Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Colture Arboree ed Agrosistemi Forestali, Ornamentali e Paesaggistici

Ciclo XXV

Settore Concorsuale di afferenza: 07/B2

Settore Scientifico disciplinare: AGR/03

EFFECTS OF GLOBAL WARMING ON BERRY COMPOSITION OF cv. SANGIOVESE: BIOCHEMICAL AND MOLECULAR ASPECTS AND AGRONOMICAL ADAPTATION APPROACHES

Presentata da: **Dr. Nooshin Movahed**

Coordinatore Dottorato: Prof. Luca Corelli Grappadelli

Relatore: Dr.ssa Ilaria Filippetti

Correlatori: Prof. Andrea Masia
Dr.ssa Sara Zenoni
Dr.ssa Chiara Pastore

Esame finale anno 2013

Contents

1. GENERAL INTRODUCTION

1.1. Grape ripening

Grape is a non-climacteric fruit and in non-climacteric fruits, ethylene does not appear to have a major role in ripening. Little is known about the factors that regulate the ripening process of grapes. However, it seems likely that plant growth substances are involved (Coombe, 1992). Grapes exhibit a double sigmoid pattern of development (Fig. 1) with the two distinct phases of growth separated by a lag phase (Coombe, 1992). The ripening phase of grape berry development occurs during the second period of berry growth and results from expansion of existing pericarp cells (Hardie et al., 1996). During the ripening phase a number of major physiological and biochemical changes occur simultaneously in the grape berry, and these determine the quality of the fruit at harvest. The onset of ripening (Veraison), signals the beginning of significant changes in metabolism which include accumulation of sugar, softening of berries, synthesis of anthocyanins, metabolism of organic acids, accumulation of flavour compounds and changes in the level of growth substances (Fig. 1). These changes are coordinated within each berry, but individual berries, even those on the same bunch, do not ripen synchronously (Coombe, 1992); this results in significant variation between berries, even at harvest. This variability represents the significant effect of berry environmental conditions on berry composition.

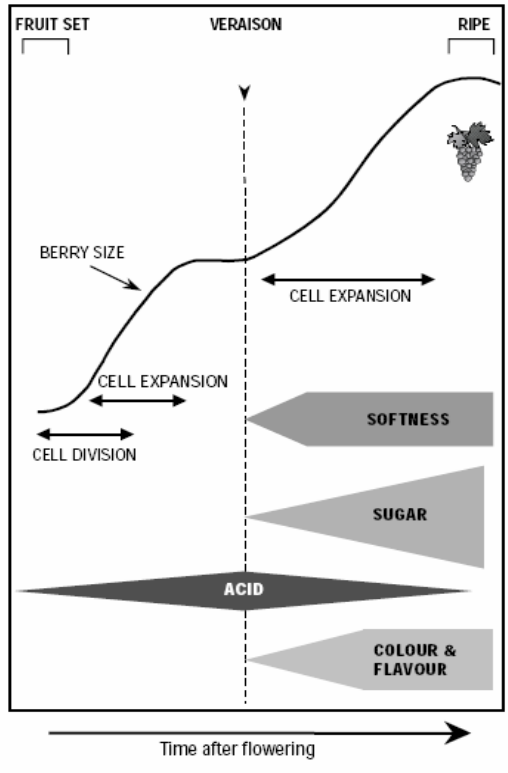

Fig. 1. Schematic representation of grape berry development (Robinson & Davies, 2000).

1.2. Grape berry composition

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. °Brix is directly related to the sugar content (g/L) and potential titratable alcohol (vol %); and about 55% of grape berry °brix converses to alcohol (Fournand et al., 2006). 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, the predominant sugar transported in the phloem, accumulates in grape berry in form of glucose and fructose. 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 (Pastore, 2010). The main role of sugars in grape berry is 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

Organic acids are some important flavor compounds in both grapes and wines, and the composition and content of organic acids are closely related to the organoleptic quality of grapes and wine (Wen et al., 2009). 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. As to Dokoozlian, 2000, green berries, together with leaves, are the main grape organs able to produce organic acids.

1.2.3. Phenolic compounds

Phenolic compounds of grape are divided into non-flavonoids and flavonoids groups (Robinson, 2006).

1.2.3.1. Non-flavonoids

Hydroxycinnamic acids, benzoic acids and stilbenes belong to the non-flavonoids group (Waterhouse, 2002).

1.2.3.1.a. Hydroxycinnamates

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); and Caftaric acid is the predominant hydroxycinnamates in grape (Kennedy et al., 2006).

1.2.3.1.b. Benzoic acids

Benzoic acids are the minor components 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 in both skin and seeds (Pastore, 2010).

1.2.3.1.c. Stilbenes

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 & Fregoni, 2001). 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) (Gatto et al., 2008).

1.2.3.2. Flavonoids

Flavonoids are a class of plant secondary metabolites which are synthesized from Phenylalanine or Tyrosine, condensed with a malonyl-CoA via the phenylpropanoid pathway. This class comprises more than 6,500 known compounds, many of which are of great biological and commercial importance. In nature, they play important roles in resistance to stresses both abiotic (such as excess light and high temperature) and biotic (such as resistance to microbes) (Mol, 1998; Winkel-Shirley, 2002); moreover, they are of central importance in the ecological relationships with pollinator insects and in nodule formation (Downey et al., 2006). They comprise both transparent compounds

such as the flavanols, and brightly coloured ones, such as phoblaphenes, anthocyanins, and polymeric proanthocyanidins (Robinson, 2006). All flavonoids share a common chemical structure represented by a C15 skeleton (C6-C3-C6), (Fig. 2).

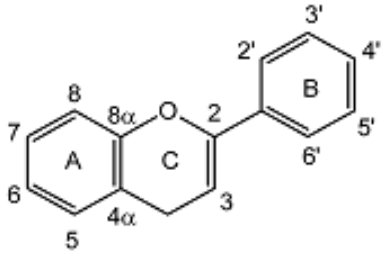

Fig. 2. Chemical structure of flavonoid molecule (Aron & Kennedy, 2008)

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). The B ring can further be hydroxylated, ortho-dihydroxylated or vicinal-trihydroxylated (Pastore, 2010). Other flavonoids modifications include methoxylation, *O*-glycosilation of hydroxyl groups or C-glycosilation of flavonoid skeleton carbon atom. Moreover, flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules (Aron & Kennedy, 2008). Different classes of flavonoids are commonly detected in grape such as anthocyanins, flavonols and flavan-3-ols (tannins or proanthocyanidins) (Fig. 3). 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.

1.2.3.2.a. 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, 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.

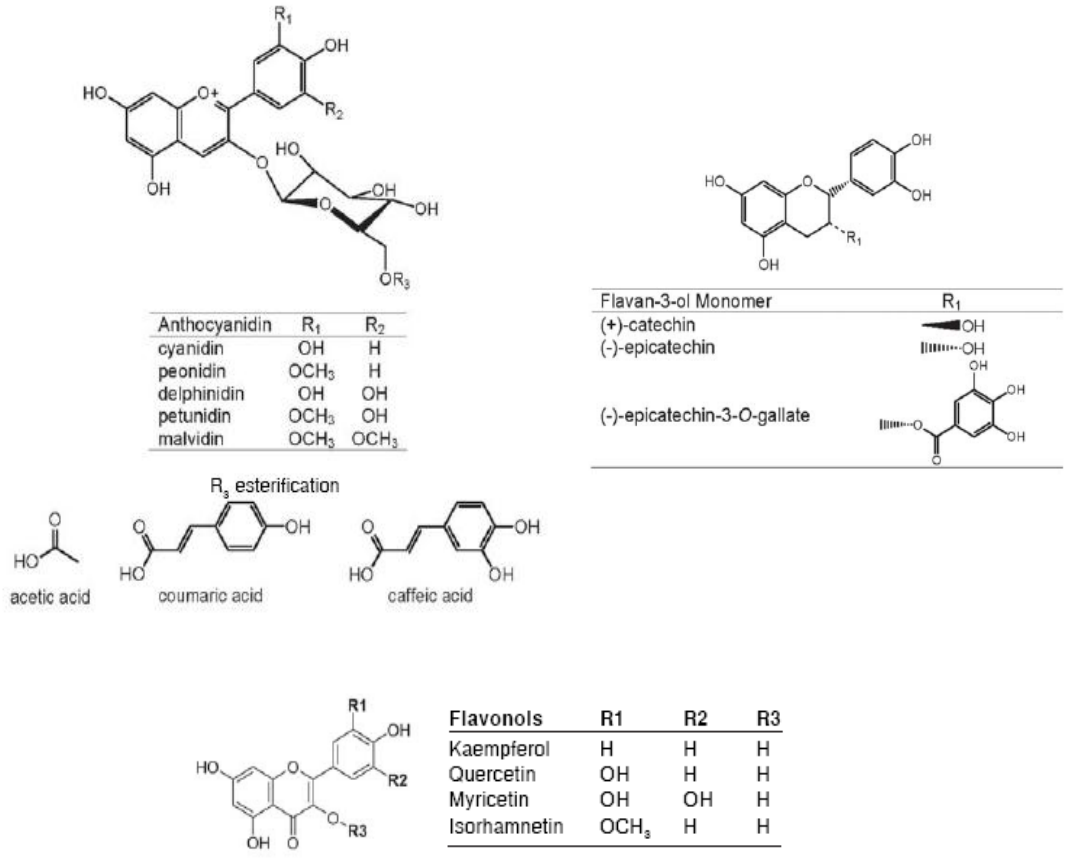

Fig. 3. Chemical structure of the three main classes of flavonoids present in red grape berry (Adams, 2006).

1.2.3.2.b. Flavan-3-ols and tannins

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 like epicatechin and epigallocatechin which constitute the chain of the polymers ("extension" subunits) and catechin which 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).

Flavonoids in the seeds accumulate not only in the external part, but even in the inner layers. Grape seeds are the primery 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. The composition of seed tannins is different comparing to the skin one; in so far that, in seed tannins generally

epigallocatechin is not present and epicatechin gallate is the major constituent of “extension” subunits (Adams, 2006).

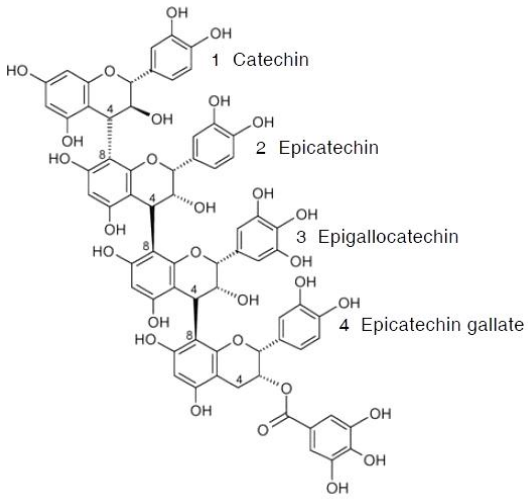

Fig. 4.This hypothetical condensed tannin is made up of four sub-units: catechin, epicatechin, epigallocatechin, epicatechin gallate. The first subunit (catechin) is bound to the second (epicatechin) by an interflavan bond between carbon 4 of catechin and carbon 8 of epicatechin. The first three subunits are referred to as "extention" subunits and only the epicatechin gallate has free 4 positions and is thus re-ferred to as a "terminal" unit (Adams, 2006).

1.2.3.2.c. Flavonols

Grape and wine contain different classes of polyphenols. Flavonols are flavonoids that are found in higher plants, usually in glycosidic forms. They are products of the flavonoid biosynthetic pathway, which also give rise to anthocyanins and to condensed tannins in grapevines (Stafford, 1990 and Darne, 1993). The main flavonols reported in grape berries are quercetin-3-O-glucoside and quercetin-3-O-glucuronide (Price et al., 1995; Cheynier et al., 1986 and Downey et al., 2003).

Flavonols are also involved in the stabilization of the flavilium form of anthocyanins in young red wines through co-pigmentation (Boulton; 2001). The highest flavonol concentrations in grapes were found at flowering, followed by a decrease as the grapes increased in size. Subsequently, a significant level of flavonol biosynthesis was observed during berry development and the greatest increase in flavonols per berry can be observed 3-4 weeks post-veraison (Downey, et al., 2003).

In red grapes, the main flavonol is quercetin (mean) 43.99%), followed by myricetin (36.81%), kaempferol (6.43%), laricitrin (5.65%), isorhamnetin (3.89%), and syringetin (3.22%). In white grapes, the main flavonol was quercetin (mean) 81.35%), followed by kaempferol (16.91%) and

isorhamnetin (1.74%). The delphinidin-like flavonols myricetin, laricitrin, and syringetin were missing in all white varieties, indicating that the enzyme flavonoid 3′,5′−hydroxylase is not expressed in white grape varieties (Mattivi et al., 2006).

It is interesting to observe the analogies between the hydroxylation of position 5′, which leads to the formation of the anthocyanidin delphinidin from cyanidin and the flavonol myricetin from quercetin. In addition to this, O-methylation of position 3′ of anthocyanidin cyanidin, which leads to the formation of peonidin, corresponds to the O-methylation, which converts flavonol quercetin into isorhamnetin. Finally, O-methylation of positions 3′ and 5′ of anthocyanidin delphinidin, which leads to the formation of petunidin and malvidin, corresponds to O-methylation, which converts flavonol myricetin into laricitrin and syringetin (Fig. 3).

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 (Boss et al., 1996) (Fig. 5, 6). The enzymes involved in flavonoid biosynthesis are associated at the cytoplasmic surface of the endoplasmic reticulum and represent a multi-enzyme complex.

1.3.1. Synthesis of the early products of the phenylpropanoid pathway

1.3.1.1. PAL

PAL is a key enzyme, ubiquitous in plants, which catalyzes one of the first steps in phenylpropanoids biosynthesis (Fig. 5), the hydrolysis of ammonia from phenylalanine to produce cinnamic acid. Cinnamic acid is the precursor of all phenolic compounds. Recently the subcellular localization of PAL was found in grape; 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 grape there are probably present 15-20 PAL isoforms (Sparvoli et al., 1994).

1.3.1.2. C4H

The cinnamic acid produced by PAL is converted to *p*-coumaric acid towards the action of cinnamate 4-hydrolase (C4H) (Fig. 5). C4H belongs to the cytochrome P450 monoxygenases

family. The enzymes of this family are able to catalyse exothermic reactions that are irreversible. 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).

1.3.1.3. "4CL"

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 (Fig. 5). 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 multi-gene family. It's very interesting to note that the activities of PAL, C4H and 4CL are really correlated and change concomitantly (Chen et al., 2006).

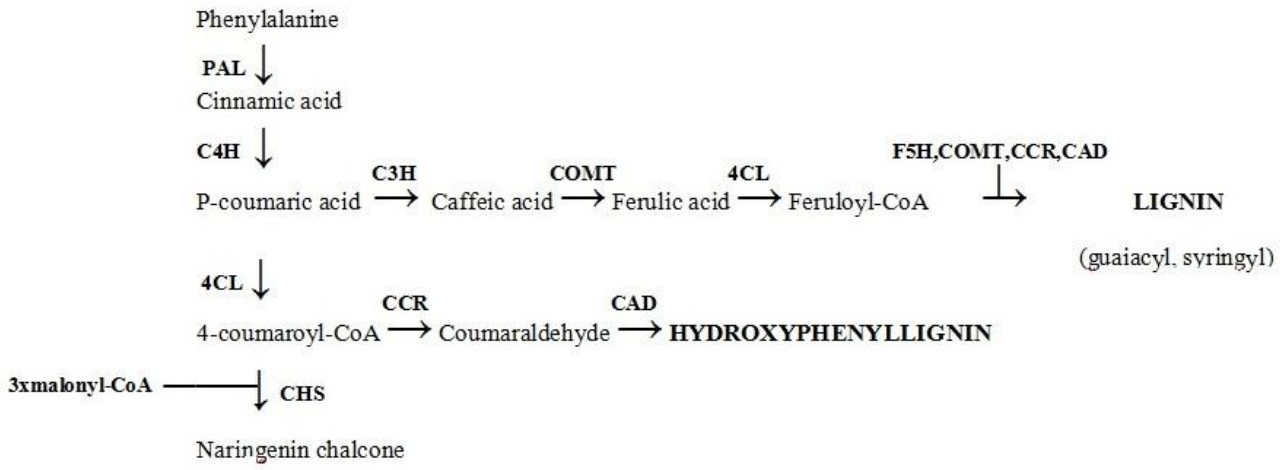

Fig. 5. The anthocyanin synthesis pathway in plants (Deluc et al., 2008)

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

1.3.2.1. CHS

The first enzyme involved specifically in flavonoids biosynthesis is calchone synthase (CHS) (Fig. 5, 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, belongs to a large super-family of proteins, the family of type III Polykethide Synthase.

1.3.2.2. CHI

Naringenin calchone is converted to flavanones (naringenin) to form the flavanoid core towards a stereo-specific ring closure catalysed by chalchone isomerase (CHI) (Jeong et al., 2004). 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 (Fig. 6).

1.3.2.3. F3H, FLS, F3'H, F3'5'H

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 two copies of F3'H and several copies of F3'5'H are present 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 (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 (Chua et al., 2008).

1.3.2.4. DFR

FLS 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 (Pastore, 2010).

1.3.2.5. ANR, LAR, LDOX, and UFGT

Leucocyanidin and leucodelphinidin are respectively the substrates to the next step of the proanthocyanidin and anthocyanin biosyntheses, which involve either anthocyanidin reductase (ANR or BAN), 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 respectively (-)-epicatechin and (-)-epicatechin-3-O-gallate (Gagné et al., 2009).

As FLS, also LDOX belongs to a family of 2-oxoglutarate-dependent oxygenase (Pastore, 2010). 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 only a single copy of LDOX gene is present in grapevine (Gollop et al., 2001). The anthocyanidins, cyanidin and delphinidin, produced towards the action of LDOX are then stabilized through the addition 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. 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.

1.3.2.6. OMTs

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 (Pastore, 2010).

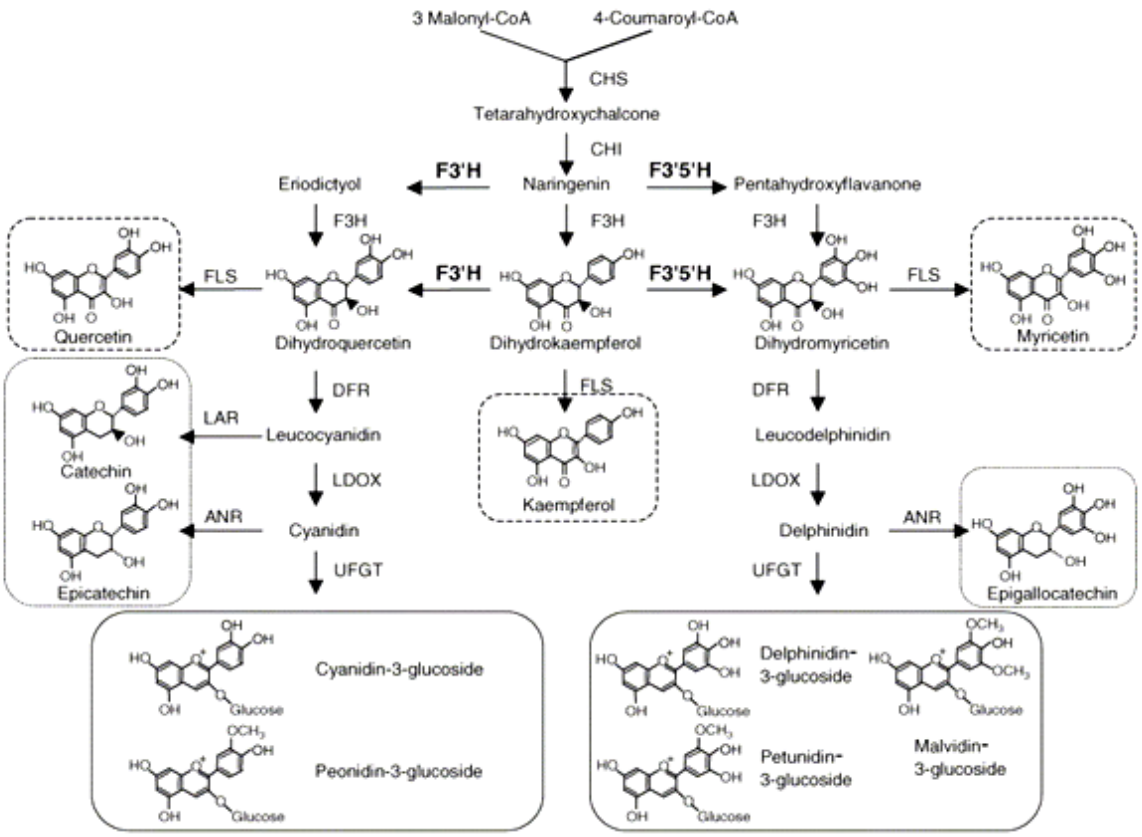

Fig. 6. Diagrammatic representation of the flavonoids biosynthesis pathway; Enzymes abbreviation are as given in the text (Jeong et al., 2006).

1.4. Flavonoid gene expression during berry development

The pattern of flavonoid gene expression (Fig. 7) 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 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., 1996). 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 a 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).

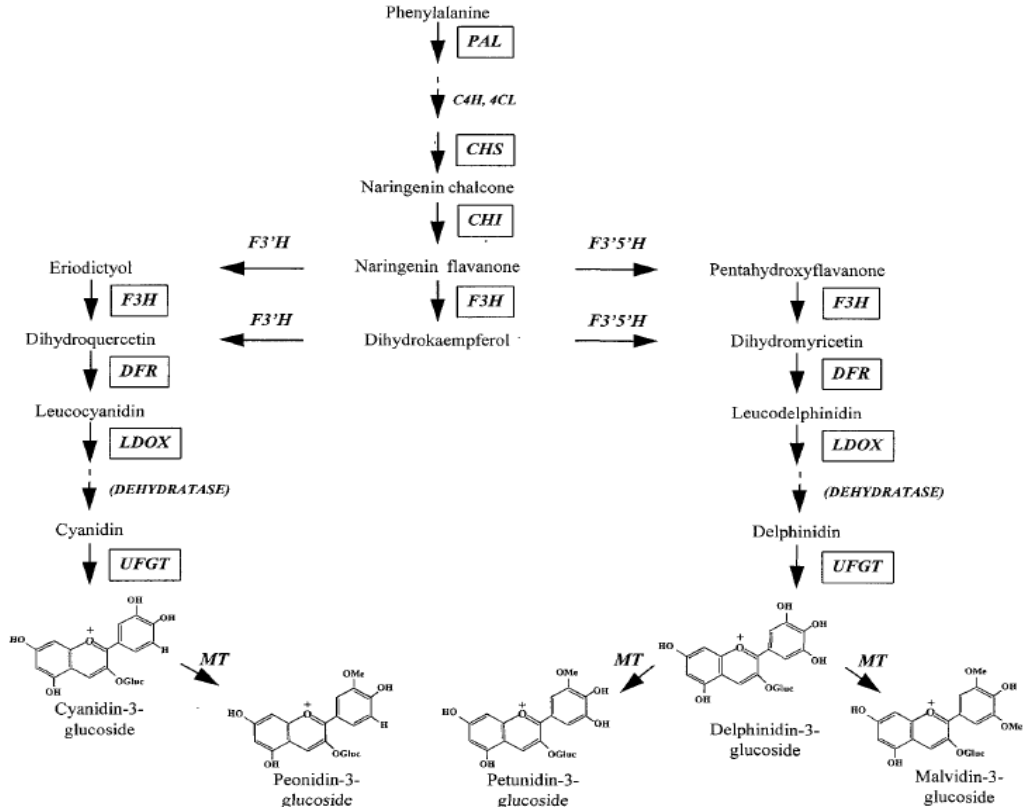

Fig. 7. Anthocyanin biosynthetic pathway (Boss et al., 1996)

1.4.1. Regulatory genes of anthocyanin biosynthesis pathway

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 & Schwinn, 2003) and then full characterized in *Arabidopsis* (Broun, 2005). From these 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. The first experience on MYB genes in grapevine was done on the cultivar Kyoho, which belongs to *Vitis labruscana* (*V. Labrusca x V. Vinifera*). A cDNA library has been constructed with cDNA of ripe berries 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 and VlMYBA2) and two for MYBB (VlMYBB1-1 and 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 and 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. In the grape varieties that have pigmented berry skin, at least one functional VvMYBA1 allele, redVvMYBA1 (rVvMYBA1), and one functional VvMYBA2 allele, redVvMYBA2 (rVvMYBA2) have to exist (Walker et al., 2007).

In nature, the color 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 primery determination of anthocyanin production in berries appeared to be controlled by a single dominant locus (Barrit & Einset, 1969). Among the 68 unigenes identified, a cluster of four VvMYBA genes (VvMYBA1, VvMYBA2, VvMYBA3, and VvMYBA4) was considered essential to determine the genetic bases of the quantitative variation of anthocyanin content in berry skin. 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.

2. Global Warming

Since the beginning of the pre-industrial era around 1750, the atmospheric concentration of greenhouse gases has increased by about 30% because of the combustion of fossil fuels and changes in land-use practices. Increased atmospheric concentrations of greenhouse gases tend to warm the atmosphere (Watson et al., 1990). The earth's climate, which has been relatively stable during the past 10,000 years, is now changing (Fig. 8). The earth's surface temperature in this century is warmer than any other centuries during the 600 years; and the last few decades have been the hottest in this century (Mann et al., 1998).

It is clear that global warming will adversely affect human health, ecological systems, and socio-economic sectors including human settlements and agriculture (Movahed & Maeiyat, 2009) and

particularly viticulture. Climate is a pervasive factor in the viability of all forms of agriculture and is never more important than with the cultivation of grapes for the production of wine (Jones et al., 2005). Global warming and viticulture are interrelated processes, both of which take place on a global scale.

3. Global warming and viticulture

In the last decades, the majority of the world's highest quality wine-producing regions have experienced growing season warming trends. In these regions, future global warming would exceed a threshold, as a result of which the balanced fruit ripening required for existing varieties and wine styles would become progressively more difficult. While improved winemaking knowledge and husbandry practices contributed to the better vintages, it was shown that climate had and will likely always have a significant role in quality variations (Jones et al., 2005).

In northern Europe, climate change may produce positive effects on viticulture through introduction of new varieties (Fig.9), higher production and expansion of suitable areas for cultivation. Nonetheless, the disadvantages may be an increase in the need for plant protection, the risk of nutrient leaching and the turnover of soil organic matters (Lough et al., 1983; Jones et al., 2005). However, in southern areas the disadvantages will predominate. The possible increase in water shortage and extreme weather events may cause lower harvestable yields, higher yield variability and a reduction in suitable areas. These effects may reinforce the current trends of intensification of viticulture in Northern and Western Europe and extensification in the Mediterranean and Southeastern parts of Europe (Lough et al., 1983; Jones et al., 2005). In general, global warming is projected to have significant impacts on conditions affecting viticulture including temperature, carbon dioxide, precipitation, increasing demand for irrigation to meet higher evaporation, increasing the incidence of pests, and through direct temperature effects on production quality and quantity and the interaction of these elements (Fraser, 2008).

3.1. The effects of global warming on berry ripening

The maturation of grapevine berries is strongly influenced by the microclimate around the fruit. Global atmospheric warming trend can affect physiological functions involved in fruit maturation (Jones et al., 2005; Mori et al., 2007). Acidity, anthocyanins and flavonols concentrations in berries are clearly linked to berry temperature (Haselgrove et al., 2000; Bergqvist et al., 2001; Spayd et al., 2002; Downey et al., 2004); while sugar content is strongly influenced by berry transpiration (Rebucci et al., 1997). Daily average temperature at the time of ripening is used to estimate potential

temperature impacts on quality. 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, the optimum temperature for berry growth and ripening is among 20°-25°C (Dokoozlian, 2000).

The Huglin index enables different viticulture regions of the world to be classified in terms of the sum of temperatures required for vine development and grape ripening, (Huglin, 1978). Specifically, it is the sum of mean and maximum temperatures above +10°C (the thermal threshold for vine development). Different grape varieties are thus classified according to their minimal thermal requirement for grape ripening. For example, the HI is 1600 for Riesling and 1900 for Cabernet Sauvignon (Shultz, 2000), (Fig.9). As a result of global warming, the Huglin index is increasing (Shultz, 2000), (Fig.8) so that the average ripening occurs about 1–2 months earlier, that leads to degraded fruit and wine quality (Lough et al., 1983; Kenny & Harrison, 1992; Butterfield et al., 2000; Bindi et al., 1996; Jones et al., 2005).

