster compactness was detected compared to the other treatments. Nevertheless, ED presented the greater homogeneity in berry dimension within each cluster while more variability was recorded among the four thesis in terms of sugars concentration.

After véraison defoliation (LD), no effects were found in berry composition in terms of sugars content. LD berries showed the lower titrable acidity, probably due to the malic acid degradation induced by cluster microclimate changes. It has to be noticed that, despite leaf removal induced an increase in cluster light exposure, few differences were found concerning the expected increase of cluster temperature. In our conditions, during berry ripening all treatments and C were affected by over-heating (temperature >30°C) for most part of the ripening period. This is generally considered a critical temperature value for metabolic changes occurring during berry ripening (Spayd et al., 2002, Yamane et al., 2006), even if this limit may depend on cultivar (Tarara et al., 2008).

Flavonoids amount and composition at harvest were strongly affected by the treatments.

CT showed higher anthocyanin values compared to C, probably as a consequence of the increase in leaf area/crop weight ratio. CT and ED anthocyanic profile was always similar to C, while LD was characterized by higher 3'-substituted percentage as a consequence of cyanidin 3-O-glucoside concentration increase and malvidin 3-O-glucoside concentration reduction.

Flavonols accumulation was, as expected, positively regulated by increase in cluster light exposure (Downey et al., 2004) and so in defoliated berries of both ED and LD treatments the highest flavonols concentration values were detected. The assessment of flavonol composition showed a specific increase in quercetin flavonols derivatives following flavonol increase, as previously reported by Spayd et al. (2002).

The results obtained in Sangiovese subjected to véraison defoliation can be confirmed in the second experiment in Cabernet Sauvignon, Nero d'Avola and Raboso Piave in terms of berry composition and anthocyanin. A cv-dependent effect was instead highlighted in flavonol composition response. In those varieties genetically characterized by high 3'-substituted anthocyanin content (Sangiovese and Raboso Piave), an increase in quercetin derivatives was found following véraison defoliation. In Cabernet Sauvignon and Nero d'Avola, which present high levels of 3'5'-substituted anthocyanin, no differences were found in flavonol composition.

Microarray analyses, carried out with a NimbleGen array assembled on the basis of the 12X *Vitis Vinifera* genome sequence (Jaillon et al., 2007), allowed to discriminate between treatments and C only at end of véraison. This seems to be the key stage of transcriptional rearrangement during berry ripening.

CT transcriptional profile confirmed the acceleration observed by physiological and biochemical analyses in berry ripening by means of an increase in expression of genes involved in sugars storage which usually follows sugar utilization as energy source. All these evidences associated with a decrease in gene response to oxidative stress, which usually corresponds to the final ripening phases (Pilati et al., 2007), could further confirm an advanced stage of ripening for CT. Sugars increase positively influences anthocyanin structural and transcriptional regulatory gene, but the main effect was registered among genes involved in anthocyanin transport and storage, which were strongly up-regulated in CT compared to all treatments.

A rather different situation on transcriptional profile at the same stage of ripening (end of véraison) has been found in ED, where it was possible to notice an up-regulation of many genes involved in sugars utilization as energy source compared to all treatments. This is compatible with the decrease in berry ripening rate identified at this stage at sugars level. Among genes involved in flavonoid biosynthesis, despite the up-regulation of genes specifically involved in anthocyanin biosynthesis and probably linked to increased source/sink and light effects, no differences were detected in terms of total anthocyanins compared to all treatments. As

expected, light induced up-regulation of specific genes linked to flavonol synthesis correlated to ED flavonols profile.

Véraison microclimatic changes imposed to LD berries did not affect gene involved in carbohydrate metabolism. Response to stress of LD berries resulted in the up-regulation of genes involved in typical very close to increased temperature reaction. As previously seen for ED, light induction of anthocyanin biosynthesis genes, did not affect total anthocyanin amount, while flavonol-biosynthesis genes were up-regulated.

The results of this research confirmed the main role of source/sink ratio in conditioning sugars metabolism and revealed also that carbohydrates availability is a crucial issue in triggering anthocyanin biosynthesis.

More complex is the situation of pre-bloom defoliation, where source/sink and cluster light increase effects are associated to determine final berry composition. It results that the application of pre-bloom defoliation may be risky, as too much dependent on seasonal conditions (rain and temperature) and physiological vine response (leaf area recovery, photosynthetic compensation, laterals regrowth). Early induced stress conditions could bring cluster at véraison in disadvantage to trigger optimal berry ripening processes compared to untreated vines. This conditions could be maintained until harvest, if no previously described physiological recovery occurs. Certainly, light exposure increase due to defoliation treatments, showed a positive and solid effect on flavonol biosynthesis, as in our conditions temperature was not so different among treatments.

Except the last aspects, that could be confirmed also for véraison defoliation, microclimatic changes by themselves seemed not able to induce any modifications in berry composition. Further studies are necessary to understand if the peculiar anthocyanic and flavonols composition detected in véraison defoliation could play an important role in both color intensity and stability of wines.

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