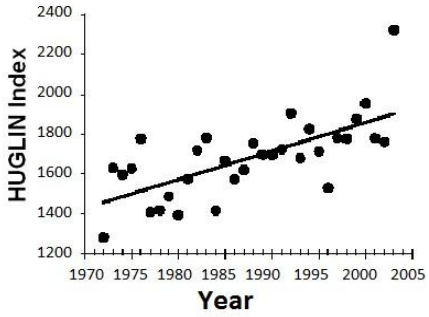

Fig. 8. Huglin index from 1972 to 2005 (Shultz, 2000).

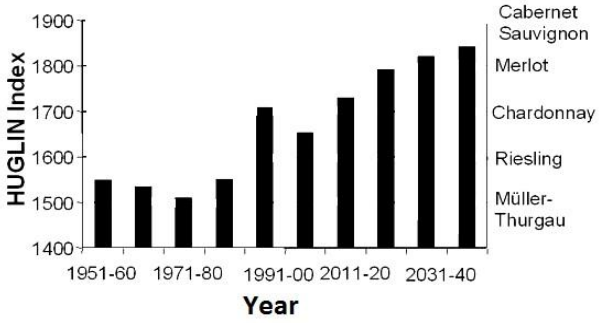

Fig. 9. Huglin index for some grape varieties (Shultz, 2000)

In general, 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 pathways stop or are significantly reduced (Shaked-Sachray et al., 2002; Vaknin et al., 2005). Biosynthesis of flavonoids (Fig. 7) is affected by many endogenous and exogenous factors (Jones et al., 2005). Among these factors, site's microclimate seems to have the greatest influences on flavonoid content (Downey et al., 2006). For instance, changes in solar radiation or temperature rise will have a direct impact on grape composition and thus flavor via alteration of secondary metabolites such as flavonoids, amino acids and carotenoids (Roby et al., 2004). Acidity, sugar content, anthocyanins and flavonols concentrations in berries are clearly linked to berry temperature (Haselgrove et al., 2000; Bergqvist

et al., 2001; Spayd et al., 2002; Downey et al., 2004; Rebucci et al., 1997). It has been suggested that light exposure, which contribute to higher temperature, could control phenols biosynthesis; It could also shift or modify flavonoids composition (Pereira et al., 2006; Downey et al., 2004; Bucchetti, 2004; Guidoni et al., 2004; Bucchetti et al., 2007; Filippetti, 2009; Spayd et al., 2002; Haselgrove et al., 2000). As to Pastore, (2010), late defoliation in grape (that contributes to the higher light exposure and consequently higher temperature) could decrease titratable acidity. In addition, a tendency to decrease in total anthocyanins, and modifications in flavonoids composition, such as decrease of cyanidin, quercetin and kaempferol, and increase of malvidin level, was observed.

Among the factors that determine grape wine quality and market value, berry skin coloration is one of the most important factors. Grape coloration is attributable to anthocyanin accumulation in the skin; and temperature is an important factor that affects anthocyanin accumulation in grape (Mori et al., 2005b; Mori et al., 2007; Winkler et al., 1962; Jones et al., 2005; Kliewer, 1970; Buttrose et al., 1971; Kliewer and Torres, 1972; Yamen et al., 2006; Spayd et al., 2002). It is reported that high diurnal temperature generally decrease total anthocyanin amount (Yamane et al., 2006; Mori et al., 2007a). This effect seems to be particularly significant after véraison and it can be affirmed that high diurnal temperature generally decreases anthocyanin amount (Winkler et al., 1962; Yamane *et al.*, 2006; Mori *et al.*, 2007a). The results of the stable isotope-labelled tracer experiments, to examine the effect of temperature on the turnover of anthocyanin in the skin, indicated that, the total anthocyanin contents were highest in berries cultured at 25°C and lowest at 35°C and confirmed the loss of anthocyanins in response to high temperature. In the berries cultured at 35°C, the total content of $^{13}C^{-}$ labeled anthocyanin was markedly reduced, while there was no decrease in labelled anthocyanin content in the skin of berries at 15°C and 25°C (Mori et al., 2005).

The results of the study on the effect of temperature on anthocyanin composition has indicated that the amount of all anthocyanins, except malvidin 3-O glucoside, was reduced by high temperature (Romero and Bakkar, 2000; Morais et al., 2002; Mori et al., 2007). In general, methoxylation, glycosylation, and acylation lead to an increase in the thermal stability of anthocyanin (Jackman and Smith, 1996). High temperature increases the degradation rate of anthocyanins; but it has been assumed that this degradation rate depends on the anthocyanin structure (Jackman and Smith, 1996; Sipiora and Gutierrez-Granda, 1998; Mori et al., 2007). It is reasonable that only malvidin derivatives, which are highly methylated anthocyanins, were accumulated and were more stable than other anthocyanins (Mori et al., 2007). The decrease in anthocyanin accumulation under high temperature may result from factors such as anthocyanin degradation as well as the inhibition of mRNA transcription of the anthocyanin biosynthetic genes (Mori et al., 2007). Temperature might

affect not only the synthesis but also the stability; therefore, the decrease in anthocyanin concentration at elevated temperatures might result from both decrease in synthesis and increase in degradation (Shaked-Sachray et al., 2002; Vaknin et al., 2005).

In *Vitis vinifera* berries, the most important pigments are derived from delphinidin, cyanidin, petunidin, peonidin and malvidin (Castellarin et al., 2006). Although the corresponding anthocyanins can be formed by the formation of acetic, caffeic or p-coumaric acid esters, they are usually formed by the addition of a 3-O glucose. It is therefore unsurprising that one of the genes most up-regulated during the anthocyanin accumulation in grape berries is UFGT (Walker et al., 2007). In the flavonoids biosynthesis pathway, anthocyanins are the last products of phenylpropanoid pathway. It has been determined that anthocyanins are synthesized from phenylalanine through an anthocyanin biosynthesis pathway (Fig. 7) regulated by enzyme activities (Hrazdina et al., 1984) and gene expressions (Boss et al., 1996). Some anthocyanin biosynthetic pathway genes, including a large number of isogenes phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), leucoantho-cyanidine dioxygenase (LDOX) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT), etc.- have been cloned (Sparvoli et al., 1994). It has already been established that in many plants the gene expression of the enzymes involved in anthocyanin biosynthesis is affected by temperature. In general, the expression of the anthocyanin biosynthetic genes was induced by low temperature in different specious (Mori et al., 2005b, 2007; Yamane et al., 2006; Christie et al., 1994; Shvarts et al., 1997; Lo Piero et al., 2005; Leyva et al., 1995; Shaked-Sachray et al., 2002 and Dela et al., 2003). Very interesting is also the effect of different night temperature on anthocyanin biosynthesis. Warm night contributes to decrease of anthocyanin content of grape berry skin. The enzymatic activities of PAL and UFGT in the skin of Darkridge berries, grown under high (30/30 °C) night temperature conditions were decreased significantly after veraison (Mori et al., 2005; Mazza and Miniati, 1993; Kliewer and Torres, 1972).

Flavonoids protect plants against various biotic and abiotic stresses. Their oxidation reactions only occur after senescence or an environmental stress. This oxidation plays a role in defending the plant against various biotic and abiotic stresses (Treutter, 2006; Buchanan-Wollaston and Morris, 2000; Munne´-Bosch and Alegre, 2004). Since, grape berries grown under high temperature would receive oxidative stress (Mori et al., 2007), Flavonoids oxidation (eg. Anthocyanin degradation) occurs in order to defend the plant. This reaction in plants is mainly catalyzed by polyphenol oxidases (catechol oxidases and laccases) and peroxidases (Pourcel et al., 2006). It was also reported that among the candidates for anthocyanin degradation enzyme, such as polyphenol oxidases (PPOs) and peroxidases (PODs), peroxidases are involved in active anthocyanin degradation of Brunfelsia

calycina flowers (Vaknin et al., 2005; Welinder et al., 2002). While, polyphenol oxidases increased the rate of anthocyanin degradation in fruit extracts and juices (Kader et al., 1997- 1998- 1999; Sarni et al., 1995). PPOs are not likely candidates for degrading anthocyanins in living tissues because of their location in plastids (Vaknin et al., 2005). Peroxidases in vacuoles have also been found in grape cells and would be involved in anthocyanin degradation in the presence of H2O2 (Calderon et al., 1992). H2O2 levels in plant tissues have been shown to increase in response to heat stress (Dat et al., 1998). Flavonoid-PODs reaction might function as a mechanism for H2O2-scavenging, and therefore plant cell detoxification (Pourcel et al., 2006). Gene chips micro ray analysis showed that grape berries grown under high temperature would receive oxidative stress (since genes encoding peroxidases and some oxidoreduction enzymes were induced (Mori et al., 2007). The indication that oxidative enzymes are involved in this process is the fact that treatment with reducing agents (such as glutathione and DTT), and protein synthesis inhibitors (like cycloheximide and anisomycin), both caused inhibition of anthocyanin degradation (Vaknin et al., 2005). In addition, the effect of temperature on fruit, foliage and flower pigmentation has shown that fading of Aster flowers color due to high temperature could be prevented by stabilizing the pigments (Shaked-Sachray et al., 2002; Nissim-levi et al., 2003; Oren-Shamir and Nissim-levi, 1997a, 1997b, 1999; Vaknin et al., 2005). These findings suggest that anthocyanin degradation plays a crucial role in pigment concentration under low rate of bisynthesis. Peroxidase activity is specific and involved in the pigment degradation (Vaknin et al., 2005). Mechanisms of flavonoid oxidation lead to brown polymers (Pourcel et al., 2006; Walker and Ferrar, 1998; Yoruk, R. and Marshall, 2003; Jiang et al., 2004). The browning of peel of litchi fruits has been correlated with the rapid degradation of red anthocyanin pigments; this process is associated with enzymatic oxidation of phenolics by polyphenol oxidases (PPO) and/or peroxidases (POD) (Jiang et al., 2004). Although the effects of temperature on grape berry composition and particularly on the content of anthocyanins in skins have been studied intensively, (Kliewer, 1970; Buttrose et al., 1971; Kliewer and Torres, 1972; Spayd et al., 2002; Mori et al., 2005b; Yamane et al., 2006), the mechanisms responsible for the poor coloration of berry skin at high temperatures have not been completely understood; and no report has demonstrate the enhancement of anthocyanin degradation due to high temperature in grape tissues. Plant responses to high temperature involve morpho-anatomical, physiological, biochemical and molecular 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 reported, anthocyanin biosynthesis is inhibited by higher temperature and this is confirmed by molecular studies. High temperature decreased mRNA

accumulation of structural anthocyanin biosynthesis genes and MYBA1 in cv. Aki Queen (Yamane et al., 2006). In Pinot Noir vines, instead, the decrease in anthocyanin content due to high temperature treatment cannot be linked to a decrease in anthocyanin biosynthesis, as no significant differences in gene expression were found. These results may contribute to suggest that the reduction in anthocyanin biosynthesis may be also due to enzyme inhibition or anthocyanin degradation rather than a transcriptional effect (Mori et al., 2007).

4. GENERAL AIMS OF THE THESIS

The present study is mainly focused on wine grape quality, with the general aim of evaluating the impacts of high temperature condition on grape quality. From 1950, the majority of the world's highest quality wine-producing regions have experienced warming trends in growing season and this condition is projected to have significant impacts on grape wine production quality and yield. In particular, high temperatures after veraison can negatively affect grape composition expecially skin anthocyanin accumulation in black berry varieties. On these bases the present thesis aims to analyse two trials both on cv. Sangiovese, which is the most cultivated variety in Italy, characterized by peculiar anthocyanins profile.

The first trial is to appraise the effects of high temperature condition on flavonoids (anthocyanins, flavonols and condensed tannins) accumulation and particularly the mechanisms responsible for lower anthocyanin content in berries ripened under elevated temperature, via assessment of the main berry biochemical compounds during ripening and of the activities of the putative key enzymes which are involved in anthocyanins biosynthesis (such as UFGT and PAL) and their degradation like peroxidases and polyphenol oxidases (PODs and PPOs). In addition, the expression of some genes responsible for flavonoids biosynthesis and degradation was assessed via Real time PCR. A further step was developed to recognize the best candidate genes responsible for anthocyanin degradation under such stress condition throught a biotechnological approach of genes transformation from grape to Petunia flower via *Agrobacterium tumefaciens*.

Due to global warming in the last decades and in several warm wine regions a too high level of sugar, and subsequent wine alcohol excess, low acidity, and, in black varieties, unbalanced phenolic ripening with insufficient berry skin colour has been shown at harvest. The aim of the second trial is to evaluate the effects of management techniques, applied in the field on the canopy in post-veraison (i.e. trimming), on limiting the carbohydrate availability for cluster ripening to slow down the accumulation rate of soluble solids in berries and to avoid the “uncoupling” of sugar accumulation versus phenolic synthesis during ripening.

The present work started from the data obtained in a preliminary evaluation in 2009 and, both in 2010 and 2011, the quantity and quality of different flavonoids as anthocyanins, flavonols and tannins were measured during ripening in berries of treated and control vines. One of the main aims of the second trial is to evaluate if the evolution of anthocyanins and skin and seed tannins is conditioned by the rate of sugar accumulation.

Chapter one/ First part

Biochemical and molecular approaches to study the effects of temperature on grape composition in cv. Sangiovese (*Vitis vinifera* L .)

1. INTRODUCTION

In the last decades, the majority of the world's highest quality wine-producing regions have experienced warming trends in growing season and this condition is projected to have significant impacts on grape production quality (Jones at al., 2005). The optimum temperature for berry growth is among 20°-25°C (Dookozlian, 2000). Generally, higher temperatures during grape ripening lead to an increase in the rates of sugar accumulation and organic acid degradation. Flavonol content is reported to be more influenced by UV exposure rather than temperature (Spayd et al., 2002); and little is known about the effects of temperature increase on skin and seed proanthocyanidin especially when applied after veraison.

In black berry varieties, grape coloration is due to anthocyanin accumulation in the skin which could be negatively affected by temperature (Mori et al., 2005 & 2007; Kliewer & Torres, 1972; Tomana et al., 1979). But the mechanism responsible for colour reduction in berry skin at high temperature is not completely clear. While some researches in red table grape have determined that high temperatures suppressed the expression of anthocyanin biosynthetic genes (Yamane et al., 2006), some other studies (in Cabernet Sauvignon) did not confirm this effect as mRNA accumulation of anthocyanin biosynthetic genes and enzyme activity of UDP-Glucose Flavonoid Transferase (UFGT) were not inhibited under high temperature (Mori et al., 2007). Therefore it can be hypothesized that temperature would affect not only the synthesis but also the stability of anthocyanins (Shacked-Sachray et el., 2002; Vaknin et al., 2005; Mori et al., 2005) and chemical or enzymatic degradation of anthocyanins can be an answer to high temperature conditions involving, in the second option, polyphenol oxidase and peroxidase (Mori et al., 2007; Vaknin et al., 2005). Moreover, temperature has been shown to have an effect on anthocyanins composition and, some authors suggested a highest thermal stability for the tri-substituted anthocyanins (particularly malvidin derivatives) due to their chemical structure (Mori et al., 2007, Cohen et al., 2012).

So, it is possible to hypothize different responses to high temperature between distinct grape cultivars according their peculiar anthocyanin composition.

To date, no evidence has demonstrated the effects of elevated temperature on anthocyanin accumulation and composition of cv. Sangiovese, characterized by monoglucoside anthocyanins with high percentages of hydroxylated anthocyanins (mainly cyanidine-3-glucoside).

On these bases, the aim of the present research is to evaluate the effects of high temperatures on flavonoids (anthocyanins, flavonols and condensed tannins) accumulation during ripening of Sangiovese berries. And in particular, the mechanisms responsible for modification of the anthocyanins content in berries ripened under elevated temperature conditions, would be studied via

assessment of the activities of the putative key enzymes which are involved in anthocyanins biosynthesis (such as Phenylalanine ammonium lyase (PAL) and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) and their degradation (like polyphenol oxidases (PPOs) and peroxidases (PODs). In addition, the expression of some genes involved in flavonoids biosynthesis and degradation would be appraised via Real time PCR.

2. MATERIALS AND METHODS

The study was conducted in 2010 and 2012 on respectively five and three years old uniform potted plants of *Vitis vinifera* cv. Sangiovese. The vines, grafted on SO4 rootstocks were grown in pots of 30 liter containing a mixture of sand and soil (1: 1). In both years, before the experiment, the number of shoots was standardized (around 9 shoots in 2010 and 6 in 2012). In addition, in order to have a uniform leaf area in all the vines, the tip of each shoot was removed when 20 main leaves were produced. Before veraison, (27th of July 2010 and 19th of July 2012), 8 and 12 uniform vines were selected in 2010 and 2012 respectively on the bases of size of shoots and the bunch number. The selected vines were assigned to the two treatments: LT (Low Temperature) and HT (High Temperature).

In the LT treatment the vines were placed from pre-veraison to harvest in a plastic tunnel where the minimum temperature was about 17 °C and the mean and maximum air temperatures were maintained with a cooler below 26° and 35°C.

In the HT treatment the air temperature was similar to the ambient as the vines were distribuited in a plastic tunnel which was open in the basal part and covered only the canopies to maintain the light conditions similar to the LT vines. In HT tunnel, during the experimental period of 2010 the minimum temperature was about 18° and the mean air temperature was 26 °C, while in 2012 they were measured to be about 19° and 28 °C respectively. The maximum air temperaure could instead exceed to 43°C and 44°C in 2010 and 2012 sequentially.

Both tunnels were made of polyethylene film (MOP, Bologna, Italy) that did not alter the spectral composition of light; while the percentage of transmission was decreased by a maximum of 12 % within the visible range.

All the vines were daily watered automatically and well supplied with nutrients.

2.1. Temperature monitoring

Berry skin temperature was monitored in 10 selected clusters of the plants in low temperature tunnel (LT) and 10 clusters of the vines in high temperature tunnel (HT) in both years. In each of these two

treatments, temperature data were recorded from one week before veraison of both years until harvest towards 10 T- type thermocouples (RS component, MI, Italy) positioned in the sub-cuticle tissues of berry skin. Each probe was then connected to a CR10X data logger (Campbell Scientific Ltd., Leicestershire, United Kingdom), registering temperature data every 20 minutes and each one hour in 2010 and 2012 sequentially. The air temperature inside the tunnels were also monitored and recorded each hour during the same period by air temperature sensors (TL20, 3M, Milan, Italy).

2.2. Berry sampling

Berry sampling was conducted from 1 week before the veraison to the harvest in both years of the trial. In total 45 berries per each 8 vines and 30 berries per each 12 vines were sampled every 8 and 10 days in 2010 and 2012 respectively. The 180 sampled berries for each of the two treatments were pooled separately and divided into 4 biological replicates of 45 berries each to perform biochemical and molecular analyses. The berries of the first group (15) were weighed and immediately used for °Brix, titratable acidity and pH measurment. All the other sampled berries were immediately frozen in liquid nitrogen and kept in the freezer (-80 °C) until the analyses to evaluate the quality and the quantity of the anthocyanins and flavonols (10 berries), skin and seed tannins (10 berries), and to assess the gene expression and the enzymatic activity (10 berries).

2.3. °Brix, titratable acidity and pH

The sampled berries were crushed and the must were sieved and used 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).

2.4. Analyses of anthocyanins and flavonols

The berries of each replication were sampled and weighted before the extraction of anthocyanins and flavonols. Then the skin was carefully removed from the pulp and immerged in 50 mL of HPLC-grade methanol (Carlo Erba Reagents, Milano, Italy); and were kept in dark for 24 hours. The day after, the extract was separated from the skin residues and conserved at (-20^{o}C) until the anthocyanins analysis.

To analyze the total content of each flavonol aglycon, an aliquot of 5 mL of the same methanol extract was completely dried under vacuum. To achieve the acid hydrolyzation of flavonols glucosides, the pellet was re-suspended 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 ^{o}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 re-suspended in 1mL of methanol and was kept at (-20^{o}C) 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 mmx4mm (5µM), 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 methanol extract was filtered through 0.22 µm, 13mm PTFE syringe-tip filters (Teknokroma, Barcelona, Spain) and an aliquot of 270 µL was diluted to final volume of 1mL 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 ^{o}C and they were quantified at 520 nm with a calibration curve with malvidin-3-glucoside (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 Extrasynthese (Genay, France).

2.5. Tannins analysis

To extract proanthocyanidins, frozen berries samples (skins and seeds) were ground separately to a fine powder under liquid N_2. 0.1 g aliquots were separated and extracted in darkness in 2 mL screw-top Eppendorf tubes for 24 hours with 70% acetone containing 0.1% ascorbate (1.0 mL) to prevent oxidation of polyphenols (Kallithraka et al., 1995). Samples were then centrifuged (15 min, 13,000 x g) and two 400 µL aliquots of the supernatant were transferred to fresh tubes and dried down under vacuum at 35ºC for 120 minutes.

One of them was re-suspended in 100 µL methanol acidified with 1% HCl then neutralized with 100 µL sodium acetate (200 mM, pH 7.5) for the analysis of free monomers. The other aliquot underwent acid-catalyzed cleavage of the proanthocyanidins in the presence of excess phloroglucinol following the method of Kennedy and Jones (2001). Briefly, the dried sample was re-suspended in 100 µL of phloroglucinol buffer (0.25 g ascorbate, 1.25 g phloroglucinol, 215 µL conc. HCl, 25 mL methanol) and incubated at 50ºC for 20 minutes, then neutralized with 100 µL of sodium acetate (200 mM, pH 7.5) and centrifuged (15 min, 13,000 x g). A 200 µL aliquot each of the acetone extract, the acetone extract after acid catalyzed cleavage and the residue after cleavage, was then transferred to Sun-broker 250 µL reduced volume HPLC auto-sampler vials. Samples were run on a Waters 1525 instrument equipped with a diode array detector (DAD), using a reversed-phase column Phenomenex™ (Castel Maggiore, BO, Italy) RP18 250 mmx4mm (5µM), with precolumn. A separate reversed-phase HPLC method was developed for the cleaved and uncleaved proanthocyanidin samples reading absorbance at 280 nm. Uncleaved samples, solvent A, 0.2% phosphoric acid, solvent B, 4:1 acetonitrile:0.2% phosphoric acid (gradient of solvent B: zero min, 0%; 5 min, 10%; 40 min, 10%; 55 min, 17%; 65 min, 19%; 75 min, 19%; 80 min, 100%; 85 min, 100%; 86 min, 0%). Cleaved samples, solvent A, 0.2% acetic acid, solvent B, methanol (gradient of solvent B: zero min, 1%; 40 min, 1%; 120 min 30%; 120.1 min, 100%; 125 min, 100%; 126 min, 1%). For both methods, 25 µL of sample was injected and run at 30ºC with a flow rate of 1 mL/min. Concentrations of free monomers and hydrolysed terminal subunits were determined from standard curves prepared from commercial standards of catechin, epicatechin, epigallocatechin and epicatechin-gallate obtained from Extrasynthese (France). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy & Jones 2001). The evaluation of skin and seeds tannins was performed only on the samples of the year 2010.

2.6. Real-time PCR analyses: RNA extraction and RNA evaluation

Real-time PCR analyses were performed for four and five selected stages of berry ripening, in 2010 and 2012 respectively, from beginning of veraison, to the end of harvest. Total RNA was isolated from 200 mg and 500 mg, in 2010 and 2012 respectively, of the powdered berry skin tissue using spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturers 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). Total RNA was treated with 1 U µg-1 RQ1 DNase (Promega,

Milan, Italy). First-strand cDNA was synthesized using 1 µg of RNA, 1µL $(dT)_{15}$ primer (500 µg/mL) and 1 U of GoScript™ reverse transcriptase (Promega). Quantitative RT-PCR was carried out on a Step One Plus™ (Applied Biosystems, Foster City, California, USA). Each reaction (10 µL) contained 188 nM of each primer, 2µL of diluted cDNA (1:50), 5µL U of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, California, USA). Thermal cycling conditions were 95 °C for 10 min followed by 95 °C for 15 s, 55 °C for 1 min for 40 cycles, followed by a melting cycle which contains 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec. Each cDNA sample was analyzed in triplicate. Primers pairs for Dihydroflavonol 4-reductase (DFR), Leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) were retrieved from literature (Goto-Yamamoto et al., 2002); primers for VvMYBA1 were from Jeong et al., (2004); for Leucocyanidin reductase (LAR) and Anthocyanidin reductase (ANR or BAN) from Bogs et al., (2005) and for Peroxidase (POD6) from Mori et al., (2007). Primers for Flavanone 3-hydroxylase (F3'Hb), Flavonoid-3'5'-hydroxylase (F3'5'Hk and F3'5'Hi), Phenylalanine ammonium lyase (PAL), Flavonol synthase (FLS) and Peroxidases (POD1 to POD5) were newly designed on the original DNA sequences to amplify 150–250 bp gene fragments (Table. 1).

Table. 1: Sequence of primers used for Real time PCR amplification

	Primer Forward	**Primer Reverse**
DFR	5'-GAAACCTGTAGATGGCAGGA-3'	5'-GGCCAAATCAAACTACCAGA-3'
LDOX	5'-AGGCTCTACTCTCCAAATGA-3'	5'-GAAGCTTGAAACACAGACCA-3'
UFGT	5'-AATCTGAGAGCCCTAAGAGA-3'	5'-GGTGGTACAAGCAACAGTTC-3'
MYBA 1	5'-TAGTCACCACTTCAAAAAGG-3'	5'-GAATGTGTTTGGGGTTTATC-3'
LAR	5'-TCTCGACATAAATGATGATGTG-3'	5'-TGCAGTTTCTTTGATTGAGTTC-3'
BAN	5'-CAATACCAGTGTTCCTGAGC-3'	5'-AAACTGAACCCCTCTTTCAC-3'
PAL	5'-ACATACCTAGTGGCACTCTG-3'	5'-TCGCAGAATCTCGATGGATG-3'
F3'Hb	5'-TCCATCACCAAGTCTTCTAC-3'	5'-GCGGAGAAGCTGAACATGGA-3'
F3'5'Hi	5'-CAGACTGCATACGCACTCTG-3'	5'-ATGCCGGATGGAGTTGAGAT-3'
F3'5'Hk	5'-CCAGGCCTAGCTACTATATG-3'	5'-GCATATGGCATGGTGGTAGA-3'
FLS	5'-CTGCACTATCCAGAGATGAG-3'	5'-TTGCCGCTTCAACAACAACA-3'
POD 1	5'-TCTGAGTAAGAGCCCACACACTCT-3'	5'-GAGACCTGAGCAGATAAATACAAAAATAAA-3'
POD 2	5'-AAAGTTGTTTGGCTGTGG-3'	5'-AATCCCTCCAAACAATCA-3'
POD 3	5'-TGCTCTAAATGCCTGAAAAATG-3'	5'-ATCATATAGAAAAGTGACAGCAACCT-3'
POD 4	5'-CAACTTCACACCAACGGGAAA-3'	5'-GCAGCTGAGCAAGAAATGCTT-3'
POD 5	5'-TCGGTGAGGAACTCCGATA-3'	5'-AGCTTCCTTGTCCTCTTCCA-3'
POD 6	5'-AACTTGAGACACAACAGCATAAATAAATC-3'	5'-AGGACCAAATCACAGGATGATAAAG-3'
ACT	5'-ATTCCTCACCATCATCAGCA-3'	5'-GACGAGCCCCTCCTACTAAAACT-3'

2.7. Enzyme assay

2.7. 1. PAL activity assay

The extraction was performed according to the method of Mori et al., (2005) with some modifications. The following procedures for protein extraction were conducted at 4° C. For each replication, 0.2 g of berry skin was ground with a mortar and pestle in liquid nitrogen until a fine powder was obtained.

The skin powder was homogenized with 1.5 mL of a 100 mM Sodium borate buffer (pH 9) containing 0.075 g of PolyVinyl-PolyPyrrolidone (PVPP) and 100 µL of a 200 mM Dithiothreitol. After incubation in ice for 20 minutes, centrifugation was done at (4° C, 15 min, 10,000 x g).

Then 1mL of the supernatant was desalted by passing through a NAP-10 column (Sephadex G-25, Amersham Biotech) equilibrated with 1.5 mL of the same buffer.

For the analysis of PAL activity, the reaction mixture consisted of 0.5 mL of Phenylalanine and 0.5 mL of the enzyme solution. The assay mixture was incubated in 37° C for 60 minutes. The reaction was terminated by adding 0.5 mL of HCl acid (18%). The quantity of the product, namely Trans-cinnamic acid, was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1} at 290 nm. One unit of PAL was expressed as synthesis of 1 mol of Trans-cinnamic acid per minute, and PAL activity was expressed as µkat g-1 protein.

2.7. 2. UFGT activity assay

The extraction was performed according to the method of Mori et al., (2007) with some modifications. The following procedures for protein extraction were conducted at 4 oC. For each replication, (0.2 g) of berry skin was ground with a mortar and pestle in liquid nitrogen until a fine powder was obtained. The skin powder was homogenized with 1.3 mL of a 250 mM TRIS–HCl buffer (pH 7.5) containing 10 mM Polyethylene glycol 3400, 150 µL of 10 mM Na-diethyldithiocarbamate, 15 µL of 200 mM Dithiothreitol, and 2 µL of 2-mercaptoethanol and was incubated for 20 min on ice. After centrifugation of the homogenate at 10,000 x g for 20 min, The supernatant was passed through a NAP-10 column (Sephadex G-25, Amersham Biotech) equilibrated with a 1.5 mM TRIS–HCl buffer (pH 7.5).

The desalted crude extract was used as the enzyme solution in the following enzyme assay, using delphinidin and cyanidin as substrates. The method of Mori et al., (2007) was employed with some modifications for the analysis of UFGT activity. For the first assay, the reaction mixture consisted of 0.2 mL of the previous extraction solution (containing TRIS–HCl buffer (pH 7.5), Polyethylene glycol 3400, Na-diethyldithiocarbamate, Dithiothreitol, and 2-mercaptoethanol), 50 µL of 1mM

Cyanidin, 50 µL of 100mM UDP-glucose, and 0.2 mL of the enzyme solution. The assay mixture was incubated for 5 min at 37° C. The reaction was terminated by adding 150 µL of 5% HCl. The quantity of the product, namely Cyanidin-3-glucoside was measured using its extinction coefficient of 26900 M^{-1} cm^{-1} (at 520 nm / pH: 1).

Therefore, one unit of UFGT was defined as the production of 1 mol of Cyanidin-3-glucoside per second, and UFGT activity was expressed as pkat g-1 protein.

For the second assay, the reaction mixture consisted of 0.2 mL of the same extraction solution (containing TRIS–HCl buffer (pH 7.5), Polyethylene glycol 3400, Na-diethyldithiocarbamate, Dithiothreitol, and 2-mercaptoethanol), 50 µL of 1mM Delphinidin, 50 µL of 100mM UDP-glucose, and 0.2 mL of the enzyme solution. The assay mixture was incubated for 5 min at 37° C. The reaction was terminated by adding 150 µL of 5% HCl. The quantity of the product, namely Delphinidin-3-glucoside was measured using its extinction coefficient of 26000 M-1 cm-1 (at 520 nm / pH: 1).

Therefore, one unit of UFGT was defined as the production of 1 mol of Delphinidin-3-glucoside per second, and UFGT activity was expressed as pkat g-1 protein.

2.7. 3. Guaiacol PODs activity assay

Guaiacol PODs were assayed as described by Ushimaru et al., (1997), using pyrogallol as the electron donor for the reaction. The plant material (skin) was extracted in cold buffer (200 mM sodium phosphate, 5 mM sodium EDTA, 1% PVPP, pH 7.0) and incubated on ice for 30 min. After centrifugation at (10,000 × g, 30 min, 4 °C), the desalted supernatant was used for the assay. The reaction mixture (2.5 mL) included 2.2 mL of the 10 mM sodium phosphate buffer, pH 7.0, 100 µL of the 2.5 mM H_2O_2, 50 mM pyrogallol, and 100 µl extract; H_2O_2 and pyrogallol were prepared fresh just before use. Absorbance (λ=430 nm) was taken after 5 min incubation at room temperature, and referred to a blank with no extract added. One unit of Guaiacol peroxidases is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of pyrogallol per minute, under the conditions described by Chen and Asada (1989) and PODs activity was expressed as kat g-1 skin fresh weight. An absorbance coefficient of 2.47 mM-1 cm-1 was assumed for calculations.

2.7. 4. PPOs activity assay

PPOs were assayed as described by Ushimaru et al., (1997), using pyrogallol as the electron donor for the reaction. The plant material (skin) was extracted in cold buffer (200 mM sodium phosphate, 5 mM sodium EDTA, 1% PVPP, pH 7.0) and incubated on ice for 30 min. After centrifugation at

(10,000 × g, 30 min, 4 °C), the desalted supernatant was used for the assay. The reaction mixture included 2.8 mL of the 100 mM sodium phosphate buffer, pH 7.0, 1 mM pyrogallol, and 100 µl extract; the pyrogallol was prepared fresh just before use. Absorbance (λ=430 nm) was taken after 5 min incubation at room temperature, and referred to a blank with no extract added. One unit of Polyphenol oxidases is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of pyrogallol per minute, under the conditions described by Chen and Asada (1989) and PPOs activity was expressed as kat g-1 skin fresh weight. An absorbance coefficient of 2.47 mM-1 cm-1 was assumed for calculations.

2.8. Vegetative and Yield measurements

In both years, immediately after harvest, all the leaves of each vine of the both tunnels were removed and their areas were measured using a leaf area meter (LI3000, LiCor Inc. Lincoln, Neb., USA) along with the number of nodes of each single shoots.

The grape bunches were harvested when the total soluble solids of berries had reached to approximately 22 ^{o}brix. All bunches were removed, counted and weighed in both treatments to determine the yield of each vine.

2.9. Statistical analyses

Data were submitted to analysis of variance and means were separated by the Student-Newman-Keuls test by SAS statistical software (SAS Institute, Cary, North Carolina, USA). The experiments were conducted with four replications from each treatment and the values were the means of four replications.

3. RESULTS AND DISCUSSION

3.1. Berry Temperature

The berry temperature in both tunnels was influenced by the two air temperature regimes (HT and LT). The average berry temperature in the LT tunnel during the ripening period of 2010 was recorded to be about 20 ^{o}C while it was about 24° C in HT. In 2012 the average berry temperature in both treatments were recorded to be about 2° higher than in 2010.

The maximum berry temperature in both years showed the highest differences between the two treatments as it was maintained below 35 °C in LT and it might exceed to 42 °C in correspondence of several heat weaves during the ripening in HT (Fig.10).

The analyses of the number of hours exceeding the 30 and 35°C showed significant higher values in both years in HT than in LT (Table 2). In particular, the number of hours over 35° C was null in LT berries while it was about 70 and 157 hours in HT berries in 2010 and 2012 respectively. Moreover, despite between the two years only few differences were found in mean and maximum berry temperatures, 2012 berries spent more hours at high temperature than in 2010 regardless from the different treatments and the temperature fluctuations were higher during the ripening period of 2010 (Table 2). In addition, in 2010, the percentage of the average berry temperature more than 30 °C was about four folds more in the HT tunnels as compared to LT one; while in 2012 this percentage was six folds higher.

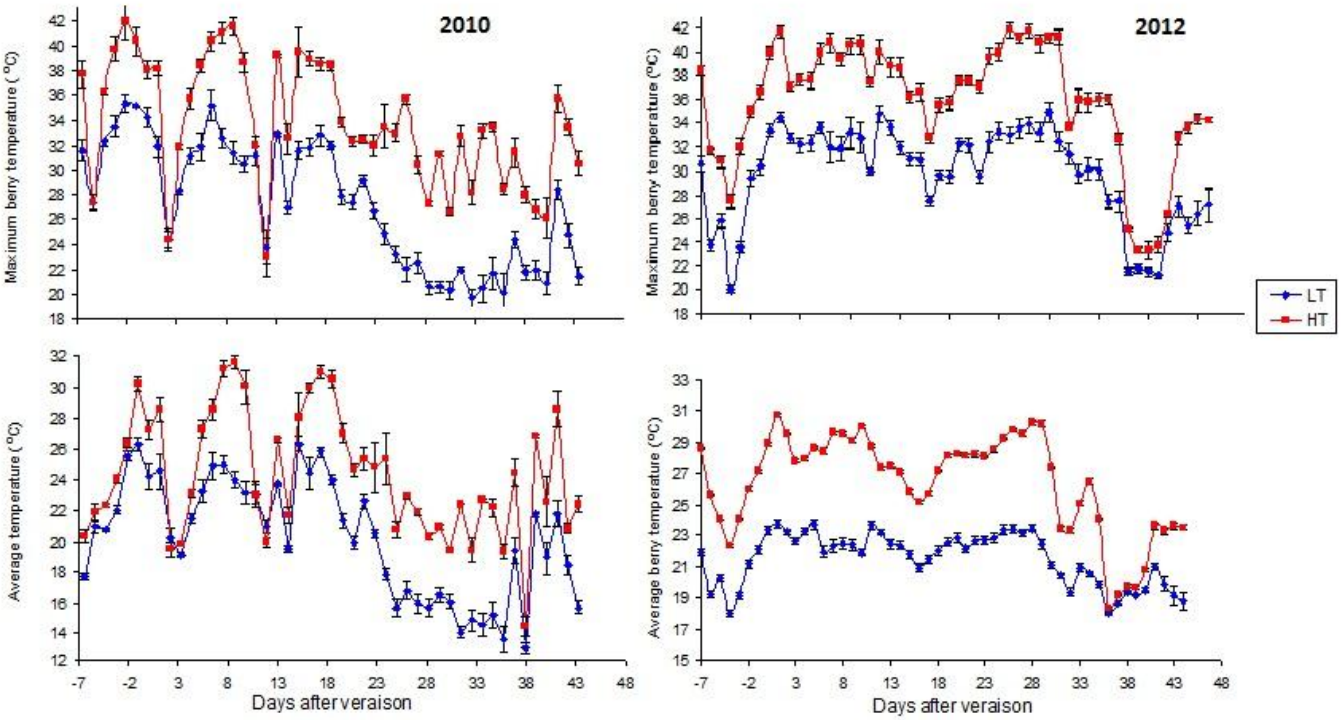

Fig. 10. The average and maximum daily berry temperature (^{o}C) grown under high and low temperature conditions during ripening in 2010 and 2012.

Table. 2: The percentage and the number of hours that the average of berry temperature was higher than 30° and 35°C in both treatments during the experimental period in the years 2010 and 2012.

Year	Treatment	Average berry temperature > 30°C (%)	Average berry temperature > 35 °C (%)	Average berry temperature > 30 °C (N° hours)	Average berry temperature> 35 °C (N° hours)	Total experimental time (N° hours)
2010	**HT**	16.3%	6 %	192 hr	70hr	1176 hr
	LT	4.7 %	0 %	55 hr	0	
2012	**HT**	31%	12.50%	392 hr	157 hr	1257 hr
	LT	7%	0%	91 hr	0	

3.2. Leaf area and Yield

The plant materials used in this trial were not the same in the two years. In 2010 and 2012, respectively, five years old and three years old uniform potted vines were used for the treatments. Therefore, the comparison could be done only between the treatments in each year; and the year effect would not be considered.

In both years before the experiment, the vines were uniformed for the number of shoots and clusters in both treatments. But the main, lateral and total leaf areas per vine were significantly higher in LT vines respect to HT (Table 3).

In 2010, the yield and its components were not significantly different between the LT and the HT vines (Table 4), while in 2012 the yield was 36% more in LT as compared to HT ones. Since the cluster number per vine and the berry weight were similar in this year, this result is mainly due to a higher number of berries per cluster which imply that the cluster weight was higher in LT vines (Table 4).

It seems that the higher temperature in HT may be responsible for drying out the basal leaves in HT at the end of ripening and these conditions, which have been assessed also by visual observation, caused a lower average leaf area compared to LT, as the leaf area estimation was assessed after harvest. Besides, the warmer condition of 2012 has been contributed to drop of some berries due to the dehydration and drought, in the very last period of ripening in HT. Based on these considerations, it is likely that HT thermal condition did not show negative physiological effect on vegetative growth and yield, as the treatment was imposed at veraison, and we registered a "secondary" effect due to the leaves and berry drop in the very last part of ripening.

Table 3. *Leaf area of grapes grown under high (HT) and low (LT) temperature conditions during ripening of the years 2010 and 2012.*

Year	Treatments	Main Leaf area (cm^2/vine)	Lateral Leaf area (cm^2/vine)	Total leaf area (cm^2/vine)
2010	**LT**	11742.5	10578.5	22321
	HT	7168.2	8062.9	15231.1
		*	*	*
2012	**LT**	13460	5690	19150
	HT	6715	3742	10457
		*	*	*

Table 4. *Yield and yield components of grapes grown under high (HT) and low (LT) temperature conditions during ripening of the years 2010 and 2012.*

Year	Treatments	N° Cluster/ Vine	Yield/ Vine (gr)	Cluster weight (gr)	Berry weight (gr)	Berries per cluster (n)	LA / Yield per vine (cm^2/ gr)
2010	**LT**	11	1666.6	151.5	2.4	63	13.4
	HT	12	1824.5	152.0	2.1	72	8.35
		ns	ns	ns	ns	ns	*
2012	**LT**	6	861.6	143.6	2.2	65	22.2
	HT	5	535.5	107.1	2.1	51	19.6
		ns	*	*	ns	*	ns

3.3. ^{o}Brix, pH, Titratable acidity

The results indicated that in 2010 a similar trend in the percentage of Total Soluble Solids in berries grown under low and high temperature was measured, and no significant differences was observed among the two treatments in titratable acidity (TA) and pH. However, in 2012 the advanced ripening in HT grapes was associated with higher level of ^{o}Brix during ripening; nevertheless, they reached the same level at harvest. Moreover, it seems that the elevated temperature contributed to degradation of titratable acids and increase in pH (Fig. 11). There are contradictory reports on the effects of high temperature on sugar accumulation in grape during different stages of berry development. Several papers (Matsui et al., 1986; Sepúlveda and Kliewer, 1986; Kliewer et al., 1977) reported a general delay in ripening which is attributed to the impact of heat on the photosynthetic supply of sugar. Photosynthesis of grapevines is reduced at temperatures above 35 ^{o}C (Kriedemann 1968; Ferrini et al., 1995; Schultz 2003; Yu et al., 2009; Zsófi et al., 2009) and generally the restrictions to photosynthesis are caused by stomatal limitation. As to Sepúlveda *et al*. (1986), prevention of the soluble sugar accumulation in berries by high temperature cannot be accounted for an inhibition of the phloem transport between the leaves and the bunchs. In contrary, Soar et al., (2009) reported an increase in photosynthesis and stomatal conductance when Shiraz vines were heated for short periods. Moreover, in some other researches sugar accumulation was not influenced by temperature increase (Yamane et al., 2006, Greer and Weston, 2010).

In general, 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. Metabolic changes occurring during grape berry development (Taureilles-Saurel et al., 1995), together with light and temperature, are the main parameters controlling the malate synthesis/degradation rate. 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 (Kliever et al., 1967). These results are in accordance with the results of this study and what was observed on the effects of elevated temperature in Cabernet Franc and Chardonnay grapes where the pH increased and TA decreased. Also the effects of higher temperature induced by veraison defoliation had similar effects on pH and titratable acidity due to the malic acid degradation (Guidoni et al., 2004; Pastore, 2011).

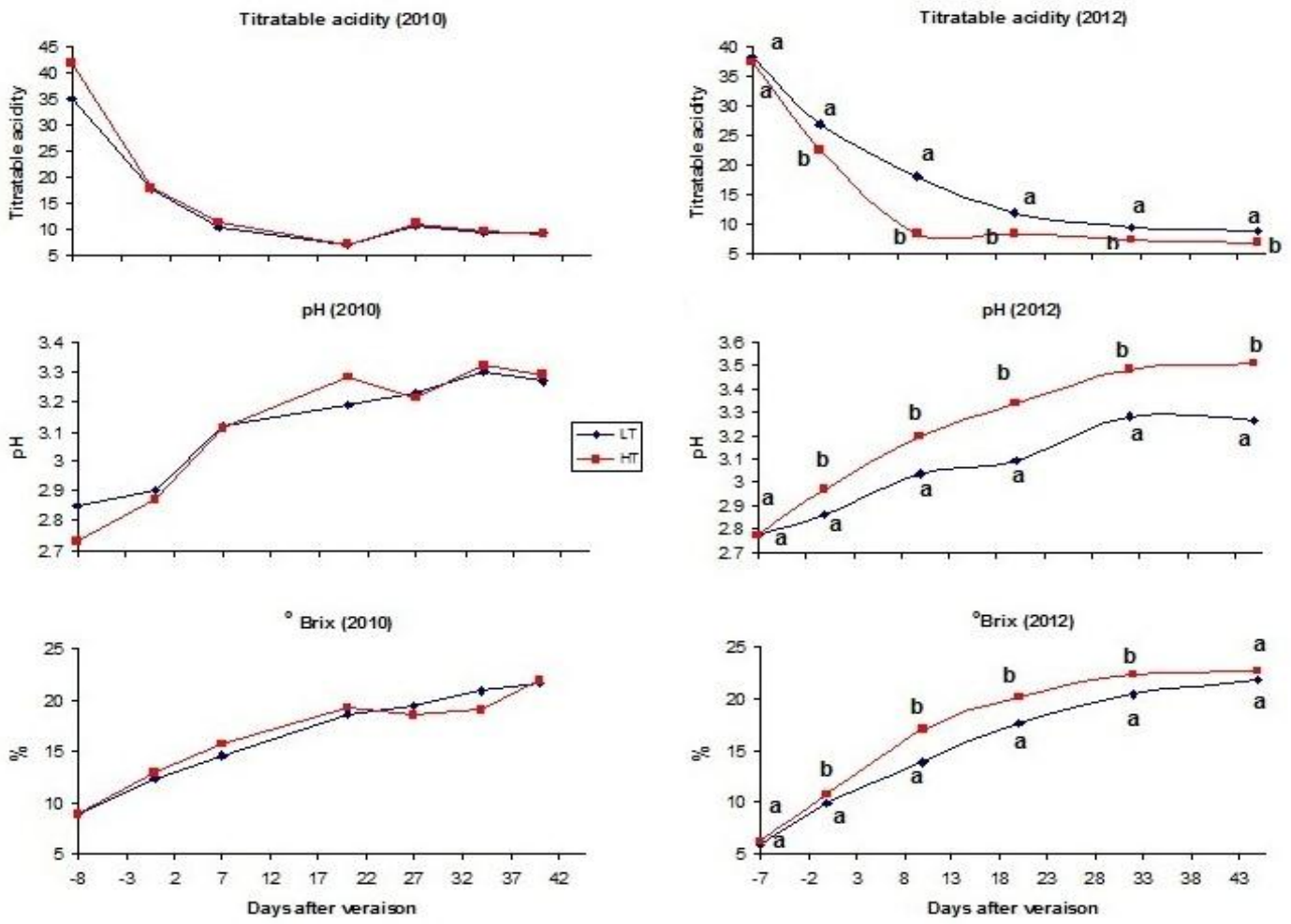

Fig. 11. The trend of °Brix, titratable acidity and pH of grapes grown under high (HT) and low (LT) temperature conditions during ripening of the years 2010 and 2012.

3.4. Biosynthesis of flavonoids

3.4. 1. Anthocyanins accumulation during ripening

In both years, the anthocyanin level in the skin of Sangiovese (Fig. 12) started to increase after veraison until harvest in both treatments. Nonetheless, in HT berries was significantly reduced respect to LT ones. As an illustration, in 2010 the level of total anthocyanin at harvest was decreased about 65% in the skin of the grapes grown under high temperature conditions, as compared to LT, and at the harvest time of the year 2012 this reduction was 50 %. In agreement with the results on other grape varieties (Darkridge grape, Cabernet Sauvignon, Tokay, Japanese table grape Kyoho, Mori et al., 2005 & 2007; Kliewer & Torres, 1972; Tomana et al., 1979), we found that in

Sangiovese, high berry temperature (over 35°C) after veraison prevented anthocyanin formation in grapes and greatly reduced the coloration as compared to fruits ripened at lower temperatures.
According to HPLC analysis of the both years, as expected, the major individual anthocyanin in the berry skin of both LT and HT treatments was malvidin-3-glucoside (Fig. 12).

Anyway, no differences in anthocyanin profiles were recorded at harvest between HT and LT grapes in 2010 (Table 5). In 2012 at harvest the percentage of malvidin -3-G was higher in LT compared to HT (Table 5).

Previous studies on Cabernet Sauvignon reported the higher thermal stability of malvidin-derivatives anthocyanins due to the high level of methoxylation and acylation (Mori et al., 2007; Romero and Bakker, 2000; Morais et al., 2002); but it is in contrast to our findings in this respect on Sangiovese. It has to be noticed that in Sangiovese, the anthocyanin composition is quite different from Cabernet Sauvignon and it is characterized by the high level of cyanidin-3G and by the absence of p-coumarated and acetated malvidin derivatives anthocyanins.

From the data of our two-year trials it is not possible to confirm the differences in thermal stability of the five monoglucoside anthocyanins of Sangiovese based on their chemical structure. And by assessing solely the anthocyanins quantity and profile**,** it was not possible to understand if the reduction of anthocyanins under high temperature is due to biosynthetic activity reduction or to a mechanism of anthocyanins degradation. Therefore, additional analyses were required in order to obtain more comprehensive information about this issue.

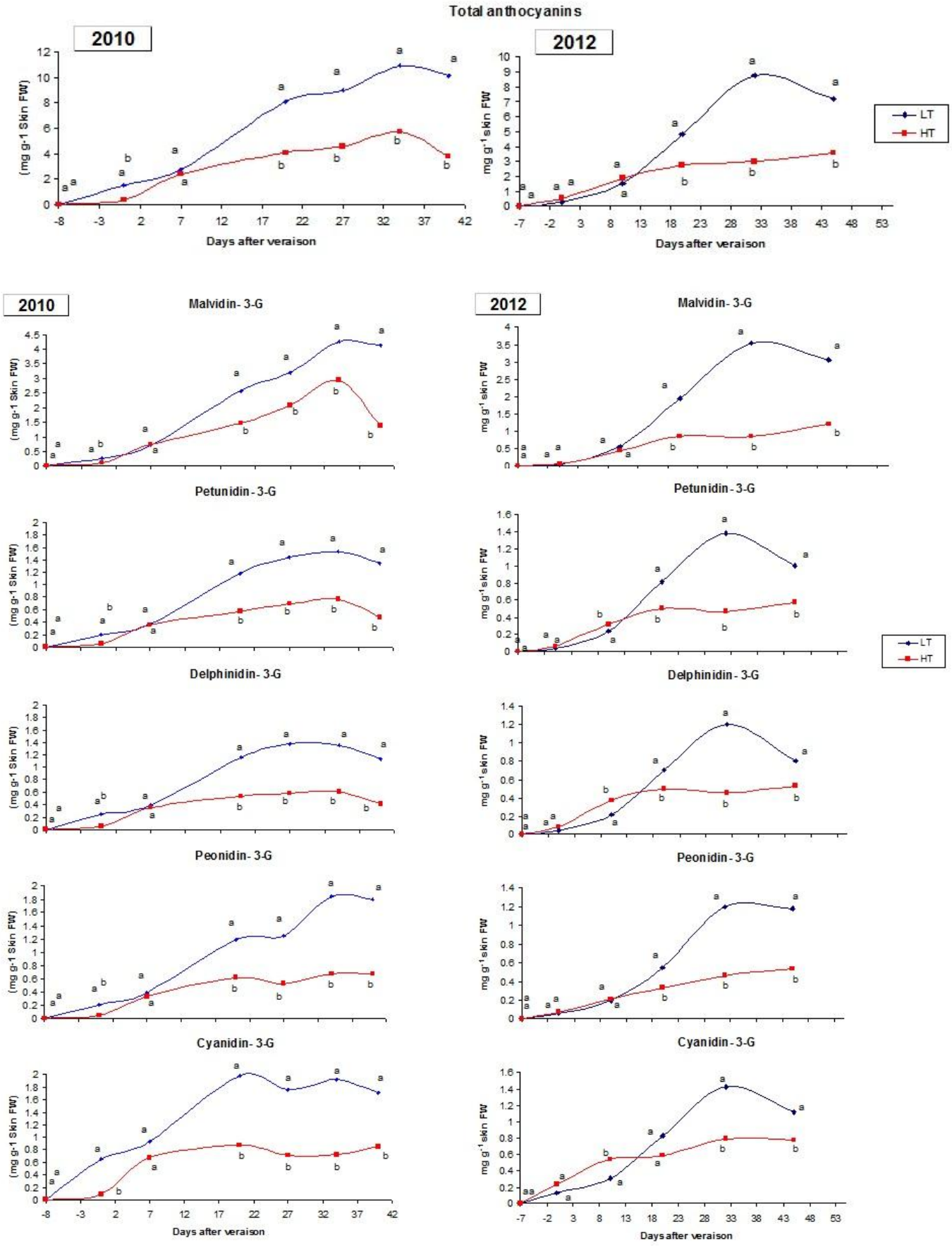

Fig. 12. The trends of total and individual anthocyanins concentrations in the skin of Sangiovese berries grown under high (HT) and low(LT) temperature conditions during ripening in the years 2010 and 2012.

Table 5. The anthocyanin profiles at harvest in the skin of Sangiovese berries grown under high (HT) and low (LT) temperature conditions during ripening in the years 2010 and 2012.

		Anthocyanin profile at harvest				
		Malvidin-3-G	**Petunidin-3-G**	**Delphinidin-3-G**	**Peonidin-3-G**	**Cyanidin-3-G**
2010	**LT**	36%	13%	11%	18%	22%
	HT	41%	13%	11%	18%	17%
		ns	**ns**	**ns**	**ns**	**ns**
2012	**LT**	43%	14%	11%	16%	16%
	HT	33%	16%	14%	16%	21%
		*****	**ns**	**ns**	**ns**	**ns**

3.4. 2. Flavonols accumulation during ripening

In the both years, the levels of flavonols in HT and LT berries increased gradually after veraison, and significant decrease was observed in the level of total and individual flavonols in HT berries as compared to LT ones in 2012 (Fig. 13).

In general, the highest level of flavonols in grapes was found at flowering, followed by a decrease as the grapes increased in size. Subsequently, a significant level of flavonol biosynthesis is in general observed during berry development and the greatest increase in flavonols per berry can be detected 3-4 weeks post-veraison (Downey, et al., 2003).

Flavonols accumulation in wine grape tissues has been studied extensively. While several authors reported a solid correlation between fruit light exposure and flavonol glucosides content (Price et al. 1995, Haselgrove et al., 2000, Spayd et al., 2002), the effect of temperature on flavonol synthesis is not understood completely. Spayd et al., (2002) and Mori et al., (2005), did not observe any effect of temperature treatment on flavonol synthesis. To the contrary, the inhibitory effect of high temperatures on flavonol was also reported by Goto-Yamamoto et al., (2009). Since in our research we excluded the effect of different light exposure between the two treatments, the lower accumulation of flavonols in HT berries is probably due to a regulatory mechanism which rules the anthocyanins response to high temperature condition.

Moreover the analyses of each flavonol compound at harvest (quercetin, kaempferol and myricetin showed, as expected, that quercetin has the highest percentage. The flavonols profiles at harvest in the skin of Sangiovese berries grown under high and low temperature conditions during ripening were similar in the year 2010 but it was significantly different among the treatments in 2012 (Table 6).

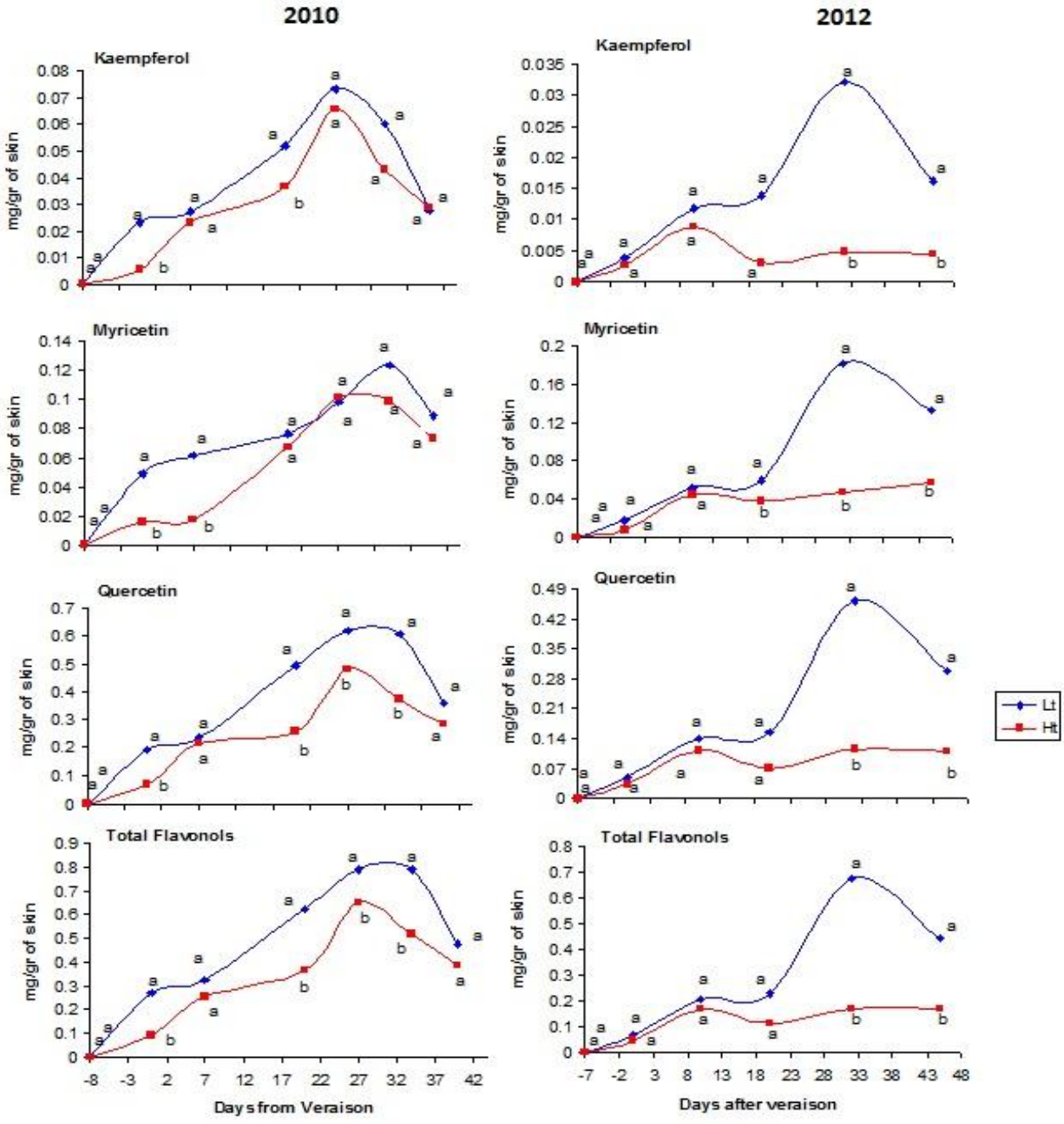

Fig. 13. The trends of total and individual flavonols concentrations in the skin of Sangiovese berries grown under high (HT) and low (LT) temperature conditions during ripening in the years 2010 and 2012.

Table 6. The flavonols profiles at harvest in the skin of Sangiovese berries grown under high (HT) and low (LT) temperature conditions during ripening in the years 2010 and 2012.

		Flavonols profile at harvest		
		Quercetin	**Kaempferol**	**Myricetin**
2010	**LT**	74.6%	6.8%	18.6%
	HT	73.5%	7.5%	18.9%
		ns	**ns**	**ns**
2012	**LT**	66.8%	3.6%	29.6%
	HT	64.2%	2.6%	33.2%
		*****	*****	*****

3.4. 3. Tannins accumulation during ripening

Tannins assessment was done in 2010. In this trial, regardless from the treatments, the amount of total and individual tannins of seed and skin decreased generally from veraison to harvest. This result is in accordance with the previous works conducted by Harbertson et al., (2002), Adams, (2006) and Kennedy et al., (2001). From this research emerges that the two temperature regimes did not affect the tannins branch of flavonoid biosynthesis. This is comprehensible considering that tannins are accumulated very early in berry development and the decline in their concentration, from veraison to harvest, only reflects the quantitative changes that take place during berry ripening (decrease of the tannins concentration in proportion to berry growth).

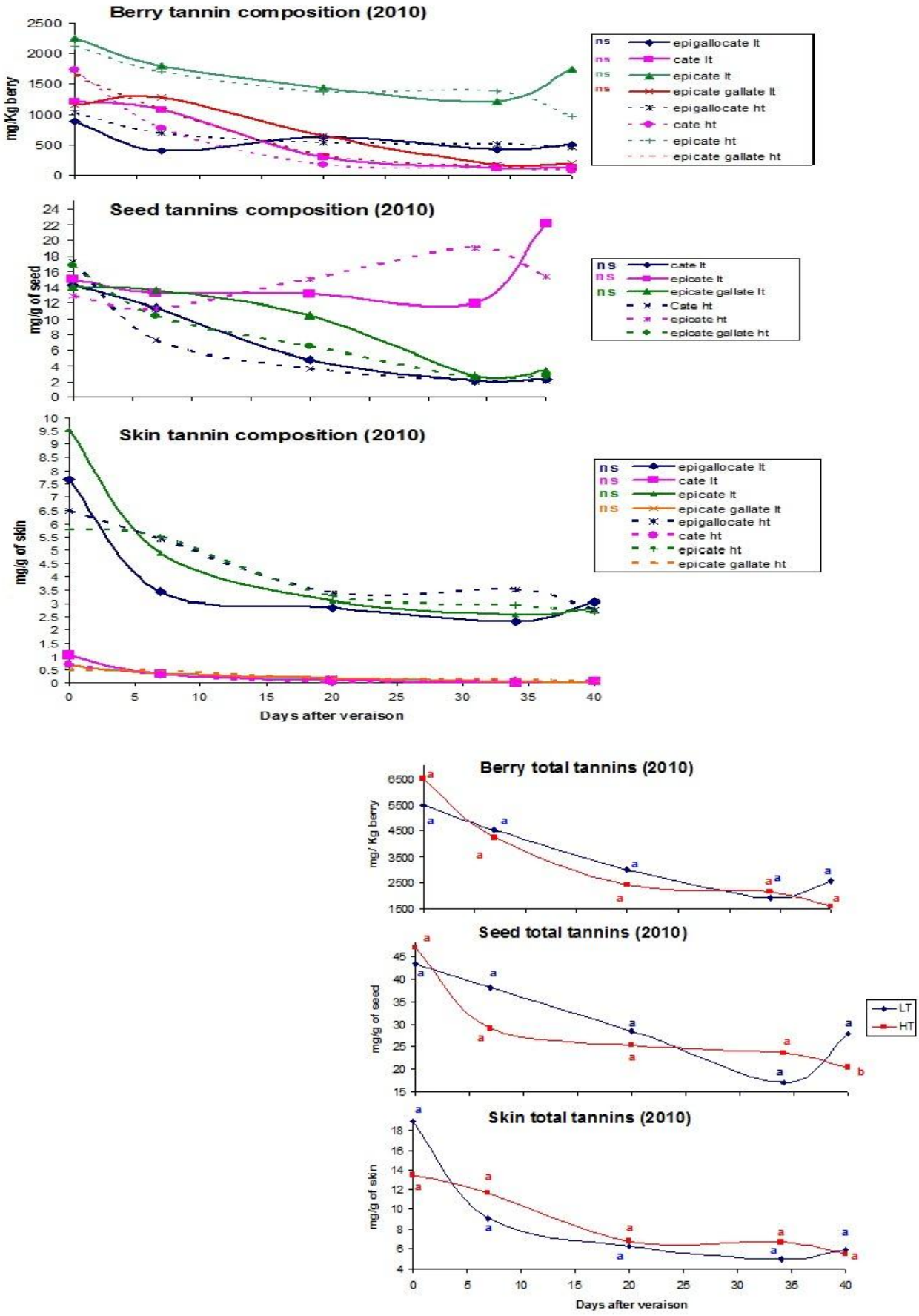

Fig. 14. The trends of total and individual tannins concentrations in the berry, seed and the skin of Sangiovese berries grown under high (HT) and low (LT) temperature conditions during ripening in 2010.

3.5. Gene expression assessment

In the present study, the expression of some genes involved in the general flavonoid biosynthetic pathway (PAL, F3'Hb, F3'5'Hk, F3'5'Hi, DFR, LDOX) and the specific genes for anthocyanins (UFGT and VvMYBA1), flavonols (FLS) and tannins biosynthesis (LAR, BAN) were studied in berries grown under two different temperature regimes (Fig. 15).

In HT berries a strong reduction of anthocyanins and flavonols concentration and no effect on tannin biosynthesis was observed and this seems to be directly related to the general inhibition of the expression of flavonoids biosynthetic genes detected in this research (Fig. 15). Furthermore, it was interesting to observe that, even if the response in terms of total flavonoid accumulation was comparable in 2010 and 2012, the flavonoid composition and the expression profile of some of the analyzed genes was quite different between the two years of the experiment (Fig. 15, Fig. 16, Table 5 and 6).

The profile of PAL gene expression was mainly similar in the both years, starting to increase in the both treatments about 15 days after veraison. PAL gene seemed to be repressed by high temperature as a considerable higher level of expression in LT berries in respect to the HT ones was recorded in both years about 37 days after veraison, even if in 2010 it was not significantly different between the two treatments. This result is in agreement with many papers in which a direct correlation between enhanced flavonoid accumulation (in particular anthocyanins) and increase in PAL gene expression is reported (Pastore et al., 2011; Ageorges et al., 2006, Ban et al., 2003, Roubelakis-Angelakis and Kliewer, 1986).

F3'5'Hs and F3'Hs are versatile enzymes that are able to accept several phenylpropanoid substrates and competitively control the synthesis of cyanidin- and delphinidin-flavonoid derivatives. Two isoforms of F3'Hs have been detected in grapevine genome until now (Castellarin et al., 2006). In this research the transcription of F3'Hb showed different profile of expression between the two years of the trial. In fact, the transcription level of F3'Hb was not affected or even induced by high temperature 20 days after veraison in 2010, but we found exactly an opposite result in 2012, where in LT berries a significant increase in F3'Hb expression was recorded 33 days after veraison in comparison to HT.

Studying the expression of F3'5'Hi and F3'5'Hk genes we observed that they were affected by high temperature during ripening in both years, showing similar transcription profiles. Starting from full veraison, when all berries have turned red, F3'5'Hi expression increased in LT berries reaching a maximum from one to two weeks after veraison in both 2010 and 2012. Also F3'5'Hk expression showed an increase in LT berries about 7 days after veraison in 2010 in comparison to HT and a strong enhancement on F3'5'Hk expression was observed in LT berries in 2012 (Fig. 15). Sixteen

copies of F3’5’Hs are present in the grapevine genome (Falginella et al., 2010) suggesting a possible role for each of them in different developmental and environmental conditions.

The different patterns of expression observed for F3’Hb and F3’5’Hs could suggest that the former may be less sensitive, in comparison to the latter, to critical temperature conditions. Considering that the berry temperature in HT tunnel was higher in 2012 than 2010 (Table 2), it seems that F3’Hb is able to tolerate thermal stress when temperature higher than 35°C are imposed for short periods, as in 2010, whereas in 2012 when HT berries spent more than two-fold hours at temperature higher than 35°C in comparison to 2010, the F3’Hb expression is repressed. From this study emerges that, at transcriptomic level, the intensity and the extent of thermal stress could not affect in equal manner the transcription of the gene that preside over the 3’4’- and 3’4’5’-OH flavonoid biosynthetic pathway in Sangiovese grape berry.

In this research, DFR and LDOX genes exhibited different patterns of expression in 2010 and in 2012, reaching maximum values in LT compared to HT 5 days after veraison in 2010 and 28 days later in 2012, thus showed an expression pattern independent from the trend of anthocyanins, flavonols and tannins accumulation and from the timing of the temperature increase application.

Our results are in agreement with previous findings reported that the expression of neither DFR nor LDOX was as strongly correlated as UFGT and VvMYBA1 with anthocyanin content (Castellarin et al., 2007) and it is comprehensible if we consider that they can produce substrates for other branches of flavonoid biosynthesis.

Instead of that, a strong correlation was found, as expected, in the expression of UFGT and of its main transcription factor VvMYBA1, with the higher anthocyanins accumulation in LT vines comparing to HT.

Flavonol synthesis involves several genes encoding flavonol synthases (FLS) which are biosynthetic enzymes converting dihydroflavonols to flavonol (Downey et al., 2003). In both years, the expressions of FLS gene were similar between LT and HT berries in the early stages, suggesting a small effect of high temperatures on the transcription of this gene during veraison. However, FLS started to be significantly higher in LT berries from mid of ripening and in 2012 a similar trend was registered excepted at harvest when a significantly higher expression of FLS was registered in HT (Fig. 16).

Among the enzyme involved in tannins synthesis, leucoanthocyanidin reductase (LAR) produces catechin, while anthocyanin reductase (BAN) converts cyanidin and delphinidin into epicatechin and epigallocatechin respectively (Xie et al., 2003 and Adams, 2006, Fig. 17). Bogs et al., (2005) found that in grape LAR is encoded by two closely related genes (LAR), whereas BAN enzyme is

encoded by a single gene (Fig.17). As LAR1 appeared to be seed specific (Bogs et al., 2005), only LAR2 and BAN were analyzed in this study in 2010. In general, timing and expression of BAN and LAR2 genes is consistent with the accumulation of proanthocyanidins in the berry (Adams, 2006). This was further confirmed in this research, where tannins analyses conducted on berry skin in 2010, showed no differences in terms of concentration and composition and, as expected, no differences were found in the transcription of LAR2 and BAN during ripening.

To better understand how the differences in gene expression could have or not influenced the anthocyanins and flavonols composition, also the timing and the coordination of flavonoid biosynthetic genes has to be considered separately for the two years.

In 2010, we detected a lack of variation in anthocyanins, flavonols and tannins composition following LT and HT treatment. Following the treatment, a coordinated transcription of F3'Hb, F3'5'Hi, F3'5'Hk, DFR, LDOX, UFGT and MybA1 was detected in LT berries, which all reached a maximum of expression 7 days after veraison (DAV). In HT, the situation was quite different as for F3'Hb and LDOX we found the highest expression 20 DAV, when the transcription of DFR and UFGT was already strongly reduced. This shift and lack of synchronization could have hampered in 2010 the increase in the accumulation of 3'4'-OH flavonoids derivatives in comparison to LT nevertheless the stability of F3'Hb transcripts detected in HT berries.

In 2012, we found a statistically significant increase in malvidin and quercetin percentages in LT berries in comparison to HT. The concomitant stronger upregulation of F3'5'Hi and F3'5'Hk, starting from véraison, in LT compared to HT berries has to contribute to the increase in malvidin concentration in LT berries in the second year of the trial. Moreover, in the same stage of berry ripening, also an increased expression of F3'Hb was recorded in LT berries and therefore the biosynthesis of 3'4'-OH flavonoids should have been enhanced too. Differently from what was previously discussed for 2010, DFR and LDOX maximum expression was delayed in comparison to the recorded peak of transcription for UGFT and VvMybA1 in both treatments. It has to be considered that FLS directly competes for its substrates with the enzyme DFR, because they share dihydroflavonols as the common substrates. On this base, probably there was a competition between the biosynthesis of flavonols and anthocyanins in the both treatments, but that contemporary increase in the expression of F3'Hb, DFR and FLS led to the rise of quercetin accumulation in LT berries as compared to HT.

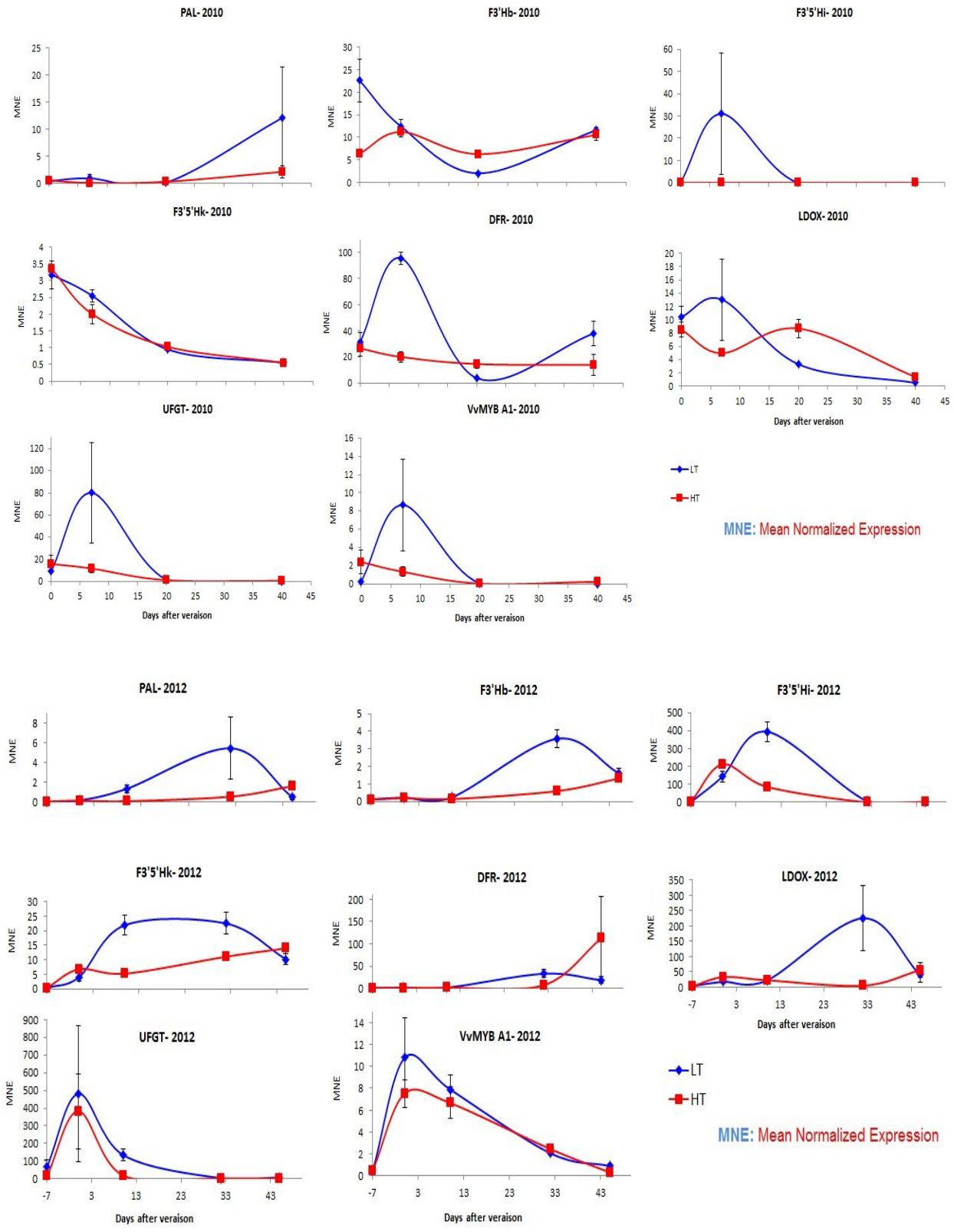

Fig. 15. Mean normalized expression of the genes involved in flavonoid biosynthetic pathway in berries grown under high(HT) and low(LT) temperature conditions during ripening in 2010 and 2012.

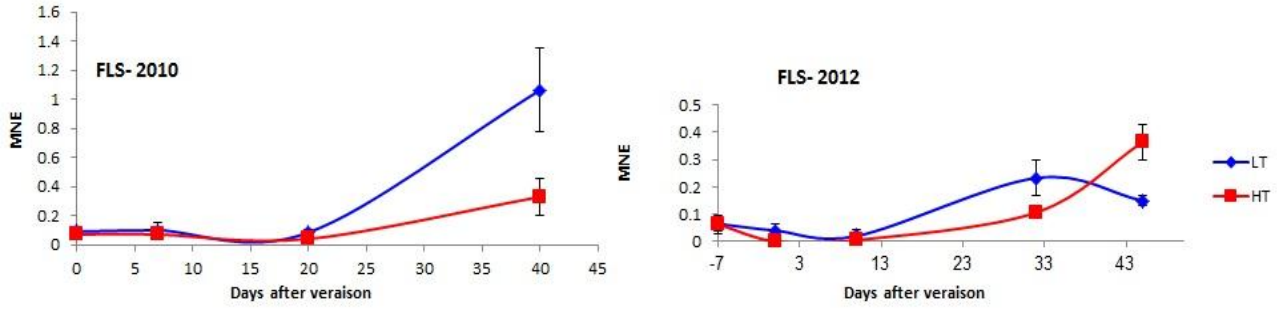

Fig. 16. Mean Normalized expression of the FLS genes involved in flavonol biosynthesis in berries grown under high(HT) and low(LT) temperature conditions during ripening in 2010 and 2012.

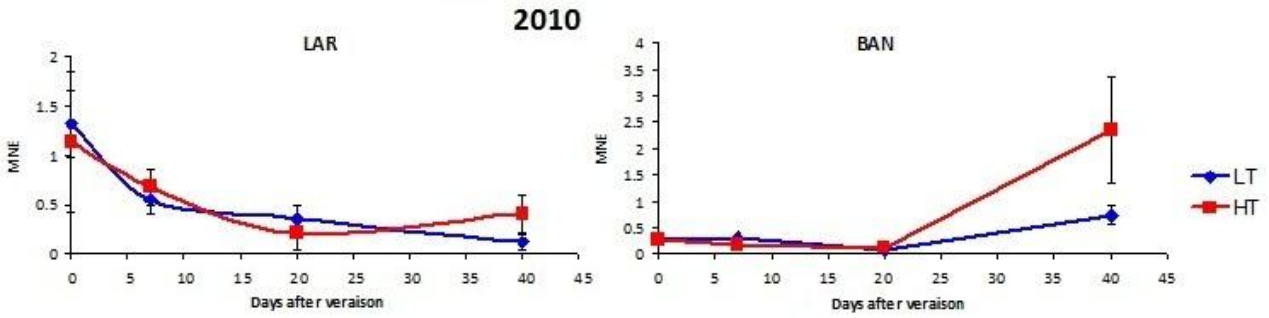

Fig.17. The mean normalized expressiom of the LAR and BAN genes in berries grown under high (HT) and low (LT) temperature conditions during ripening in 2010.

3.6. Activity of biosynthetic enzymes: PAL and UFGT

In general, the activity of PAL enzyme in both LT and HT berries increased gradually during ripening but was considerably lower in HT plants, in all sampling data from the end of full veraison to harvest (Fig. 18). This result is in agreement with the higher expression of PAL genes assessed in LT berries.

Using delphinidin and cyanidin as substrates, in both years, the activity of UFGT enzyme showed an increasing trend from the end of full veraison to harvest in both LT and HT berries (Fig. 19); nonetheless, a strong reduction was observed in HT plants compared to the LT ones during ripening. Also, irrespective from thermal conditions, UFGT activity at veraison was higher for cyanidin compared to delphinidin as substrate (Fig. 19) and this could be related to earlier expression of the F3'H genes (starting after full bloom), compared to the expression of the F3'5'H genes which begin after veraison. Basically, these results confirmed the negative effects of high temperature on anthocyanin biosynthesis at least for PAL and UFGT enzyme activities which showed a strong correlation with anthocyanin concentration and confirmed to be the key enzymes of anthocyanin biosynthesis pathway (Hrazdina et al., 1984; Mori et al., 2005; Boss et al., 1996).

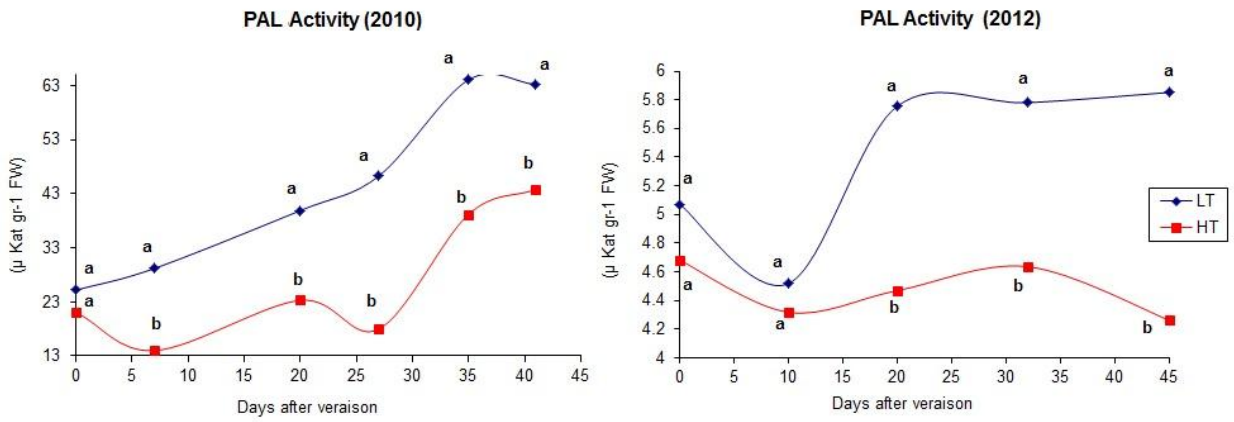

Fig. 18. The trends of PAL activity (in one minute) in the skin of Sangiovese berries grown under high (HT) and low(LT) temperature conditions during ripening in the years 2010 and 2012.

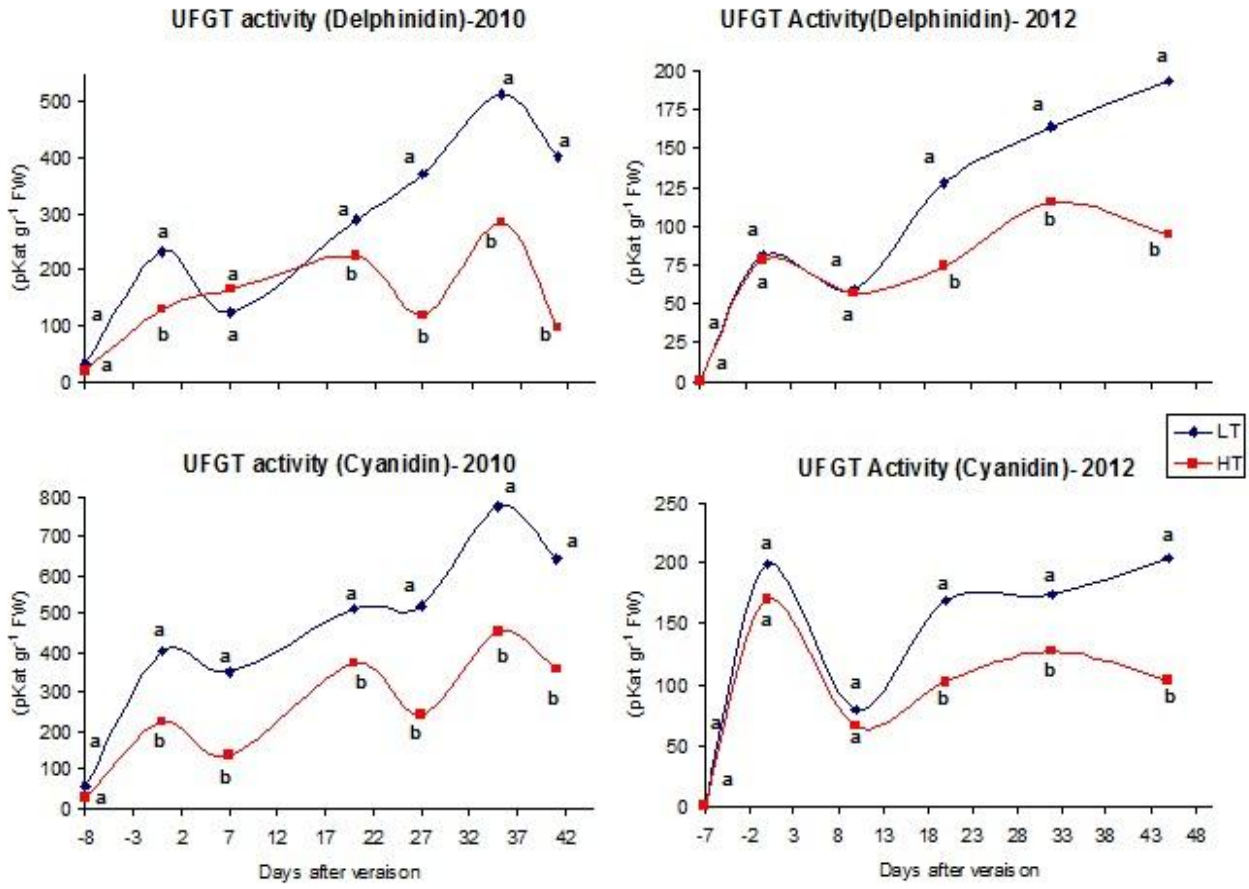

Fig.19. The trends of UFGT activity (Using cyanidin and delphinidin as substrate) in the skin of Sangiovese berries grown under high (HT) and low(LT) temperature conditions during ripening in the years 2010 and 2012.

3.7. Hypothesis for anthocyanin degradation

3.7.1. POD candidate genes

From the research conducted by Fasoli et al., (2012), it can be realized that there are many different isoforms of Peroxidases gene in different organs of grape. As to recent researchers, five POD isoforms (labeled POD1, POD2, POD3, POD4 and POD5) are showed to be more expressed in berry skin from veraison to harvest in Sangiovese (Pastore, 2010). In addition to these 5 isoforms, another one (POD6) which was reported by Mori, et al., (2007) was assessed in this thesis.

In 2010, Real Time experiment revealed that among these 6 genes that were studied in the berry skin of Sangiovese, only the expression of the genes POD1 and POD3 seem to be linked to the temperature (Fig. 20). The expression of POD1 and POD3 in HT detected to occur in different patterns during the ripening but POD 1 was expressed with a similar trend in both years.

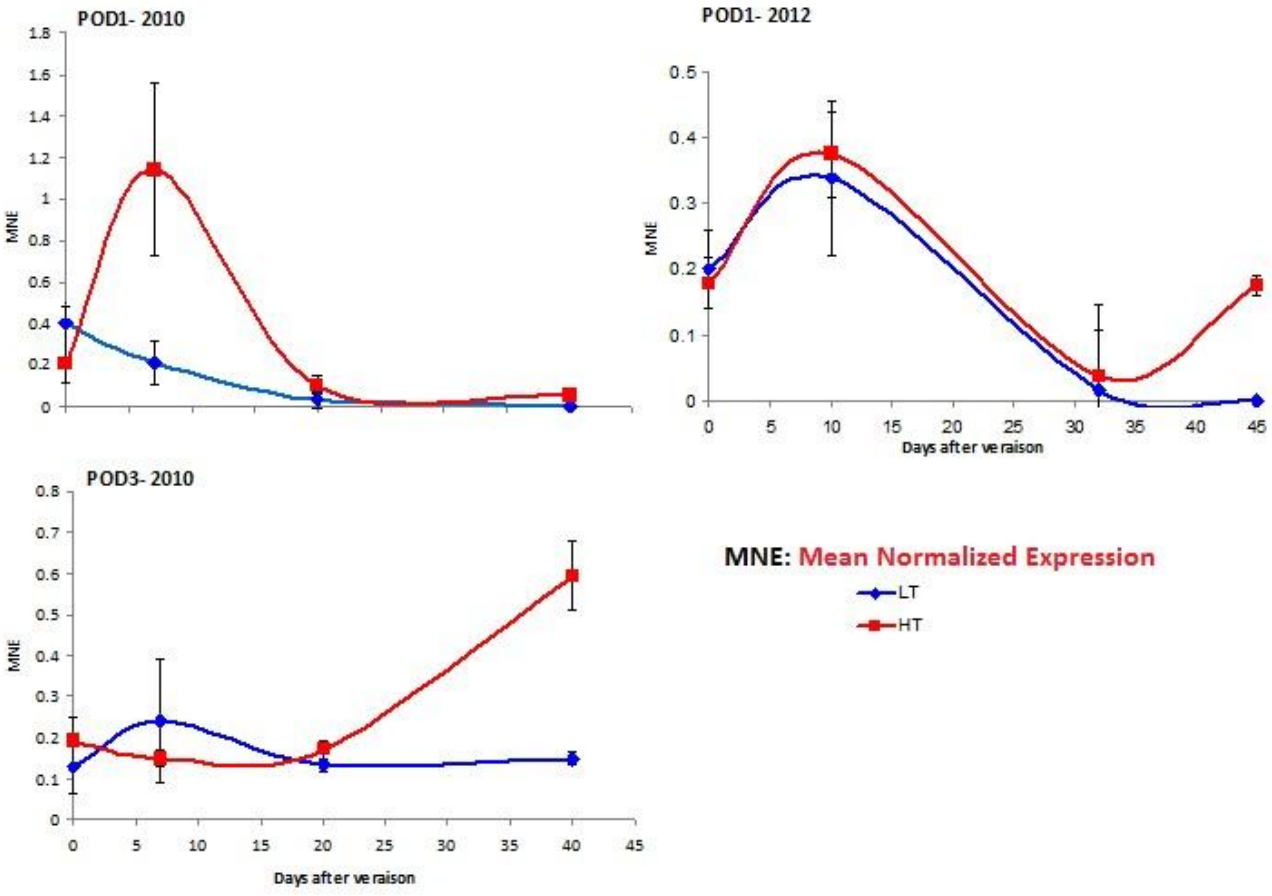

Fig. 20. Mean Normalized expression of the selected POD genes involved in anthocyanin biosynthetic pathway in the in berries grown under high (HT) and low(LT) temperature conditions during ripening in 2010 and 2012.

3.7.2. Activity of enzymes involved in anthocyanin degradation: PODs and PPOs

In the both years, the PODs activity during ripening showed a significant increase in HT berries compared to LT (Fig. 21), supporting the hypothesis of anthocyanin degradation in response to high temperature (Vaknin et al., 2005 & Mori et al., 2007).

It has been reported that peroxidase is involved in the active anthocyanin degradation of *Brunfelsia calycina* flowers (Vaknin et al., 2005) and in presence of H_2O_2 in the vacuoles of grape cells (Calderon et al., 1992). Our findings are coherent with other papers; since heat stress can contribute to H_2O_2 accumulation in plant tissue (Date et al., 1998), and so PODs-anthocyanins reaction may act as a scavenging mechanism for H_2O_2 and plant cell detoxification in high temperature conditions (Yamasaki et al., 1997; Pourcel at al., 2007).

It is interesting to note that in this experiment we could not find any PPOs activity likely due to the plastidial localization of this enzyme (Dry & Robinson, 1994) and so we demonstred the lack of direct involvement in active anthocyanin degradation under high temperature conditions in *Vitis vinifera*.

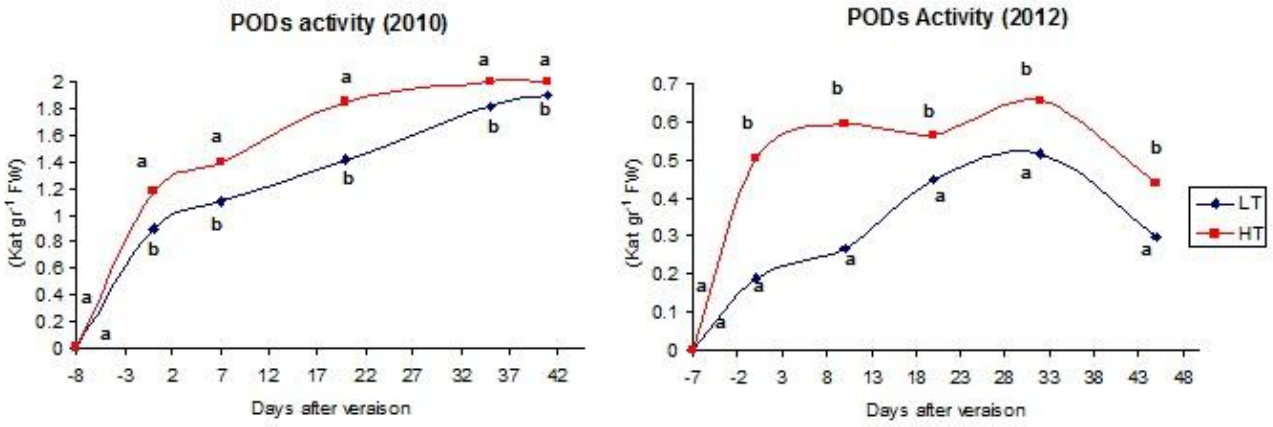

Fig. 21. The trends of PODs activity in the skin of Sangiovese berries grown under high (HT) and low (LT) temperature conditions during ripening in the years 2010 and 2012.

4. CONCLUSION

In the present study, we examined the contents of anthocyanins, flavonols and tannins, the expression of some biosynthetic genes involved in their synthesis and the activities of some related enzymes in grape berries of Sangiovese cv. grown under different thermal conditions in two different years.

Anthocyanins and flavonols accumulation in Sangiovese cv. revealed to be very sensitive to high temperature (showing a reduction over 50% in total anthocyanin concentration at harvest). Several mechanisms such as a general inhibition of the expression of flavonoids biosynthetic genes and of the activities of some flavonoids biosynthetic enzymes (PAL and UFGT) associated with a stimulation of some putative anthocyanin degradation enzymes (PODs) showed to be involved in the inhibitory effect of high temperatures on anthocyanin accumulation. A similar mechanism may be supposed for flavonols degradation.

This study showed that the temperature regimes after véraison have different effects on each flavonoid group in grape berry; because in conterary to anthocyanins and flavonols that were reduced in the skin of grapes ripened under high temperature environments after veraison, seed and skin tannins were not affected by the same conditions.

Furthermore, the warmer condition verified in 2012 compared to 2010 and the different flavonoid composition and expression profile of some of the analyzed genes in the two years, may suggest a different sensitivity to high temperature of some genes involved in anthocyanins biosynthesis.

Chapter one /Second Part

Recognition of putative POD genes involved in anthocyanins degradation under high temperature condition

1. INTRODUCTION

Petunia has played a central role in transformation research since the earliest reports of plant transformation. It was a key model system when the first definitive accounts of *Agrobacterium*-mediated transformation and direct DNA transfer established the transfer and expression of foreign genes in plants. Petunia subsequently played an important role in elucidating many of the characteristics of plant transformation. One of the key reasons for the importance of Petunia in plant transformation research has been the selection for and/or identification of genotypes well suited to growth and regeneration in culture. Ease of transformation coupled with other favorable biological characteristics, ensures that Petunia will remain a valuable model system for studies of gene function in plants (Conner et al., 2009). Moreover, *Petunia hybrida* is one of the classical subjects of investigation of plants pigmentation in which the pathway of anthocyanin biosynthesis has been analyzed genetically and biochemically (Meyer et al., 1987; Quattrocchio et al., 2006). Although the information about Petunia sequenced genome is somehow limited, it can be still used as model species in this important field of study for the obvious ease of detecting mutants for this pathway. It can also be actively employed in complementation studies with genes from other species such as grape (Venturini, 2009).

Therefore, in the second phase of this trial regarding to the results obtained from the assessment of the expression of the genes involved in anthocyanin degradation at elevated temperature, gene transformation was conducted with different isoforms of peroxidases genes from *Vitis vinifera* (cv. Sangiovese) to *Petunia hybrida* with the aim of recognizing the most effective candidate genes for anthocyanins degradation under high temperature.

2. MATERIALS

2.1. Gene selection

In the first phase of this trial, the expressions of 6 different isoforms of Peroxidases genes that were showed to be more expressed in berry skin from veraison to harvest (Fasoli et al., 2012, Mori, et al., 2007), were assessed via Real Time-PCR; subsequently, according to that results, 3 different isoformes were selected to transform *Petunia hybrida*.

2.2. *Escherichia coli* strain and culture media

The DH10B strain was provided by Invitrogen® and features the mutations recA1 endA1, presence of the gene lacZΔM15, and high transformation efficiency. Moreover, it didn't carry any antibiotic resistance genes.

2.2.1. Luria-Bertani (LB) medium

Component	Concentration (g/l)
Yeast extract	5
Tryptone	10
NaCl	10
Agar	15

Final pH: 7.5

The medium was sterilized in an autoclave (121°C for 20 minutes, 2 atm). After sterilization, antibiotic (kanamycin) was added to the solution, for a final concentration of 50 mg/l.

2.2.2. SOC medium

Component	Concentration (g/l)
Yeast extract	5
Tryptone	20

After sterilization in an autoclave (121°C for 20 minutes, 2 atm), glucose and salts sterilized by filtration (filters with ores of 0.2 μm diameter) were added to the solution at the following concentrations:

Component	Concentration
NaCl	10 mM
KCl	2.5 mM
Glucose	20 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM

Final pH: 6.8-7.0

2.3. *Agrobacterium tumefaciens* strain and culture media

For *Petunia hybrida* transformation the super virulent EHA105 *Agrobacterium tumefaciens* strain was chosen (Hellens et al., 2000). The bacteria possess a disarmed helper Ti plasmid (pTIBo542ΔT-DNA) encoding the necessary sequences for the transfer of the T-DNA region, which is carried by a second shuttle plasmid, the pK7WG2,0. The two plasmids are defined as binary vectors. The cells containing both plasmids can be selected for with the use of the following antibiotics:

Antibiotic	Concentration	Resistance
Rifampicin	50 mg/l	Genomic
Streptomycin	300 mg/l	pK7WG2,0
Spectinomycin	100 mg/l	pK7WG2,0

2.3.1. YEB Medium

Component	Concentration
Yeast extract	1 g/l
Tryptone	5 g/l
Beef extract	5 g/l
Sucrose	5 g/l
$MgSO_4$	5 Mm
Agar	15 g/l

Final pH: 7.5

The medium was sterilized in an autoclave (121°C for 20 minutes, 2 atm). After sterilization, antibiotics (streptomycin 300 mg/l, spectinomycin 100 mg/l and rifampicin 50 mg/l) were added to the solution for selective mediums.

2.4. Vectors

2.4.1. pENTR™/D-TOPO® (2580 bp)

Feature	Benefit
rrnB T1 and T2 transcription terminators	Protects the cloned gene from expression by vector- encoded promoters, thereby reducing possible toxicity in *E. coli*
attL1 and *attL2* sites	Allows sequencing in the sense orientation Bacteriophage λ-derived DNA recombination sequences that allow recombination cloning of the gene of interest from a Gateway® expression clone or *attB* PCR product (Landy, 1989)
TOPO® Cloning site, directional	Allows a fast and directional cloning
M13 forward and reverse primer sequences	Allow sequencing of the insert
Kanamycin resistance gene	Allows selection of the plasmid in E. coli
pUC origin	Allows high-copy replication and maintenance in E. coli

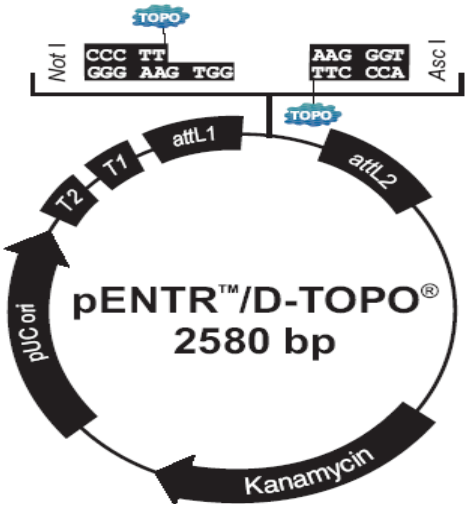

Fig. 22: a scheme of the pENTR™/D-TOPO® vector

2.4.2. pK7WG2,0

A scheme for vector pk7WG2,0 is presented in Fig. 23. It is a binary vector used as destination vector in the Gateway® technology. The T-DNA comprises the marker gene nptII which confers

resistance to kanamycin. The cloning of an exogenous genes in this position implies an LR recombination between the 35S CaMV constitutive promoter and the terminator 35S.

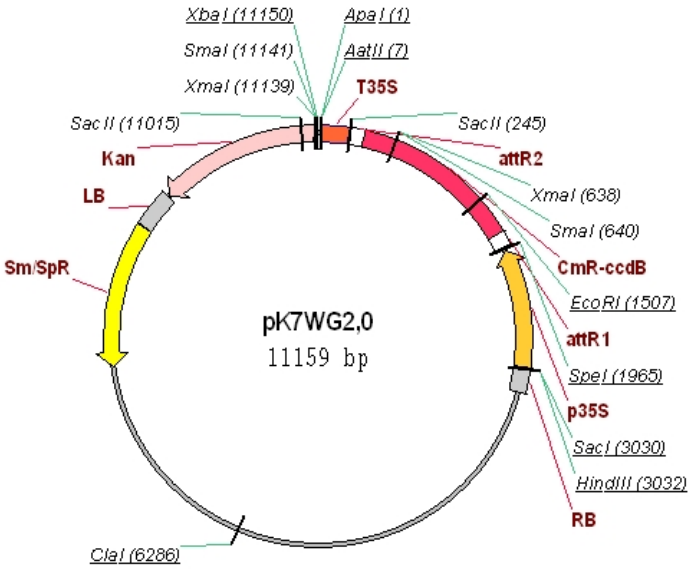

Fig. 23: Schematic representation of the pK7WG2,0 vector

2.5. Primers

The Primers pairs for POD1 to POD5 were newly designed on the original DNA sequences to amplify 150–250 bp gene fragments and the Primers pairs for the POD6 was referred to Mori et al., (2007) (Table 7).

Subsequently, according to the results of those expressions, 3 different isoformes (POD 1, POD 3 and POD 5) were selected to transform *Petunia hybrida*.

(Table 7): (POD 1-6): The primers of the selected isoforms of the Peroxidases genes for RT-PCR and (POD 1-3 cloning): The primers of the selected isoforms of the Peroxidases genes for cloning.

	primer Forward	**primer Reverse**
POD 1	5'-TCTGAGTAAGAGCCCACACACTCT-3'	5'-GAGACCTGAGCAGATAAATACAAAAATAAA-3'
POD 2	5'-AAAGTTGTTTGGCTGTGG-3'	5'-AATCCCTCCAAACAATCA-3'
POD 3	5'-TGCTCTAAATGCCTGAAAAATG-3'	5'-ATCATATAGAAAAGTGACAGCAACCT-3'
POD 4	5'-CAACTTCACACCAACGGGAAA-3'	5'-GCAGCTGAGCAAGAAATGCTT-3'
POD 5	5'-TCGGTGAGGAACTCCGATA-3'	5'-AGCTTCCTTGTCCTCTTCCA-3'
POD 6	5'-AACTTGAGACACAACAGCATAAATAAATC-3'	5'-AGGACCAAATCACAGGATGATAAAG-3'
POD 1 cloning	5'-cacc atg gca ttg atc ctc ttt tc-3'	5'-cta gtt taa ggc atc aca cc-3'
POD 3 cloning	5'-cacc atg tct ctt ctc gtt ctt ctc-3'	5'-tta aga ttc cag gag tgt gtc-3'
POD 5 cloning	5'-cacc atg aga aaa ctt atg gag gc-3'	5'-tta aag ttc agc aga agc ttc-3'

2.6. Plant materials

Petunia hyrida line W84xV30 (*P. axillaris* X *P. inflata)*, has become an attractive system for genetic, molecular and complementation studies as it possesses many desirable characteristics: colored flowers, whose dimensions (5-6 cms) allow for relative ease of dissection and separation of floral organs; rapid growth cycle (2-3 months from seeds to full bloom); ease and rapidity of genetic transformation via infection with disarmed *Agrobacterium tumefaciens*.

This species has a limited number of chromosomes (n=7) and a small genome (1.2 × 106 kb). In particular, the line W84 is not capable of expressing a Glutathione S-transferase, *PhAN9* (Alfenito et al., 1998) and hence to accumulate anthocyanins in the vacuole, leading to a fading color phenotype. However, the W84 line had proved difficult to transform in previous experiments with the GST4 gene. To overcome this problem, a new *Petunia hybrida* line was generated by crossing W84 with the V30 cultivar, and recovering the mutated offspring after self-crossing. This W84xV30 line was used in all subsequent experiments.

The peroxidases genes were isolated from *Vitis vinifera* (cv. Sangiovese) (the plant materials used in the first part of this chapter).

Fig. 24: Petunia hyrida – line W84xV30

3. METHODS

After the PCR amplification of our selected genes with proof-editing PFU enzyme, DNA purification was done directly from the PCR product and used for the TOPO®Cloning reaction following the protocol provided by Invitrogen kit.

3.1. Transformation of *Escherichia coli* by heat shock

After the TOPO®Cloning reaction, the DNA was added to the *E. coli* DH10B competent cells and the mixture left in ice for other 30 minutes. Cells were incubated at 42°C without agitation for 30 seconds, and then rapidly transferred into ice. 250 µL of S.O.C. medium provided by Invitrogen kit kept at room temperature was added to the culture, and the re-suspended cells were incubated at 37°C with agitation (200 rpm) for 1 hour. 100- 200 µl of the solution was placed on to a solid LB medium and left at 37°C overnight.

3.2. Preparation of competent cells of *A. tumefaciens*

To obtain *Agrobacterium tumefaciens* strain EHA105 cells competent for the transformation by electroporation, a single colony was inoculated into 3 mL YEB medium containing Rifampicin (50 mg/l) and incubated at 28°C overnight with vigorous shaking. When the culture reaches the exponential growth phase (usually after 12 hours, characterized by an OD_{600} equal to 0.9-1.0), 1.5 mL of solution was sampled and centrifuged at 14,000 rpm for 1 minute at room temperature. The pellet thus obtained was washed, to eliminate antibiotic, and re-suspended three times in 500 µl cold and sterile glycerol 10% (v/v); at each step the solution was centrifuged at 14,000 rpm for 1 minute at room temperature. Finally the pellet was re-suspended in 40 µl of cold and sterile glycerol 10% (v/v). The cells were ready to be electroporated.

3.3. Transformation of *A. Tumefaciens* by electroporation

A Bio-Rad device for electroporation was used for this experiment, calibrated at 25 μF, 200 Ω and 2.5 kV. 1 or 2 μL of DNA plasmid (20 ng) was re-suspended in 40 μl of competent cells. The operation was performed in ice. The mix was transferred into a pre-cooled electroporation cuvette; the cuvette was then inserted into the Bio-Rad device and subjected to a pulse of 2.5 kV/cm at 200 Ohm; immediately after the pulse, 1 mL LB without antibiotics was added in sterile conditions. The suspension was recovered and grown in a propylene tube with agitation for one hour at 28°C, so that the cells can express the resistance gene. The cells were placed in 100 – 200 μl aliquots on to YEB solid medium containing Spectinomycin (final concentration 100 mg/l), Rifampicin (final concentration 50 mg/l), and Streptomycin (final concentration 300 mg/l). The Petri dishes were incubated at 28°C for two days. The transformation efficiency of this method is 8x105 cfu/μg DNA.

3.4. Colony PCR

To detect bacterial recombinant clones which carry a fragment of interest, it is possible to perform the colony PCR consists of a single bacterial colony, for a final volume of 20 μl. A sterile tip is used to sample the colony from the solid culture medium and dissolved in the reaction mix.

3.5. Plasmid DNA extraction

To purify the plasmid DNA of colony which resulted positive by PCR, bacteria were put in 5 mL of liquid LB with the correct antibiotic to grow overnight at 37°C with agitation. The extraction of DNA plasmid was carried out on the base of "QIAprep® Spin Miniprep Kit" (Qiagen) following the manufacture's instruction.

3.6. Purification of PCR products

To purify the PCR products the protocol of the kit provided by Promega Wizard® SV Gel and PCR clean-up system was performed.

3.7. GATEWAY® cloning system

The Gateway® Technology (Invitrogen) is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways. This recombination method facilitates transfer

of heterologous DNA sequences (flanked by modified att sites) between vectors. Two recombination reactions constitute the basis of the Gateway® Technology:

Site	Length	Function
attL	100bp	Entry clone/ Entry vector
attR	125bp	Destination vector

3.8. LR Recombination reaction

LR Recombination reaction was performed following the protocol provided by Invitrogen.

This reaction facilitates recombination of an attL substrate (entry clone) with an attR substrate (destination vector) to create an attR-containing expression clone (see diagram below).

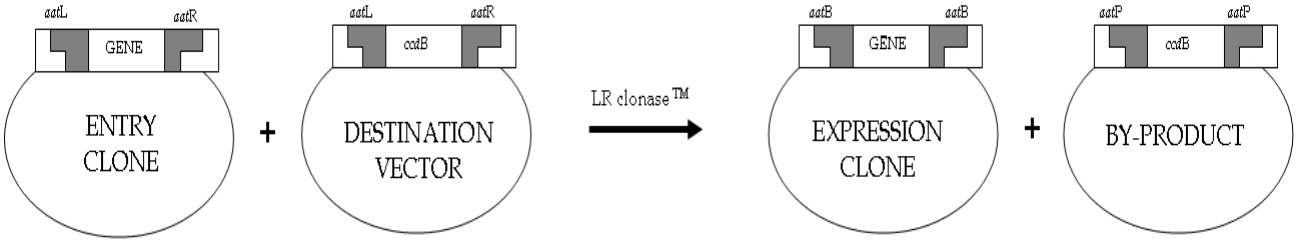

Fig. 25: a scheme of the Gateway® cloning system.

Componente	Volume
Entry clone (100 ηg)	1 µL
Destination Vector (150 ηg)	1 µL
5X LR Clonase buffer	2µL
Sterile water	6 µL

The *Entry-clone* vectors had been generated with independent ligation reactions in the pENTR™/D-TOPO® vector containing the *attL1* and *attL2* sites. It was therefore possible to perform directly the recombination reaction with the destination vector, pK7WG2,0.

The solution is incubated for 1 hour at 25°C, then 1 µl of the Proteinase K enzyme (Invitrogen) is added to the mix, which is placed for an additional 10 minutes at 37°C. At this point it is possible to perform the transformation of the competent *E. coli* DH10B cells.

3.9. TOPO cloning reaction

TOPO® cloning reaction was performed following the Invitrogen protocol for electro-competent *E. coli* cells.

Reagents	**Chemically Competent *E. coli***	**Electrocompetent *E. coli***
Fresh PCR product	5 to 4 µl	0.5 to 4 µl
Dilute Salt Solution (1:4)	--	1 µl
Salt Solution	1 µl	--
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

The mix reaction was incubated in ice for 20 minutes; then the cells were incubated at 42°C without agitation for 30 seconds, and then rapidly transferred into ice, followed by the procedure of Transforming One Shot® TOP10 Competent Cells provided by Invitrogen kit.

3.10. RNA extraction and Real time PCR

Real-time PCR was performed, to evaluate the expression of six different isoforms of peroxidases genes, for five selected stages of grape berry ripening from beginning of veraison to the end of harvest. For each sampling 3 biological replicates were analyzed. Total RNA was isolated from 200 mg of the powdered berry skin tissue using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturers 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). Total RNA was treated with 1 U µg-1 RQ1 DNase (Promega, Milan, Italy). First-strand cDNA was synthesized using 1 µg of RNA, 1µL $(dT)_{15}$ primer (500 µg/mL) and 1 U of GoScript™ reverse transcriptase (Promega). Quantitative RT-PCR was carried out on a Step One Plus™ (Applied Biosystems, Foster City, California, USA). Each reaction (10 µL) contained 188 nM of each primer, 2µL of diluted cDNA (1:25), 5µL U of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, California, USA). Thermal cycling conditions were 95 °C for 10 min followed by 95 °C for 15 s, 55 °C for 1 min for 40 cycles, followed by a melting cycle which contains 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec. Each cDNA sample was analyzed in triplicate. Gene transcripts were quantified as reported by (Pastore, 2010). The Primers pairs for POD1 to POD5 were newly designed on the original DNA sequences to amplify 150–250 bp gene fragments and the Primers pairs for the POD6 was referred to Mori et al., (2007) (Table 7).

3.11. Stable transformation of *Petunia hybrida*

The protocol is applied under a laminar flow cabinet to ensure sterility. Young, apical leaves are harvested and kept in water to avoid dehydration. They are subsequently sterilized with a solution of 0.5% sodium hydrochloride in which they are dipped for 10 minutes, followed by 5 washings of 10 minutes each in bi-distilled water. The tips, borders and petioles are removed with a sterilized scalpel, while the rest of the leaf is cut in three sections. The leaf pieces are immersed in a solution 1:10 of overnight grown *Agrobacterium tumefaciens*, transformed with a selected gene, for 20 minutes, so that the bacteria could attach to the explants, after which they are transferred to a Petri dish containing solidified Medium I (supplemented with Folic acid (1 mg/mL), NAA (0.1 mg/mL), 6-BAP (2 mg/mL); without antibiotics). Each Petri dish can hold up to 10 discs. The Petri dishes are sealed and stored in a growth chamber at 25°C under day-night regime (with a long day light period of 16 hours of light). After 2-3 days, the explants are transferred into a new Petri dish filled with medium II, which contains two antibiotics: 500 mg/l carbenicillin to stop *Agrobacterium* growth and 300 mg/l kanamycin to select the transformed explants. The explants were transferred to fresh selective plates each 2-3 weeks, and the explants were constantly checked against contamination with molds or other pathogens. After about 8 weeks, the shoots regenerated from the calluses were removed and transferred onto the rooting medium (medium III: supplemented with 500 mg/l carbenicillin and 50 mg/l kanamycin). After the roots were sufficiently developed, the medium was washed away from the roots, were put in jiffy pots and were grown in greenhouse. *Petunia hybrida* plants grow and flower in about 2 to 3 months.

3.11.1. Medium I

Component	Concentration
Sucrose	20 g/l
Glucose	10 g/l
MS-macro & micro elements with Gamborg B5 vitamins (Duchefa M0231)	4.4 g/l
Plant Agar	8 g/l

The pH is adjusted to 5.7-5.9 with HCl and KOH. The medium is sterilized in an autoclave for 20 minutes, and after it has reached 60° C the following hormones are added:

Component	Concentration
Folic acid	1 mg/l
6-BAP	2 mg/l
NAA	0,1 mg/l

3.11.2. Medium II

Medium II is obtained from Medium I with the addition of the antibiotics needed for the eradication of *Agrobacterium tumefaciens* and the selection of transformed calluses. They are as thermolabile as the phytohormones, and must therefore be injected in the medium only after it has reached a temperature lower than 60° C.

Component	Concentration
Carbenicillin	500 mg/l
Kanamycin	300 mg/l

3.11.3. Medium III

The composition of Medium III is identical to that of Medium I, but without the addition of phytohormones. Also, the following antibiotics are added to the medium:

Component	Concentration
Carbenicillin	500 mg/l
Kanamycin	50 mg/l

3.12. Agro-infiltration of petunia petals

Agro-infiltration represents recent advancements in Petunia transformation. This is a technique for transient gene expression and the development of intragenic vectors to affect gene transfer without the integration of "foreign" DNA.

One mL of the Agrobacterium culture grown overnight for inoculation of 25 mL of LB liquid medium was added to 300 µg/mL Streptomycin, 100 µg/mL Spectinomycin, 20 µM Acetosyringon and 10 mM MES and was incubated to grow overnight at 28 ^{o}C at 180 rpm. After centrifugation for 20 minutes at 12,000 rpm, the pellet was re-suspended with 15 mL of the buffer below. After adding 100 µl of the inoculums to 900 µl of the re-suspending buffer, the OD (Optical Density) was measured spectrophotometrically and the following formula was used for dilution of the inoculums up to the final volume of 50 mL:

3.12.1. Resuspending buffer

Component	Concentration
$MgCl_2$	10mM
MES	10mM
Acetosyringone	100 µM

The volume of the inoculums = [0.5 (The optimal OD) × 50 (The final volume)] ÷ [(The read OD via spectrophotometer) × 10]

Afterward, 100 µM of Acetosyringone was added to the inoculums and the mixture was incubated in room temperature for 2-3 hours.

The bacterial suspension was infiltrated into the upper layer of petals using a 1mL syringe without a needle. Infiltrated flowers were kept at 25 ^{o}C for 36 to 48 hours after infiltration.

3.13. DNA extraction from Petunia

The DNA was extracted from a *Petunia hybrida* leaf disc ground thoroughly, in 400 µl of extraction buffer. Then the extract was centrifuged and 300 µL of the supernatant was mixed with 300 µL of isopropyl alcohol. After the incubation at room temperature for 15 minutes and centrifugation, the pellet was dried at room temperature under a fuming hood and subsequently re-suspended in 100 µL of sterile bi-distillated water and incubate overnight in the refrigerator. To discard any remaining impurities, the tube was centrifuged again, and the supernatant transferred to a new sterile tube.

3.13.1. Extraction buffer

Component	Concentration
Tris HCl pH 8.0	200 mM
NaCl	250 mM
SDS	1%
EDTA	25 mM
β-mercaptoethanol	10 mM

3.14. PCR of transformed Petunia genomic DNA

PCR amplification was performed on Petunia genomic DNA in a final volume of 20 µl. As the primer forward, Promoter 35 S and as the primer reverse, the specific primer for "POD 1cloning" was used. As the negative control, the genomic DNA of the Wild type line was used as the template.

3.14.1. Reaction Mix

Component	Stock	Volume
Template DNA		1 µl
Buffer (containing $MgCl_2$)	10x/5x	4 µl
Nucleotides	10 mM	0.4 µl
Primers	20 µM	0.4 µl
Taq	5,000 u/mL	0.1 µl

3.14.2. Standard thermal cycle

Initial denaturation	94 °C	5’	
Denaturation	94 °C	40”	
Primers annealing	55°C	40”	X 35 cycles
Extension	72 °C	90’’	
Final extension	72 °C	7’	

3.15. RNA extraction from *Petunia hybrida* petal

Petunia petals from the young expanded flowers were immediately frozen in liquid nitrogen and then were kept in the freezer (-80 ^{o}C) until analyses were carried out. Total RNA was isolated from 200 mg of the powdered petal using spectrum ^{TM} 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).

3.16. cDNA synthesis RT PCR reaction

After controlling the RNA via the electrophoresis gel, total RNA was treated with 1 U µg-1 RQ1 DNase (Promega, Milan, Italy). First-strand cDNA was synthesized using 1 µg of RNA, 1µL $(dT)_{15}$ primer (500 µg/mL) and 1 U of GoScript™ reverse transcriptase (Promega). PCR amplification was

performed on the derived cDNA, for a final volume of 20 µl. As the primers, once the Primers pair of actin was used and subsequently, for the RT- PCR amplification, the specific primers of "POD 1cloning" (Table 7) were used.

3.16.1. Reaction Mix

Component	Stock	Volume
Template DNA		1 µl
Buffer (containing $MgCl_2$)	10x/5x	4 µl
Nucleotides	10 mM	0.4 µl
Primers	20 µM	0.4 µl
Taq	5000 u/mL	0.1 µl

3.16.2. Standard thermal cycle

Initial denaturation	95 °C	5'	
Denaturation	95 °C	30"	
Primers annealing	55°C	30"	X 35 cycles
Extension	72 °C	20''	
Final extension	72 °C	7'	

3.17. Total anthocyanin measurement

Petunia petals from the young expanded flowers were selected in 5 biological replicates for the transformed plants and wild type line. They were immediately frozen in liquid nitrogen, ground and the fine powder was weighted before the extraction of anthocyanins. The powder was homogenized with the solvent mixture of methanol 99% with HCl 1% at the volumes of eightfold of the powder. Then, the mixture was sondicated for 20 minutes.

This is the act of applying ultrasound energy to agitate particles in a sample. It is applied using an ultrasonic bath. All the procedures were conducted at 4 ^{o}C. After the centrifugation at 12,000 rpm,

for 10 minutes at 4 ^{o}C, an aliquot of the supernatant was diluted 1/40 and the total anthocyanin was quantified spectrophotometrically at 540 nm.

3.18. Heat shock performance

Young petunia flowers in 5 biological replicates for the transgenic plants and wild type line were selected for the heat shock. To carry out this experiment, the flowers were cut with a long pedicle and some small leaves and were placed in a falcon of water. They were kept for one hour in the incubator at 45 ^{o}C; and transferred subsequently to the room temperature. After 30 hours, the petals were frozen in liquid nitrogen, ground and the fine powder was weighted to evaluate the activity of Guaiacol Peroxidases enzyme and the total anthocyanin.

3.19. Guaiacol Peroxidases activity assay

Guaiacol peroxidases was assayed as described by Ushimaru et al., (1997), using pyrogallol as the electron donor for the reaction. The plant material was extracted in cold buffer (200 mM sodium phosphate, 5 mM sodium EDTA, 1% PVPP, pH 7.0) and incubated on ice for 30 min. After centrifugation at (10000 × g, 30 min, 4 °C), the desalted supernatant was used for the assay. The reaction mixture (1.5 mL) included 1140 µl of the 100 mM sodium phosphate buffer, pH 7.0, 60 µL of the 2.5 mM H_2O_2, 60 mM pyrogallol, and 240 µl extract; H_2O_2 and pyrogallol were prepared fresh just before use. Absorbance (λ=430 nm) was taken after 25 min incubation at room temperature, and referred to a blank with no extract added. One unit of Guaiacol peroxidases is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of pyrogallol per minute, under the conditions described by Chen and Asada (1989); and PODs activity was expressed as kat g^{-1} petal fresh weight. An absorbance coefficient of 2.47 mM^{-1} cm^{-1} was assumed for calculations.

4. RESULTS

The aim of this trial is recognizing the most effective candidate genes responsible for anthocyanins degradation under high temperature conditions during the ripening time. Among the candidates for anthocyanin degradation, peroxidases are involved in active anthocyanin degradation in living tissues (Vaknin et al., 2005; Welinder et al., 2002). In addition, the results obtained from the enzymatic activity of peroxidases at elevated temperature and the expression of this gene during ripening supports the hypothesis of its role in anthocyanin degradation. On these bases, the technique of gene transformation was conducted with different isoforms of peroxidases genes from *Vitis vinifera* (cv. Sangiovese) to *Petunia hybrida.*

4.1. Gene selection

From the research conducted by Fasoli et al., (2012), it can be realized that there are different isoforms of Peroxidases gene in different organs of grape that among them only 5 isoforms are showed to be more expressed in berry skin from veraison to harvest. In addition to these isoforms, there was another one which was reported by Mori, et al., (2007) to be expressed in grape berry skin.

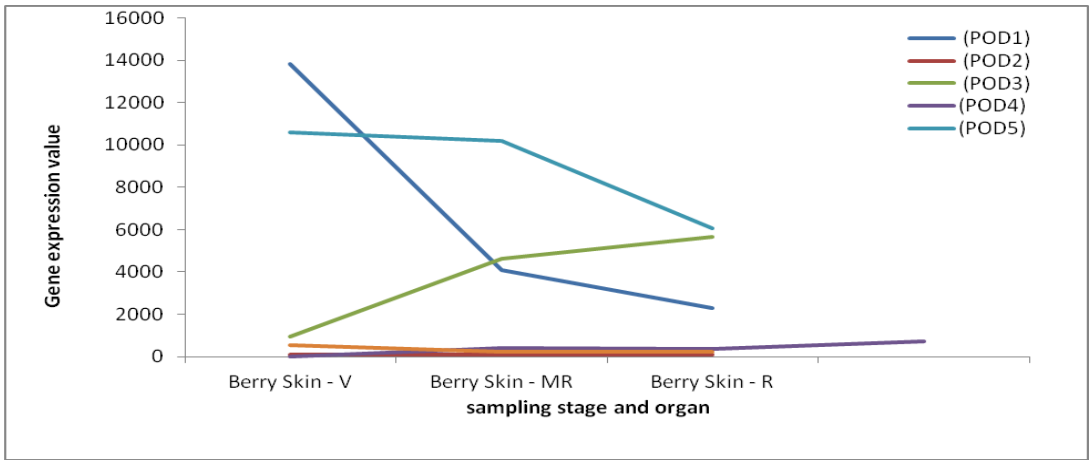

Fig. 26: The expression value of the different isoforms of Peroxidases in berry skin from veraison to harvest: (V: Véraison; MR: Mid-ripening; R: Ripening) Fasoli et al., (2012).

As to the expression of these different isoforms by Real time pcr, 3 of them were selected for the transformation to *Petunia hybrida*.

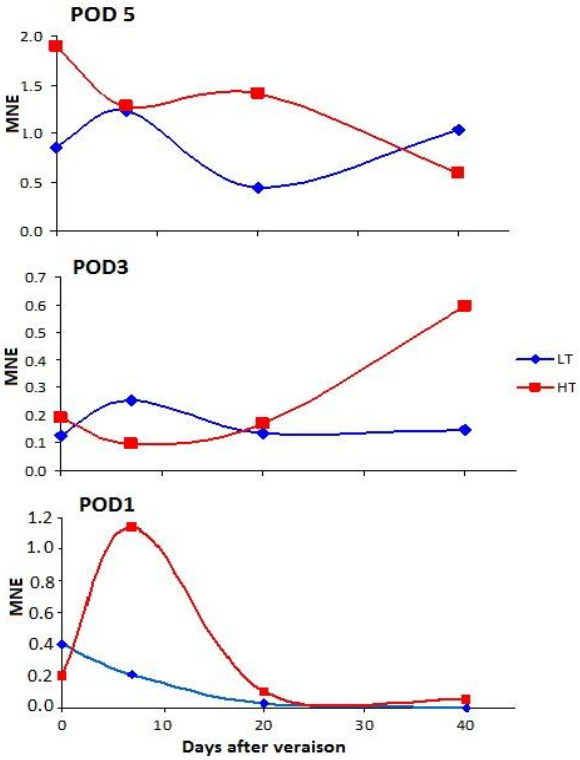

Fig. 27: Mean Normalized expression of the selected POD genes isolated from berry skin for the transformation to Petunia hybrida.

Among all the three isoforms, only the POD 1 could be successfully isolated from grape berry skin and transformed to Petunia.

4.2. The sequence of the POD1 gene

Vv14s0066g01850 (POD1)
ATG GCA TTG ATC CTC TTT TC TCTACTATTCTTTCTGGGATTAGTGAACCCTTCGGAGTCCAGGTTGAGTGTCAACTACTACCAAAAATCATGCCCAAGA TTCAGTCAGATCATGCAGGATACCATCACCAACAAGCAAATCACCAGCCCCACCACGGCTGCGGCCACTCTCCGCCTC TTCTTCCACGACTGCTTCATTGAGGGCTGCGATGCTTCCGTTCTTGTCTCCTCGACCCCCTTCAACGAGGCCGAGCGCGA TGCCGACATGAACCTCTCCCTCCCCGGCGACGGCTTCGACGTCGTTGTCCGCGCCAAGACCGCCCTCGAGCTCGCTTGC CCCGGTGTGGTCTCCTGTGCCGACATTCTCGCCGTCGCTACCCGCGATCTCGTCACCATGGTGGGAGGTCCCTTCTACA AGGTCCCGCTGGGGAGAAGAGATGGGCTGGTCTCCCGGGCGAACAGGGTTGAAGGTAACCTTCCAAGACCCACGATG TCCATTTCTCAGATAATTTCGATTTTCGCTGTGAGAGGGTTCTCGGTTCAAGAAATGGTGGCGCTGAGTGGTGCCCACA CGATTGGGTTCTCGCATTGTAAAGAATTCAGCAGCGGGATCTACAATTACAGCCGCAGTTCCCAGTCCAATCCGAGCTA CAACCCTAGATTCGCAGAGGGGTTGAGAAAAGCTTGCAGCGATTACCAGAAGAATCCTACATTGTCGGTGTTCAACGA TATAATGACTCCCAACAAGTTCGATAACATGTATTTCCAGAATCTGCCCAAGGGTTTGGGGCTATTGGCGACGGACCAT ACCATGGCTACCGATCCGAGGACGAGGCAGTTTACGGATTTGTATGCCAAGAATCAAAGCGCCTTCTTTGAGGCATTT GGTCGAGCTATGGAGAAGCTTGGCCTTTATGGGATCAAGACCGGGCGAAGAGGAGAGATTCGACGCA GG TGT GAT GCC TTA AAC TAG
Vv14s0066g01850 (POD1) genomic
AAGGCTTCTGAGTAAGAGCCCACACACTCTCCATTGCTGCCACTACCACTGGCTTTTTTTTTTATTTTTGTATTTATCTGC TCAGGTCTCTGAGCTTTGAGCTCTGAGAAACAATGGCATTGATCCTCTTTTCTCTACTATTCTTTCTGGGATTAGTGAAC CCTTCGGAGTCCAGGTTGAGTGTCAACTACTACCAAAAATCATGCCCAAGATTCAGTCAGATCATGCAGGATACCATC ACCAACAAGCAAATCACCAGCCCCACCACGGCTGCGGCCACTCTCCGCCTCTTCTTCCACGACTGCTTCATTGAGGGCT GCGATGCTTCCGTTCTTGTCTCCTCGACCCCCTTCAACGAGGCCGAGCGCGATGCCGACATGAACCTCTCCCTCCCCGG CGACGGCTTCGACGTCGTTGTCCGCGCCAAGACCGCCCTCGAGCTCGCTTGCCCCGGTGTGGTCTCCTGTGCCGACATT CTCGCCGTCGCTACCCGCGATCTCGTCACCATGGTGGGAGGTCCCTTCTACAAGGTCCCGCTGGGGAGAAGAGATGGG CTGGTCTCCCGGGCGAACAGGGTTGAAGGTAACCTTCCAAGACCCACGATGTCCATTTCTCAGATAATTTCGATTTTCG CTGTGAGAGGGTTCTCGGTTCAAGAAATGGTGGCGCTGAGTGGTGCCCACACGATTGGGTTCTCGCATTGTAAAGAAT TCAGCAGCGGGATCTACAATTACAGCCGCAGTTCCCAGTCCAATCCGAGCTACAACCCTAGATTCGCAGAGGGGTTGA GAAAAGCTTGCAGCGATTACCAGAAGAATCCTACATTGTCGGTGTTCAACGATATAATGACTCCCAACAAGTTCGATA ACATGTATTTCCAGAATCTGCCCAAGGGTTTGGGGCTATTGGCGACGGACCATACCATGGCTACCGATCCGAGGACGA GGCAGTTTACGGATTTGTATGCCAAGAATCAAAGCGCCTTCTTTGAGGCATTTGGTCGAGCTATGGAGAAGCTTGGCCT TTATGGGATCAAGACCGGGCGAAGAGGAGAGATTCGACGCAGGTGTGATGCCTTAAACTAGGTTTTTAAGGTTTTTTTT TTTTTTTTTGCATTACAAACTCCCACCGTAGTGATGATCAATCAAGGTGAATTGATGGTGAGGGGGTGGTTTCATTTGA ATTCTTTTTTTTTTTTGTTGT

4.3. Gene transformation

After amplification of POD1 gene with PFU enzyme and specific POD1 primers (Table 7, Fig. 28), the *E. coli* transformation was performed via pTOPO cloning system. The day after, a single colony of DH10B cells that was grown on solid LB was transferred into 10 mL of liquid LB containing the appropriate kanamycin antibiotic (for a final concentration of 50 mg/l) and incubated overnight at 37° and 250 rpm. Afterward, the pcr amplification of the colony was done to detect bacterial recombinant colonies which carried the fragment of interest.

Therefore, 2 plates were designed to amplify each colony two times (Fig. 29):

In the first plate, the combination of the primer forward of M13 was used with the specific primer of POD 1 reverse, and, simultaneously, in the second plate, the same colony was amplified with the primer forward of POD1 with the M13 reverse. The primers pair M13 gives the possibility to amplify the fragment 50 bp longer than our inserted gene. Consequently, only the colonies which were amplified in the correct position in the both plates had successfully carried the fragment of interest (Fig. 29).

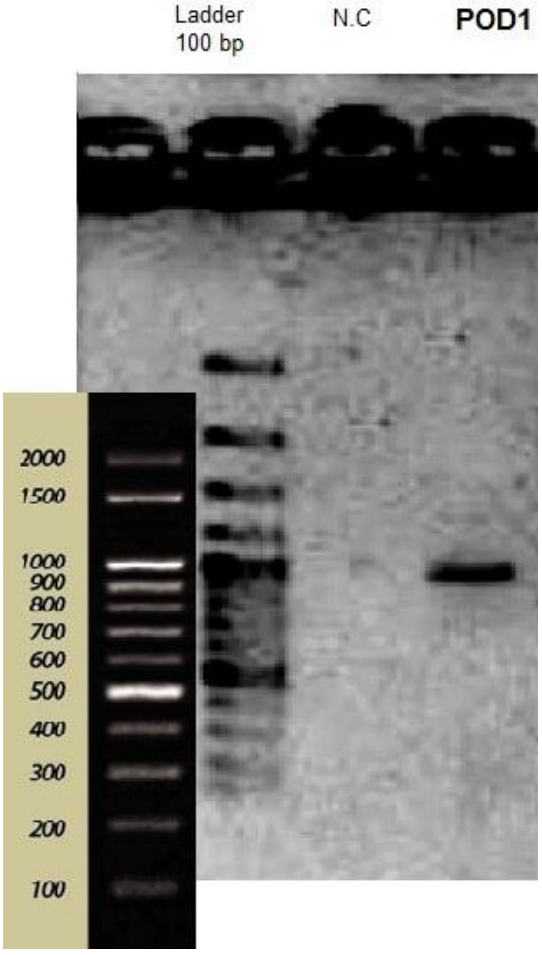

Fig. 28: Amplification of POD 1 gene by PFU.

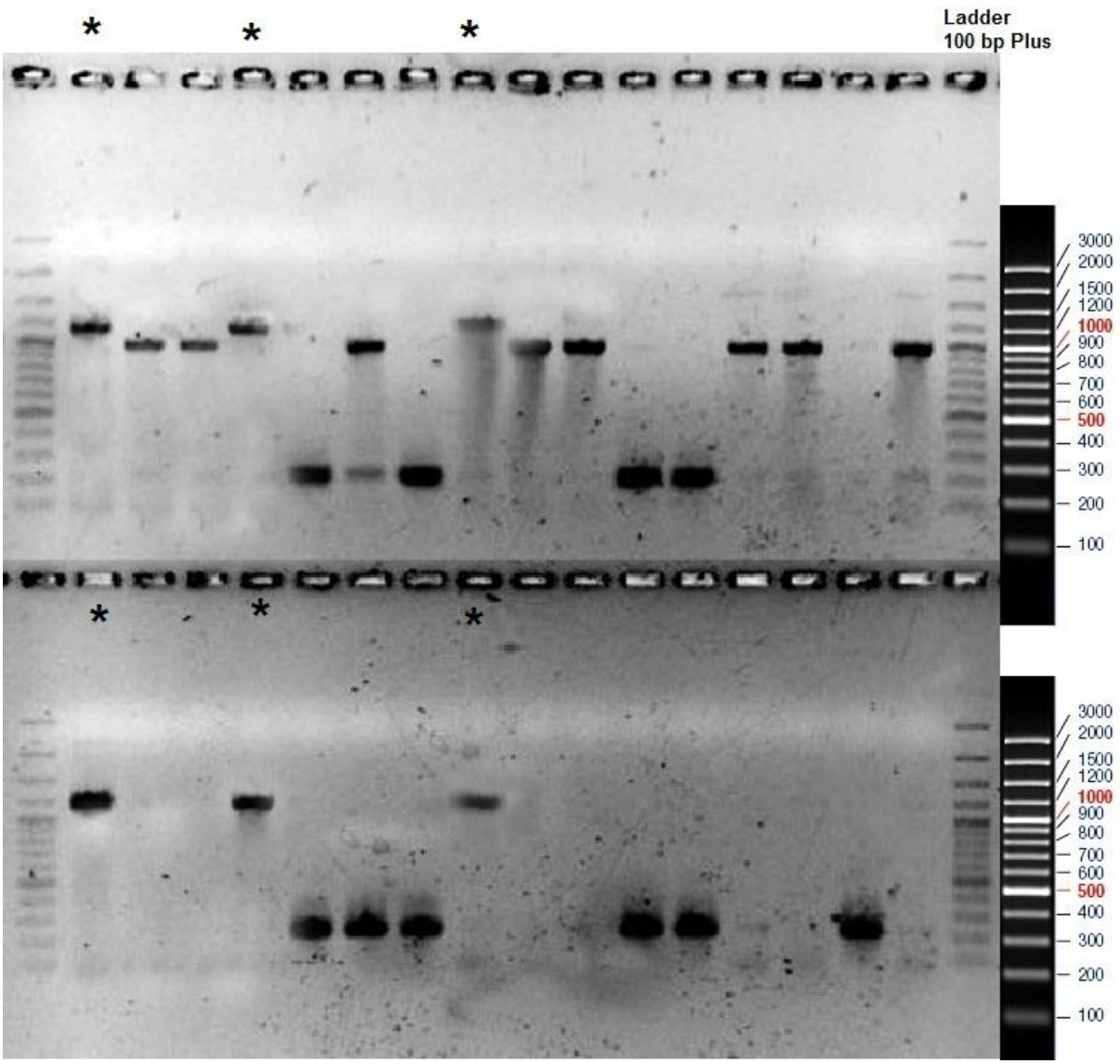

Fig. 29: Colony PCR of POD 1 gene; (The colonies marked with a star sign, in the both gels, are the ones with our fragment of interest).

After the sequencing of the insert in the plasmid, the LR recombination reaction was performed. The colony PCR was done using the "PROMOTER 35 S" as the primer forward and the primer "SONDA TER" as the reverse. The fragment 1575 bp was amplified. The colony marked with a star sign is the one with our fragment of interest (Fig. 30) and the plasmid DNA was extracted and the insert fragment of 1375 bp was amplified with the primer forward "PROMOTER 35 S" and the specific primer reverse of "POD 1cloning" (Table 7), (Fig. 31).

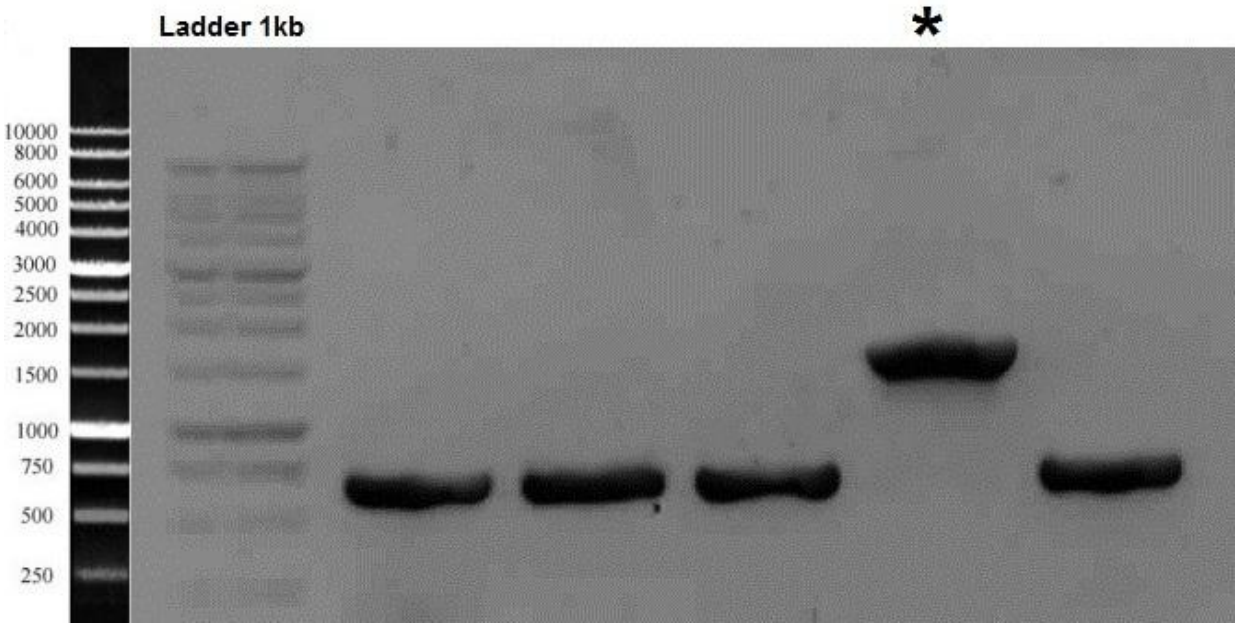

Fig. 30: The PK7WG2 Colony PCR of POD 1 gene

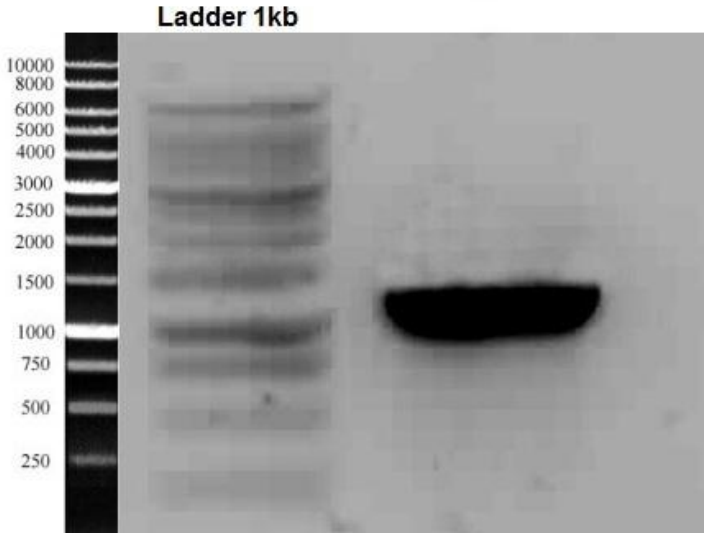

Fig. 31: The plasmid DNA (PK7WG2) control

After the transformation of *A. Tumefaciens*, the positive colonies were checked by pcr amplification of the Agro-plasmid with the primer forward "PROMOTER 35 S" and the specific primer reverse of "POD 1cloning" (Fig. 32). The plasmid DNA PK7WG2 was used as the positive control.

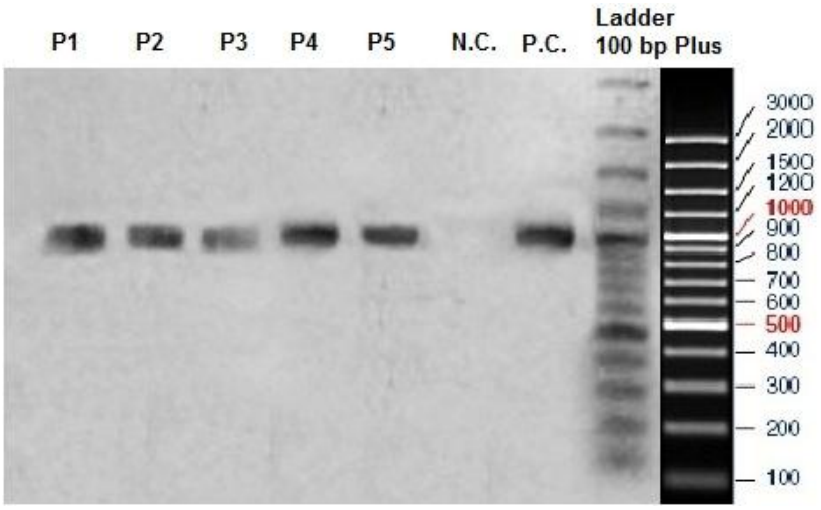

Fig. 32: The pcr amplification of the Agro-plasmid; (P.C.): Positive control; (N.C.): Negative control; (P1 - P5): Agro-plasmids

Afterward, the stable transformation of *Petunia hybrida* was done. In addition, at the same time the Agro-infiltration of petunia petals was performed and the bacterial suspension was infiltrate into the upper layer of petals. Infiltrate flowers were then kept at 25 ^{o}C for two days, but the flowers were wilted without any change observed. After 4 to 5 months, two plants derived from the two independent stable transformations grew in selective medium containing kanamycin and flowered and were propagated asexually by cutting.

To control the transformation efficiency, the DNA was extracted from a *Petunia hybrida* leaf disc of the two transgenic lines, a wild type line and also as a positive control, the plasmid DNA PK7WG2 was used. The pcr amplification was done with the primer forward "PROMOTER 35 S" and the specific primer reverse of "POD 1cloning" (Fig. 33).

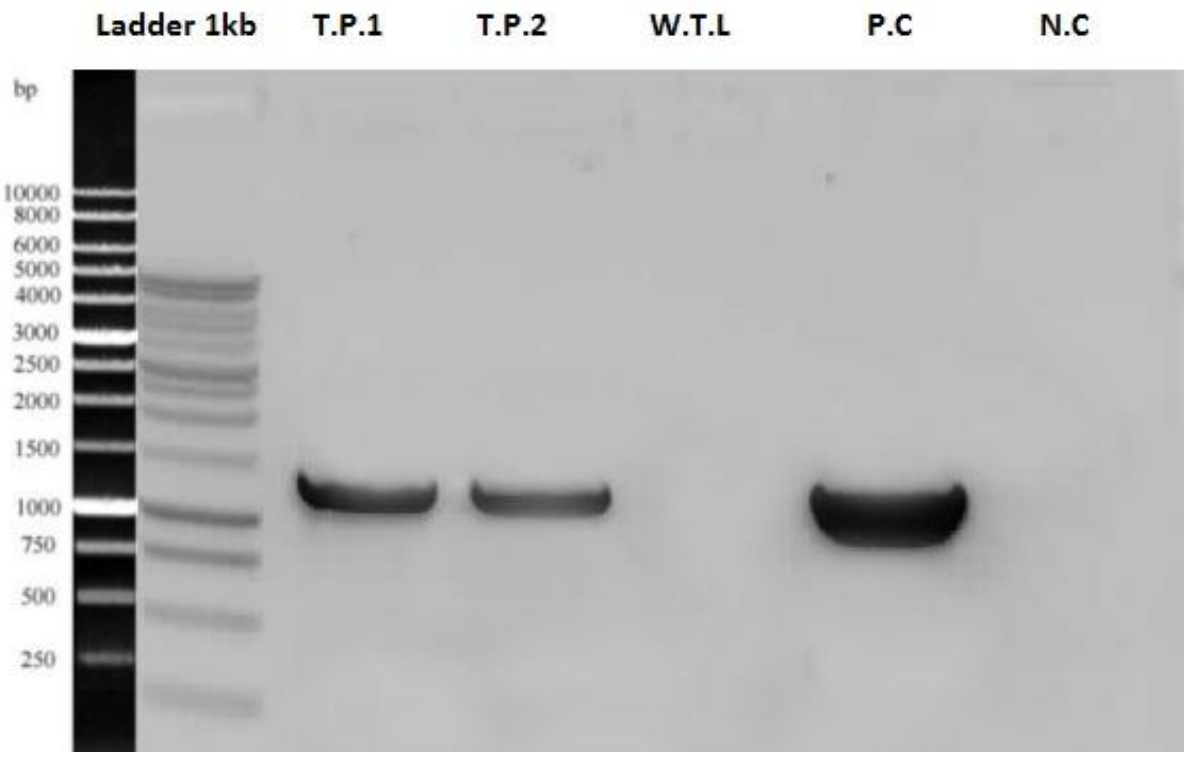

Fig. 33: The PCR amplification to control the transformation efficiency: (T.P.1): Transgenic petunia 1_{st} pot; (T.P.2): Transgenic petunia 2_{nd} pot; (W.T.L.): Wild type line; (P.C.): Positive control; (N.C.): Negative control.

As to the obtained results (Fig. 33), both plants were transformed successfully. To investigate the expression of the transgene in the two lines, RNA was extracted from the petal of the transgenic plants and from the wild type line, used as negative control, and retro transcript to cDNA. By pcr amplification performed with the primers pairs of Actin the correct retro transcription of the RNA was evaluated. Then, to analyze the expression of the transgene VvPOD1 in the petunia petal, the RT-PCR was performed with the specific primers pairs of "POD 1cloning".

According to the (Fig. 34), the fragment of 1kb was amplified and observed only in the second lane, confirming that the POD1 gene was expressed successfully in the *Petunia hybrida* line 2.

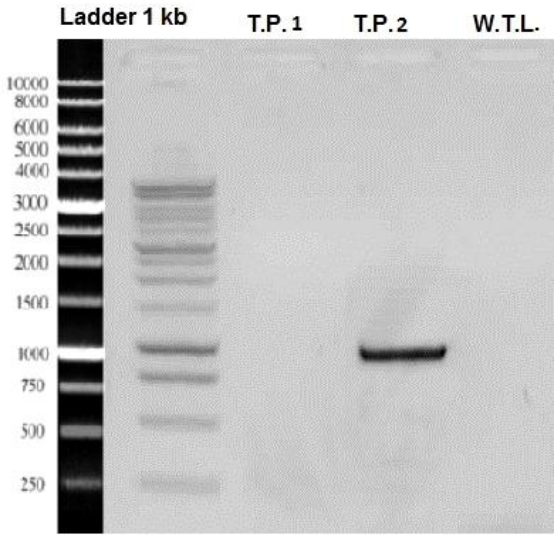

Fig. 34: The RT-PCR of POD1 gene in the transformed plant; (T.P.1): Transgenic petunia 1_{st} pot; (T.P.2): Transgenic petunia 2_{nd} pot; (W.T.L.): Wild type line.

4.4. Heat shock and the plant response

Subsequently, flowers from the transgenic line 2 (which expressed the POD1 gene) and from the wild type were selected in 5 replicates and treated with the heat shock in order to highlight the possible role of POD1 in anthocyanin degradation under stress condition.

The same number of flower replicates of wild type and the transgenic line 2 were selected as the control, i.e. not subjected to the heat shock. The petals of the treated flowers together with the control ones were grounded by liquid nitrogen and the fine powder was weighted and divided in to two, in order to evaluate the activity of Guaiacol Peroxidases enzyme and the total anthocyanin. (Fig. 35, 36).

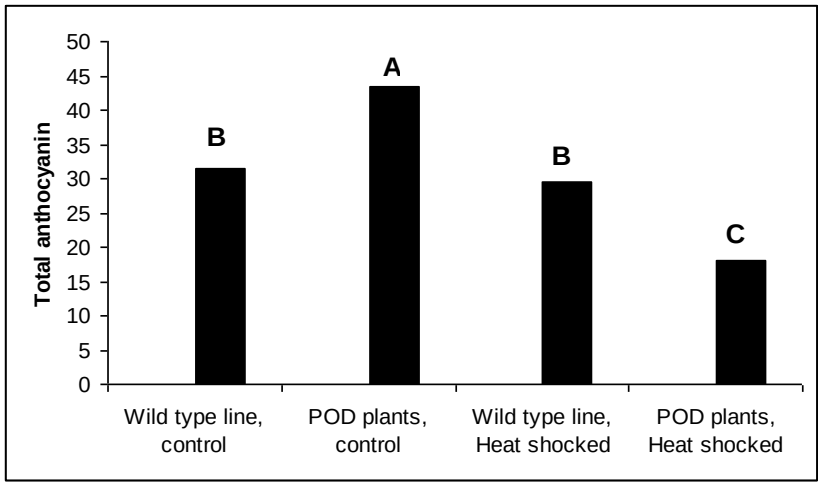

Fig. 35. Total anthocyanin content of wild type line and POD plants.

By comparing the total anthocyanin content of the wild type and the transgenic lines before and after heat treatment, we found that the total anthocyanin content of the wild type line was significantly similar in the both control and shocked treatment (Fig. 35). After the heat shock, the level of the total anthocyanin in the transgenic petunia was reduced significantly. However, the total anthocyanin content of the POD flowers in control was surprisingly highest among the others.

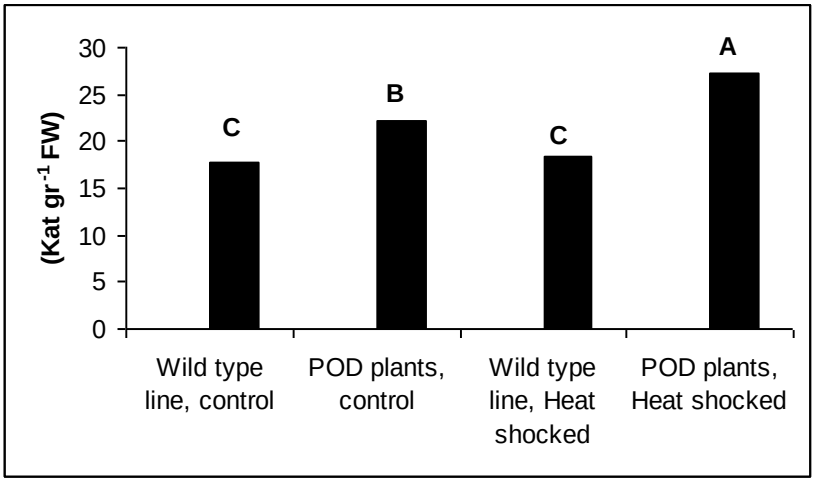

Fig. 36. The Peroxidase activity of wild type line and POD plants in the control and heat shock treatment.

The enzymatic activity of peroxidase in both wild type and transgenic line 2 was evaluated before and after the treatment.

The wild type flowers showed the same level of enzymatic activity before and after the treatment (Fig. 36). The peroxidase activity was higher in the transgenic line in both control and the shocked flowers. However, heat shock treatment contributed to significantly increase the peroxidase activity in transformed flowers (Fig. 36).

5. CONCLUSION

Most of the works studied the effects of temperature on anthocyanin accumulation, has dealt with the effects on the biosynthesis of anthocyanins (Mori, et al., 2007).

Yamane et al. (2006) discussed the possibility of the contribution of another mechanism (e.g. anthocyanin degradation) to the inhibitory effect of high temperature on anthocyanin accumulation. To date, no report has demonstrated the enhancement of anthocyanin degradation due to high temperature in plant tissue. Hence, the present study is the pioneer to show that the mechanism of anthocyanin loss in grape skins due to high temperature involves not only the inhibition of anthocyanin biosynthesis, but also its enzymatic degradation.

As to the results of this research and the literatures (Vaknin et al., 2005, Mori et al., 2007), among the different candidates, peroxidases (PODs) are reputed to be the main responsible for degrading the anthocyanin pigment in vivid tissues.

There are many isoforms of Peroxidases gene in different organs of grape that among them only 6 isoforms are reported to be more expressed in berry skin from veraison to harvest (Fasoli et al., 2012 and Mori, et al., 2007).

Therefore, in order to recognize the most important gene that encodes the peroxidase enzyme in Sangiovese grape under stress condition, selected isoforms of POD were isolated from *Vitis vinifera* (cv. Sangiovese) berries and transformed to *Petunia hybrida*. Afterward the expression of the POD1 gene and the activity of the peroxidase enzyme were assessed in the transgenic petunia and were compared to the wild type line under the stress condition.

The obtained results could confirm that the level of total anthocyanin was decreased in the living Petunia suffered from the heat stress; this was under the circumstance that the level of the peroxidase enzyme was significantly higher in those plants.

On these bases, it can be figured out that peroxidases can be considered as the responsible for anthocyanin degradation in living tissues and the decrease of total anthocyanin content in the plants faced to an abiotic stress such as the excessive high temperature. What is more, among the different isoforms of the tested peroxidases genes, the POD 1 isoform (with the sequence mentioned before) can be identified as one of the propellant in this respect.

Chapter two

Effects of late-season source limitations induced by trimming on grape composition during ripening in *Vitis vinifera* cv. Sangiovese

1. INTRODUCTION

Grape wine production showed a higher sensitivity to climate variability and the ongoing climatic changes have already caused phenological alterations in some viticultural areas and are expected to continue in the future (Jones et al., 2005; Schultz, 2000; Duchene and Schneider, 2005). Besides, over the last fifty years an overall upgrading of vineyard management techniques which lead to a general wine quality improvement has been verified. As consequence, in wine regions where heat summations largely exceed technological requirements for the grown grape varieties, a too high level of sugar, and subsequent wine alcohol excess, low acidity, alteration of aroma composition and, in black varieties, unbalanced phenolic ripening with insufficient berry skin colour has been shown at harvest.

Accumulation of sugars is probably more responsive to temperature variations than accumulation of anthocyanins; because the optimal temperature for sugars biosynthesis and transport ranges from 8 to 33 °C, while for anthocyanin pathway range from 17 to 26 °C. Moreover high temperatures (above 30-35 °C) have been the inhibitor of anthocyanin accumulation (Kliewer, 1970- 1977; Mori et al., 2007, Movahed, 2011). The relationship between tannins and high temperature is not clear but some authors reported that seed tannin concentration was less susceptible to changes in temperature conditions respect to anthocyanins (Cortell et al., 2007a, Ristic et al., 2007). Moreover while soluble solids and anthocyanins sysnthesis starts at veraison, tannins were produced several weeks before veraison and then their concentration in the berry starts to decrease following by a plateau at later berry ripening stages (Kennedy et al., 2000; 2002). In other word it seems that there are differential effects of temperature increase on the evolution of different berry traits in particular when they occure after veraison. On these bases the hypothesis is that the slowing down of sugars accumulation rate may allow to achieve full ripeness of phenolic compounds, mainly anthocyanins and tannins. As well known, fruit sugar accumulation can be decreased by grape-growers, for example via bud load and yield increases, but, for the production of high quality wine, it is not allowed to exceed foreseen thresholds by the Appellation rules.

A different approach to slow down sugar accumulation is represented by source limitation as the rate of soluble solids increase in berries is well related to the ratio between leaf area to yield (Kliewer and Dokoozlian, 2005). In particular, several researches showed that the removal around veraison of the more young and efficient part of the canopy, may be effective in reducing sugar accumulation (Reynolds and Wardle, 1989; Intrieri et al, 1983; Intrieri, 1994).

Although the relationship between sugar accumulation and leaf area to yield ratio is well documented, the evolution of some phenolic compounds in relation to leaf area efficiency and sugar

content in berries remain unclear. Sugar has been shown to stimulate anthocyanin accumulation in grape (Pirie and Mullins, 1976; Larronde et al., 1998; Hiratsuka et al., 2001; Gollop et al., 2002) but it has been also reported that anthocyanins exhibit an initial phase of rapid increase strongly correlated to sugar accumulation and a second phase where anthocyanins and sugars are uncoupled (Guidoni et al., 2008).

Moreover several studies with red wine cultivars in which crop was adjusted by means of cluster thinning, increasing the leaf area to yield ratio, resulted in improved soluble solids, anthocyanins, total phenols and colour intensity (Filippetti et al., 2007; Pastore et al., 2011;Guidoni et al., 2002; Reynolds et al., 1994).

As well known the prediction of wine colour and quality based on berry composition is a difficult task but the aim of this trial is to understand (i) if post veraison trimming is effective to slow down the rate of sugar accumulation and (ii) to verify the possible derived alterations after treatment in anthocyanins and tannins accumulation and in their compositional changes during ripening compared to control in *Vitis vinifera* L. cultivar Sangiovese.

2. MATERIAL AND METHODS

This trial was conducted in 2009, 2010 and 2011 on Sangiovese vines (*V. vinifera* L., clone 12T, grafted on 3309C) planted in 1994 in Ozzano Emilia, Bologna, Italy (44°25'N, 11°28'E). Vines were spaced 1.5 m on the row and 3 m between the rows and they were trained to Free Cordon (16 buds per vine).

Twenty-four vines in four blocks (6 vines per block) were randomLy assigned, within each block, to the following treatments:

a) Non-trimmed control (C);

b) Shoot trimming, 1 week after full veraison, which left 8 nodes on every main shoot (TRIMM).

Trimming was performed when berry sugar concentration reached around 15° -18° Brix and precisely 6th August 2009 (17 days after veraison) 10th August 2010 (17 days after veraison) and 4th August 2011 (6 days after veraison).

2.1. Vegetative and yield data

In each year, before trimming and at harvest, two regressions between shoot length (main and lateral) and their actual leaf area were calculated and used to estimate leaf area of all tested vines. At

trimming, the main and lateral leaves were removed and leaf area was measured with a leaf area meter (LI-3000A, Li-Cor Biosciences, Lincoln, Nebraska, USA). At harvest time, the crops of each individual plant were gathered separately and the number of clusters and their weights were recorded.

Weather data (mean daily air temperature and rainfall) were recorded from April to September in the both years, at a meteorological station located close to the experimental site.

2.2. Berry sampling

In each year, every ten days, from veraison to harvest, samples of 20 berries per plant were collected, pooled per each block (120 berries) and divided in three subsamples (40 each), for determinations of the following parameters:

a) Berry weight and must biochemical parameters (soluble solids, pH, and titratable acidity) on 20 berries performed immediately after sampling;

b) Skin anthocyanins on 10 berries immediately stored in -20° C and kept there until the analyses;

c) Skin and seed tannins on 10 berries immediately stored in -20° C and kept there until the analyses.

2.3. Analyses of main must biochemical parameters

The 20 sample berries were crushed and the must were sieved and used 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 Titratable Acidity (TA) data (expressed as g/l of tartaric acid equivalents).

2.4. Analyses of skin anthocyanins

The berries sampled were weighted before the extraction of anthocyanins. Then the skin was carefully removed from the pulp and immerged in 50 mL of HPLC-grade methanol (Carlo Erba Reagents, Milano, Italy); and were kept in dark for 24 hours. The day after, the extract was separated from the skin residues and conserved at (-20°C) until the analysis.

HPLC separation and quantification of anthocyanins 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 mmx4mm (5µM), with precolumn. The eluents used were the following: A= HClO4 0.3% in milliQ water; B = methanol.

The methanol extract was filtered through 0.22 µm, 13mm PTFE syringe-tip filters (Teknokroma, Barcelona, Spain) and an aliquot of 270µL was diluted to final volume of 1mL 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 (Sigma-Aldrich, ST. Louis, MO, USA).

2.5. Tannins analysis

To extract proanthocyanidins, the sampled berries (skins and seeds) were ground separately to a fine powder under liquid N2. 0.1 gr aliquots were separated and extracted in darkness in 2 mL screw-top Eppendorf tubes for 24 hours with 70% acetone (1.0 mL) containing 0.1% ascorbate to prevent oxidation of polyphenols. Samples were then centrifuged (15 min, 13,000 g) and two 400 µL aliquots of the supernatant were transferred to fresh tubes and dried down under vacuum at 35ºC for 120 minutes.

One of them was re-suspended in 100 µL methanol acidified with 1% HCl then neutralised with 100 µL sodium acetate (200 mM, pH 7.5) for the analysis of free monomers. The other aliquot underwent acid-catalysed cleavage of the proanthocyanidins in the presence of excess phloroglucinol following the method of Kennedy and Jones (2001). Briefly, the dried sample was resuspended in 100 µL of phloroglucinol buffer (0.25 g ascorbate, 1.25 g phloroglucinol, 215 µL conc. HCl, 25 mL methanol) and incubated at 50ºC for 20 minutes, then neutralised with 100 µL of sodium acetate (200 mM, pH 7.5) and centrifuged (15 min, 13000 g).

A 200 µL aliquot each of the acetone extract, the acetone extract after acid catalysed cleavage and the residue after cleavage, was then transferred to Sunbroker 250 µL reduced volume HPLC autosampler vials. Samples were run on a Waters 1525 instrument equipped with a diode array detector (DAD), using a reversed-phase column Phenomenex (Castel Maggiore, BO, Italy) RP18 250 mmx4mm (5µM), with precolumn. A separate reversed-phase HPLC method was developed for the cleaved and uncleaved proanthocyanidin samples reading absorbance at 280 nm. Uncleaved samples, solvent A, 0.2% phosphoric acid, solvent B, 4:1 acetonitrile:0.2% phosphoric acid

(gradient of solvent B: zero min, 0%; 5 min, 10%; 40 min, 10%; 55 min, 17%; 65 min, 19%; 75 min, 19%; 80 min, 100%; 85 min, 100%; 86 min, 0%). Cleaved samples, solvent A, 0.2% acetic acid, solvent B, methanol (gradient of solvent B: zero min, 1%; 40 min, 1%; 120 min 30%; 120.1 min, 100%; 125 min, 100%; 126 min, 1%). For both methods, 25 μL of sample was injected and run at 30ºC with a flow rate of 1 mL/min. Concentrations of free monomers and hydrolysed terminal subunits were determined from standard curves prepared from commercial standards of catechin, epicatechin, epigallocatechin and epicatechin-gallate obtained from Extrasynthese (France). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy & Jones 2001).

3. RESULTS AND DISCUSSION

3.1. Climatic data

The Growing Degree Days (GDD, 10 °C baseline) from April 1st to September 30th (Table 8, Fig. 37) were highest in 2011 (2054) and lowest in 2010 (1718). Moreover the average daily temperature recorded from veraison to harvest in 2011 was mostly (70% of the period) above 25° C. In addition, in 2011 the level of the rainfall from April to September was only 136 mm, whereas in 2010 the rainfall in the same period was found to be three times more (Table 8, Fig. 37.).

Table 8. The climatic conditions in 2009, 2010 and 2011.

Year	Rain from April to September (mm)	DD from April to September (°C)	Number of days with average daily temp. > 25° C from veraison to harvest
2009	269	1963	24
2010	390	1718	7
2011	136	2054	29

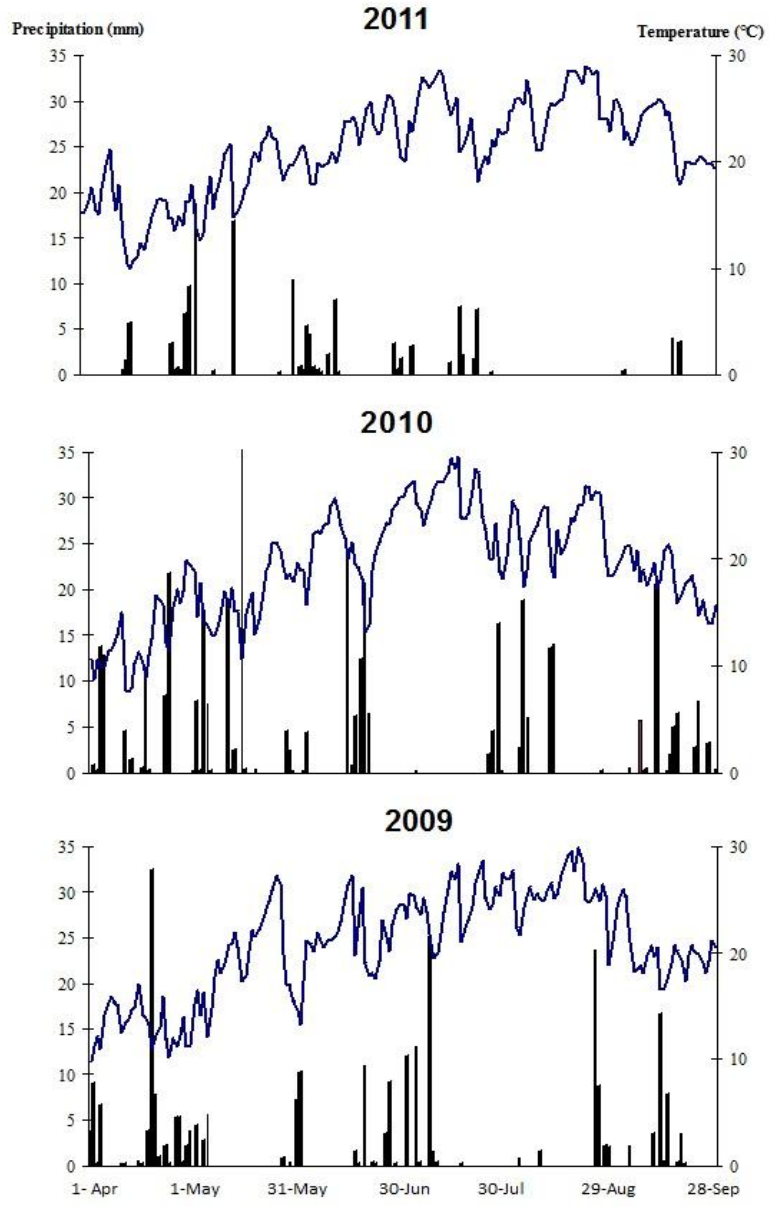

Fig. 37. The yearly recorded rainfall and daily average temperature from April 1 to September of 2009, 2010 and 2011.

3.2. Vegetative and productive traits

All vines were quite uniform in terms of leaf area before trimming in all years (data not shown). The TRIMM treatment induced a leaf area reduction around 60 % in 2009 and 2010 and around 40 % in 2011, compared to control. The trimming of the upper part of the canopy affected mostly the laterals compared to the main leaves, especially in 2010 (Table 9). It is interesting to note that in 2010 a significant higher leaf area was recorded compared to 2009, probably due to the more favourable seasonal climatic conditions during the first phase of shoots growth. No laterals re-growth was measured after treatments in all years.

Starting from a uniform bunch number per meter of cordon, yield, cluster weight and berry weight were unaffected by treatments in 2009 (Table 9). In 2010 and 2011 yield and cluster weight were significantly lower in TRIMM vines compared to control, while berry weight didn't show any difference. A year-by-year variability in terms of yield was verified. In 2010 yield was significantly higher than in 2009 and 2011 for both treatments, and, due to the setting of uniform buds and clusters number per vine, it might be almost entirely a result of cluster weight increase (Table 9). Berry weight did not differ between treatments during ripening (Fig.38) in all the tested years. Moreover, the number of berries per cluster was significantly lower in TRIMM vines compared to C in 2010 and 2011 (Table 9)

At harvest, the leaf area/yield ratio was reduced in TRIMM vines to very low values corresponding to 0.38 and 0.39 m^2/kg in 2009 and 2010 respectively, compared to 1.01 and 0.82 m^2/kg in the control vines which are considered optimal for ripening in Free Cordon (Table 9). In 2011, probably due to the low yield level registered in TRIMM vines, the leaf area yield ratio reached a value of 0.93 m^2/kg, which was not significantly different from C and that may allow an optimal ripening (Table 9).

Despite we did not evaluate the starch content in the different parts of the tested vines, it would be expected that the TRIMM vines, subjected to leaf area reduction ranging from 40 to 60 % after veraison, induced further remobilization of reserves (Candolfi-Vasconcelos et al. 1994). The possible carbohydrate reserve depletion in 2009 and 2010 induced by defoliation could be responsible for yield constraint mainly due to reduction of flower numbers per inflorescence (Scholefield et al. 1977, Bennett et al. 2005).

Table 9. Vegetative growth and yield components recorded over 2009–2011 in C and TRIMM vines.

Parameters	**2009**		**2010**		**2011**		**Year effect**	**Year x Treatment Interaction**
	C	**TRIMM**	**C**	**TRIMM**	**C**	**TRIMM**		
Main LA after trimming (m^2/m of cordon)	1.44 a	0.56 b	1.77 a	0.96 b	1.21 a	0.71 b	ns	ns
Laterals LA after trimming (m^2/m of cordon)	0.79 a	0.25 b	1.12 a	0.18 b	0.59 a	0.30 b	ns	ns
Total LA after trimming (m^2/m of cordon)	2.23 a	0.81 b	2.89 a	1.14 b	1.80 a	1.01 b	*	ns
Cluster (n/m of cordon)	10.5 a	10.6 a	11.1 a	10.2 a	10.1 a	10.2 a	ns	ns
Yield (kg/m of cordon)	2.2 a	2.1 a	3.5 a	2.9 b	1.43 a	1.08 b	**	ns
Cluster weight (g)	210.8 a	196.6 a	312.1 a	286.8 b	147.3 a	104.5 b	**	ns
Berry weight (g)	1.55 a	1.53 a	1.70 a	1.69 a	1.28 a	1.23 a	*	ns
Berries per cluster (n)	136a	128.5a	183.5a	169.7b	115.0a	85.0b	*	ns
Leaf area /yield (m^2/kg)	1.01a	0.38b	0.82a	0.39b	1.25a	0.93a	*	ns

Different letters indicate mean separation between treatments C and TRIMM within rows and years by t -test. *P = 0.05; **P = 0.01. year x treatment interaction, indicate mean separation within rows and years by t-test.

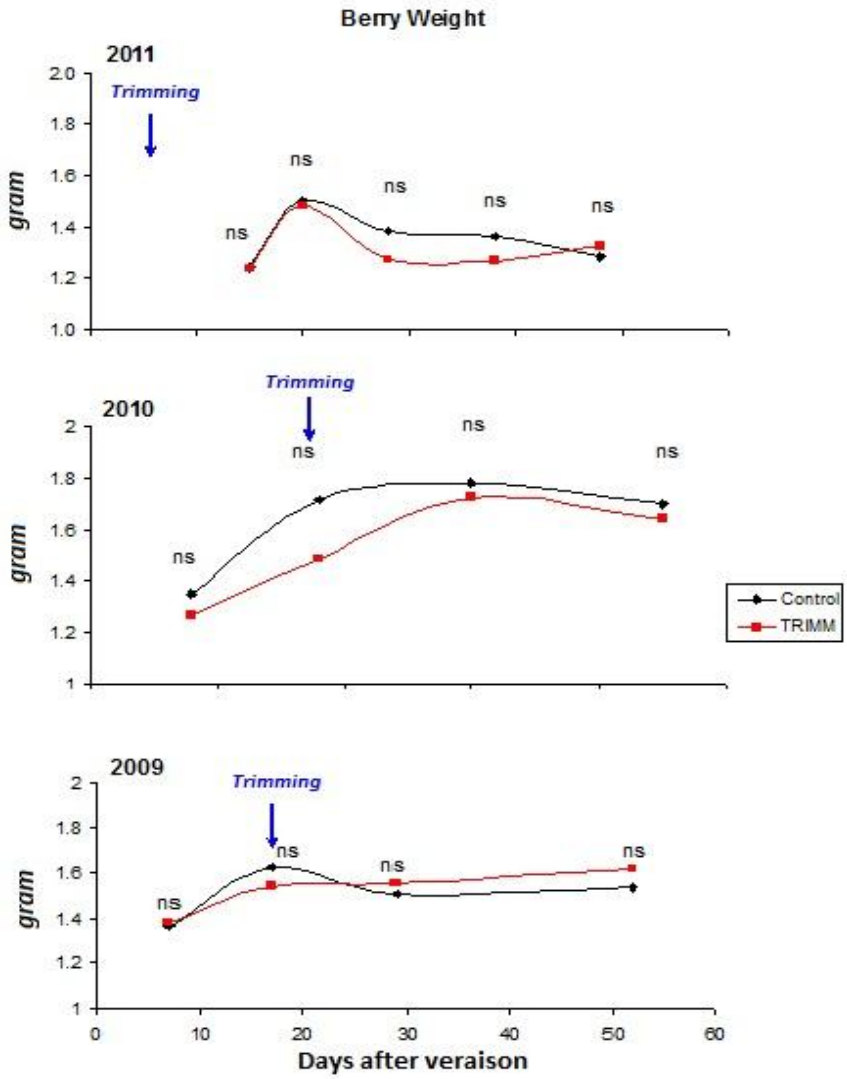

Fig. 38. Berry weight in the control and trimmed vines from veraison to harvest of the years 2009, 2010 and 2011.

3.3. Ripening analyses: Soluble solids, pH and Total Acidity

In 2009 and in 2010 TRIMM vines had significant lower soluble solids concentration (°Brix) at harvest compared to C but no significant effect was recorded in 2011(Fig. 39). TA and pH did not show any difference between treatments in all years (Table 10). Daily soluble solids accumulation rate has been reduced after trimming in 2009 and 2010 of 8 % and 16% and not at all in 2011 compared to control vines.

In 2011, the extremely hot and dry climatic conditions led to an acceleration in the accumulation of soluble solids; so that 23 days after veraison the °Brix level of C reached the 24 °Brix, (Fig. 39) while, in 2009 and 2010, this value was reached respectively 42 and 58 days after veraison. Under

such circumstances, associated with a general lower level of yield in 2011 compared to the previous years, the grapes of TRIMM vines did not show differential accumulation rate and no significant difference was recorded in berry composition compared to Control in this year. This result is in agreement with the high value of leaf area to yield ratio (Table 9), measured in TRIMM vines in 2011, which is considered adequate to allow an optimal ripening.

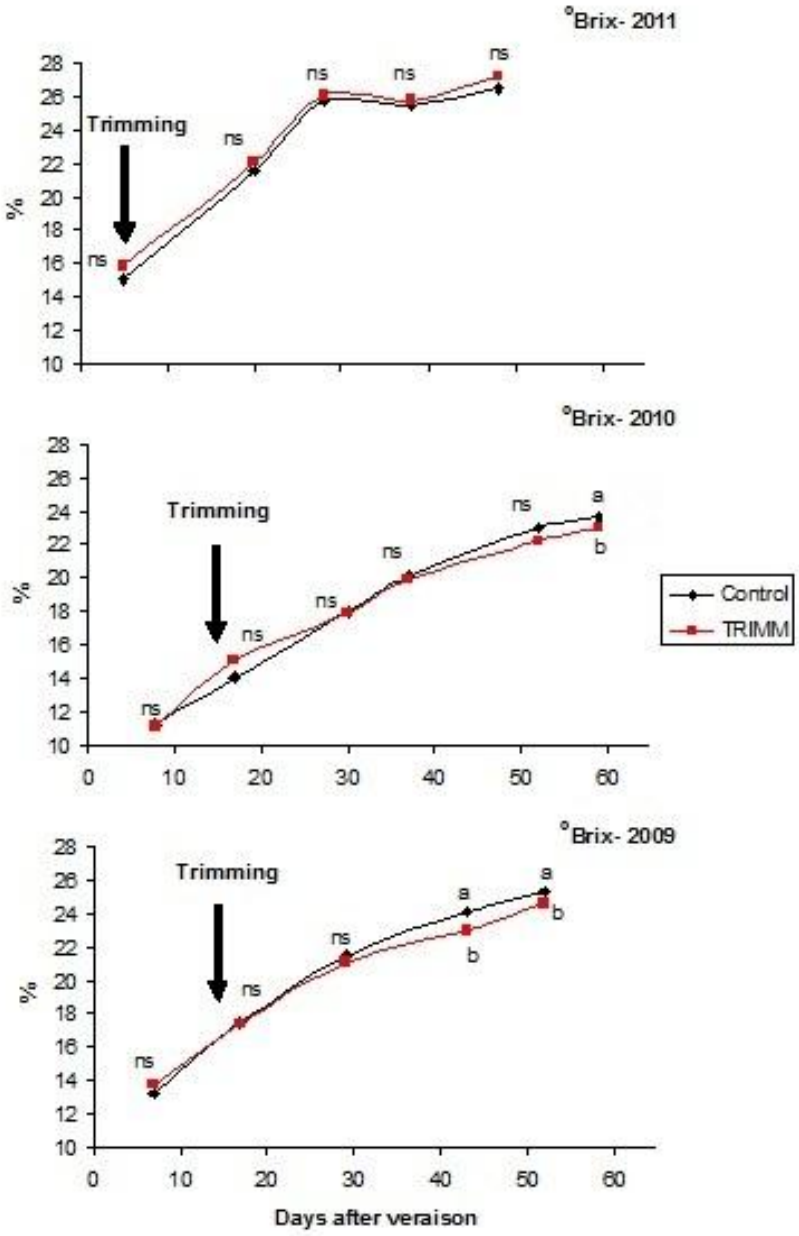

Fig. 39. °Brix in the control and trimmed vines from veraison to harvest of the years 2009, 2010 and 2011

3.4. Anthocyanins accumulation during ripenning

Total anthocyanin concentration (mg/kg) did not differ among treatments during ripening in all the experimental years (Fig.40) and no variation was registered at harvest (Table 10). Although no difference was recorded among the treatments, the anthocyanins concentrations were different among the years (Fig.40). The lowest anthocyanin value at harvest was registered to be in 2009 and the maximum was observed in 2011 (Fig.40). These results do not seem correlated with the different temperature trend in the experimental years; because in the both 2009 and 2011 the temperature exceeded to the critical value for anthocyanin accumulation for periods with similar length. Having the lowest berry weight in 2011 is probably the reason of the highest anthocyanin value registered at harvest in the grapes of both treatments as compared to the previous years (Table 9, 10).

Notably, no relation has been verified between the reduction in soluble solids accumulation, occurred in 2009 and in 2010, and variation in anthocyanins accumulation.

In particular in 2009, when the trimming was effective in slowing down the rate of sugar accumulation the correlation between total anthocyanin content (mg per berry) and berry juice soluble solids concentration showed similar curves for TRIMM and C (Fig. 41). Both treatments showed an increasing positive trend between 10° Brix to 22° Brix where there is a peak concentration and a decline in anthocyanins during the latter stage of ripening (Fig. 41). Our results are in acordance with Guidoni et al., (2008) who demonstrated that anthocyanins may accumulate in a two-phase process, with an initial phase of rapid increase and a second phase where anthocyanins and sugars are uncoupled.

As other authors pointed out, sugar accumulation was shown to be more strictly associated to thermal time, whereas anthocyanin accumulation was also affected by other sources of variations (water, yield, and climatic conditions) (Sadras et al., 2007) and in general displays a much wider range of variation than that of sugars.

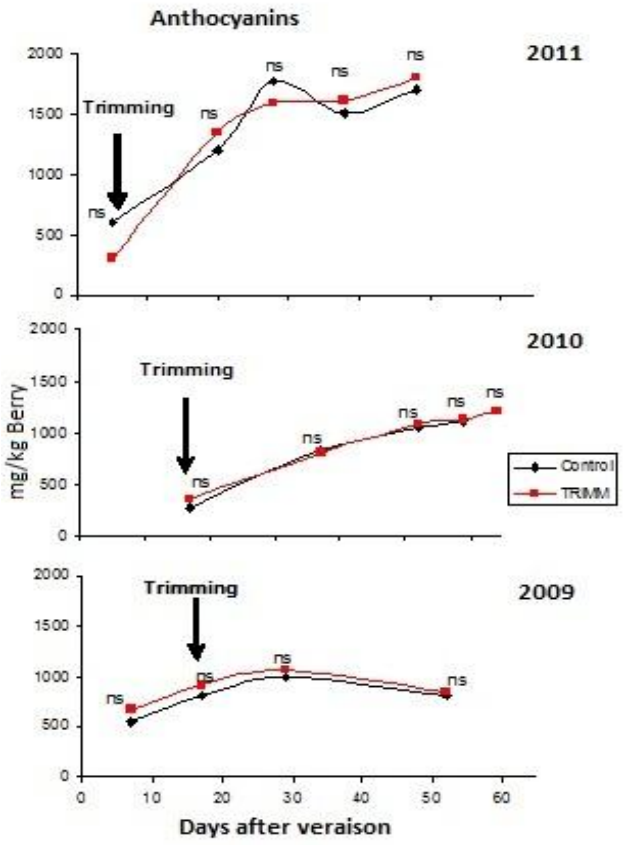

Fig. 40. The anthocyanin concentration in the control and trimmed vines from veraison to harvest of the years 2009, 2010 and 2011.

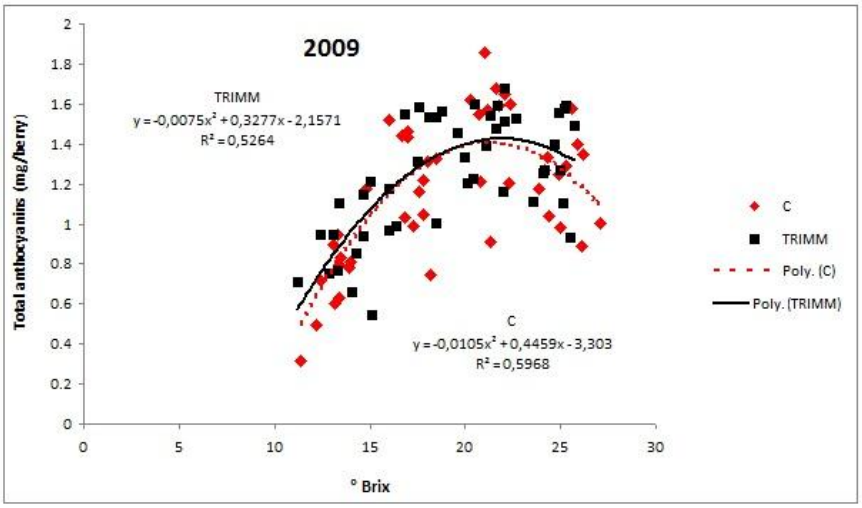

Fig. 41. The correlation between the total anthocyanin content and berry soluble solids concentration in the control and trimmed vines from veraison to harvest of the year 2009.

Besides, the analysis of the anthocyanin composition at harvest did not show significant difference between the treatments in all the three years. The percentage of delfinidid 3-G, cyanidin 3-G, peonidin 3-G, petunidin -3-G and malvidin 3-G in berries of TRIMM and C vines showed the typical Sangiovese grape profile with high level of cyanidin 3-G and malvidin 3-G (Fig. 42). It seems that the biosynthesis of each individual anthocyanin is not influenced by the variation of sugar concentration in the berries at harvest as occurred in 2009 and 2010.

Moreover, the trimming treatment did not modify cluster microclimatic conditions which are found to be responsible for anthocyanin profile changes as reported after cluster shading (Downey et al., 2004) and temperature variation (Mori et al., 2005).

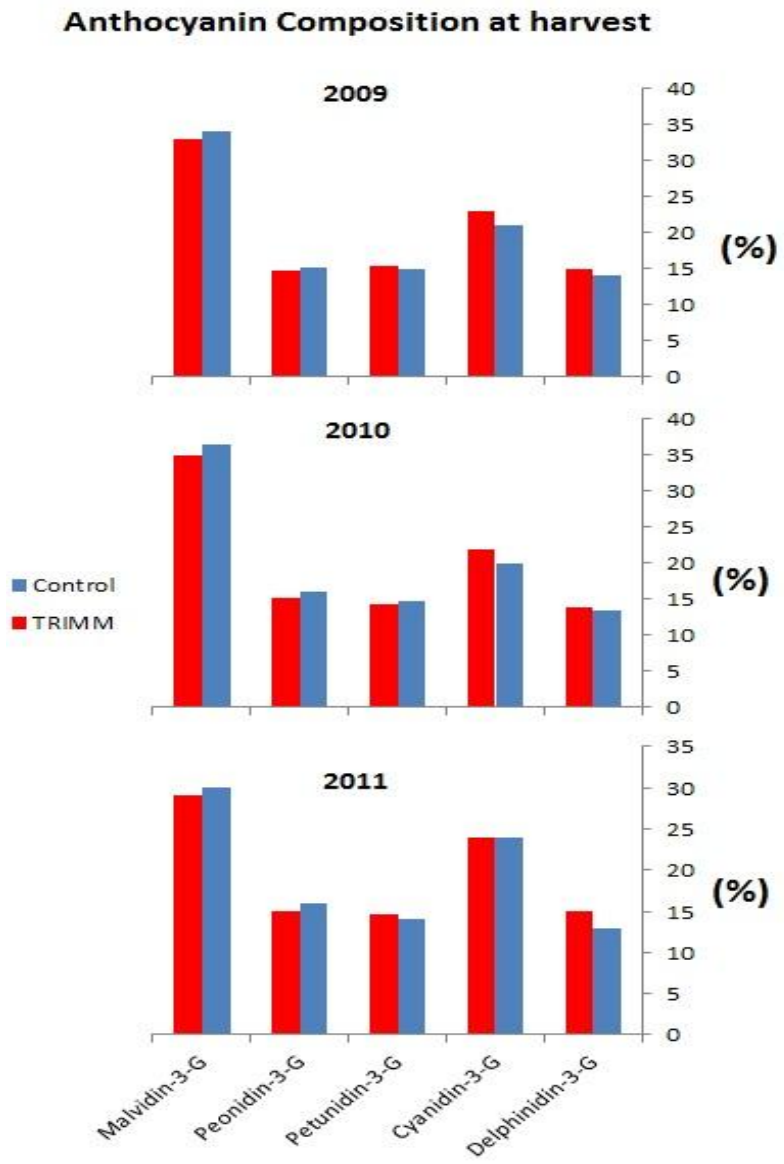

Fig. 42. The anthocyanin profile in the berry skin of control and trimmed vines at harvest of the years 2009, 2010 and 2011.

3.5. Tannins accumulation during ripenning

In all experimental years, trimming did not affect the evolution of seed and skin tannins concentration expressed as mg per kg of berries during ripening and at harvest (Table 10, Fig. 43, 45 and 47). The tannins were expressed as mg per kg of berries as no differences were recorded between treatments in berry weight during ripening (Fig. 38).

Similar to what was previously reported on different varieties (Harbertson et al., 2002, Kennedy et al., 2002; Downey et al., 2003), the levels of seed tannins were higher compared to skin tannins at veraison, but declined during ripening. The levels of berry skin and seed tannins reached to nearly equal levels at harvest (Table 10, Fig. 43, 45 and 47). By evaluating the seed monomers and polymers tannins, the similar trends were observed in all treatments during ripening and their concentration (mg/kg of berries) in seed polymers was considerably higher than in its monomers, as expected.

As well as that, the berry tannins composition was assessed from veraison to harvest in each year; and the evolution of all four flavan-3-ols (catechin, epicatechin, epigallocatechin and epicatechin-gallate) followed the same tendency between treatments (Fig. 44, 46 and 48); And among them, epicatechin is the major and catechin, is the minor constituent of berry tannins (Fig. 44, 46 and 48).

Our results assessed that the concentration and composition of each group of tannins compounds at harvest were not affected by trimming treatment after veraison also when this technique was able to reduce sugar accumulation rate. Moreover it appeared that the concentration of each flavan 3 ols (catechin, epicatechin, epigallocatechin and epicatechin-gallate) remains almost constant in the last thirty days before harvest, revealing that both tannins composition and content may be not responsable of different level of astringency in wine obtained from grapes at different degree of ripening. In other words our results seem to assess a very low effectiveness of the determinations of each compound in prediction of wine astringency traits. As previously pointed out by several authors the extractability and solubility of each group of flavonoid compounds during ripening may play an important rule to understand the relationships between berry composition, wine composition and sensory properties of wine.

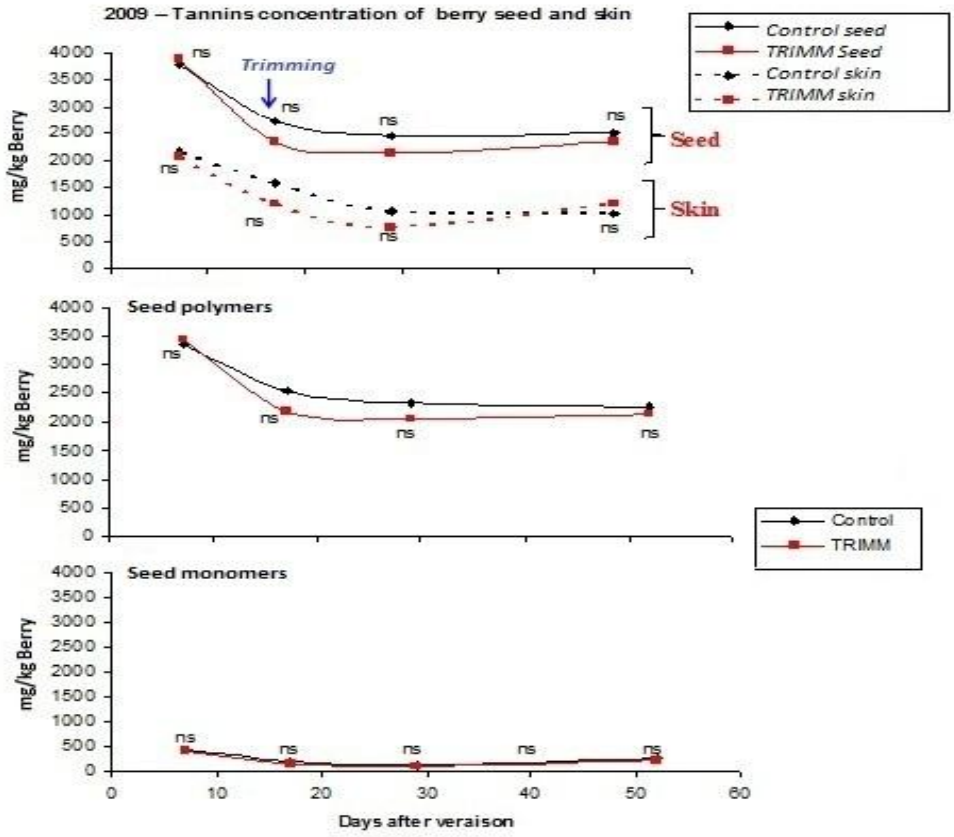

Fig. 43. The tannins concentration of berry seed and skin in the control and trimmed vines from veraison to harvest of the year 2009.

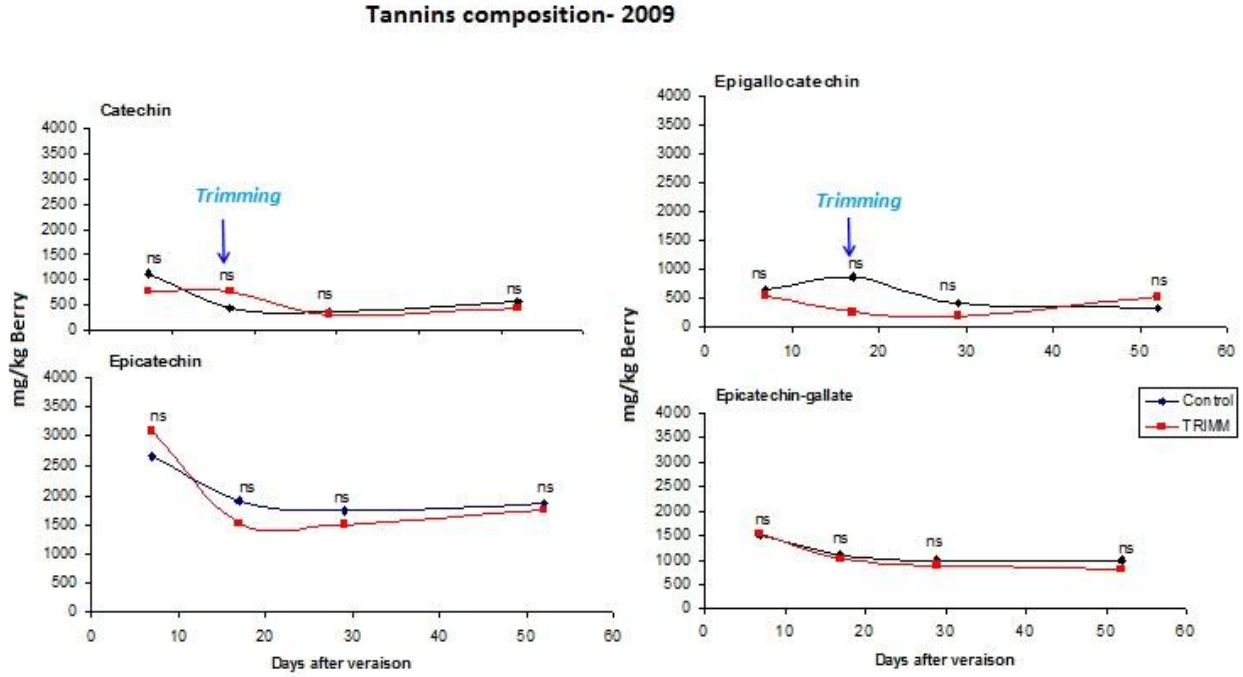

Fig. 44. The tannins composition in the control and trimmed vines from veraison to harvest of the year 2009.

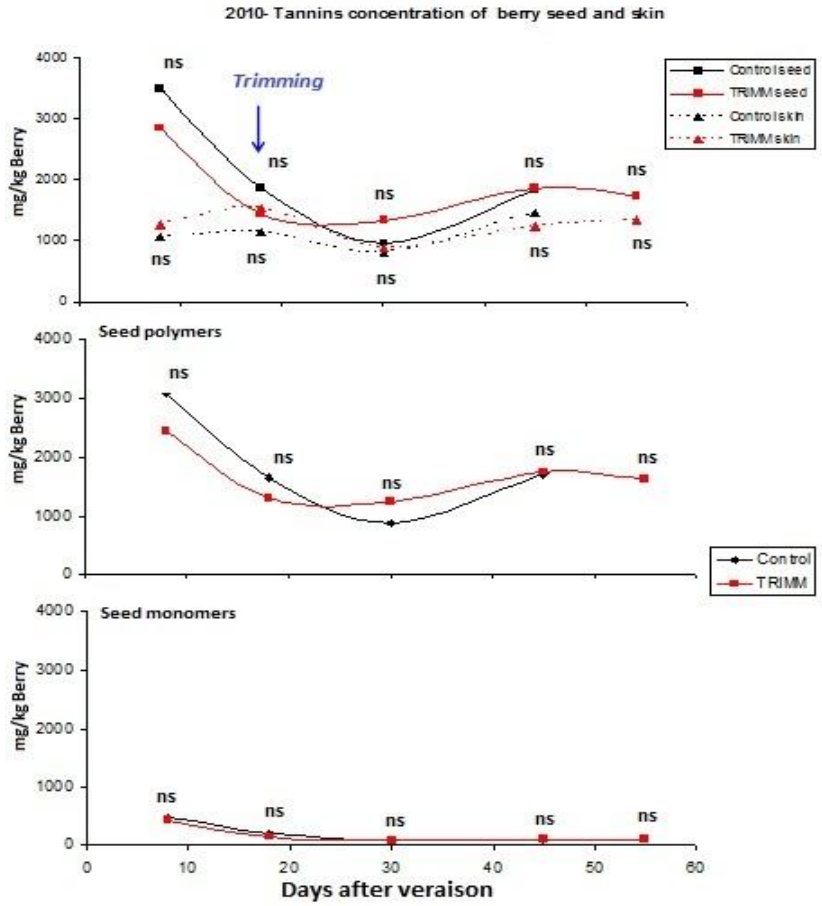

Fig. 45. The tannins concentration of berry seed and skin in the control and trimmed vines from veraison to harvest of the year 2010.

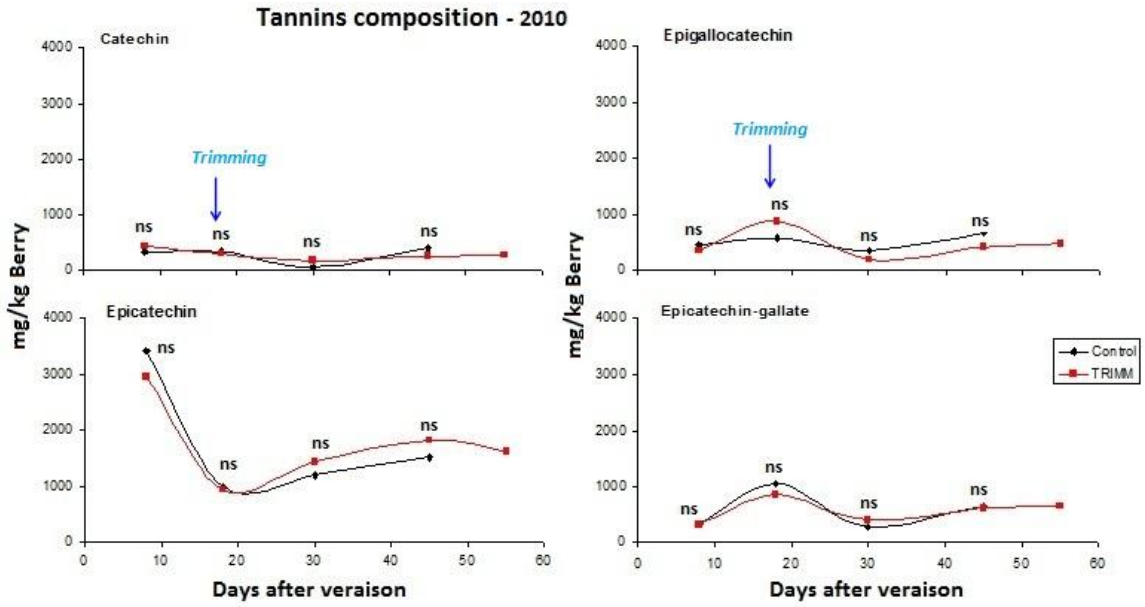

Fig. 46. The tannins composition in the control and trimmed vines from veraison to harvest of the year 2010.

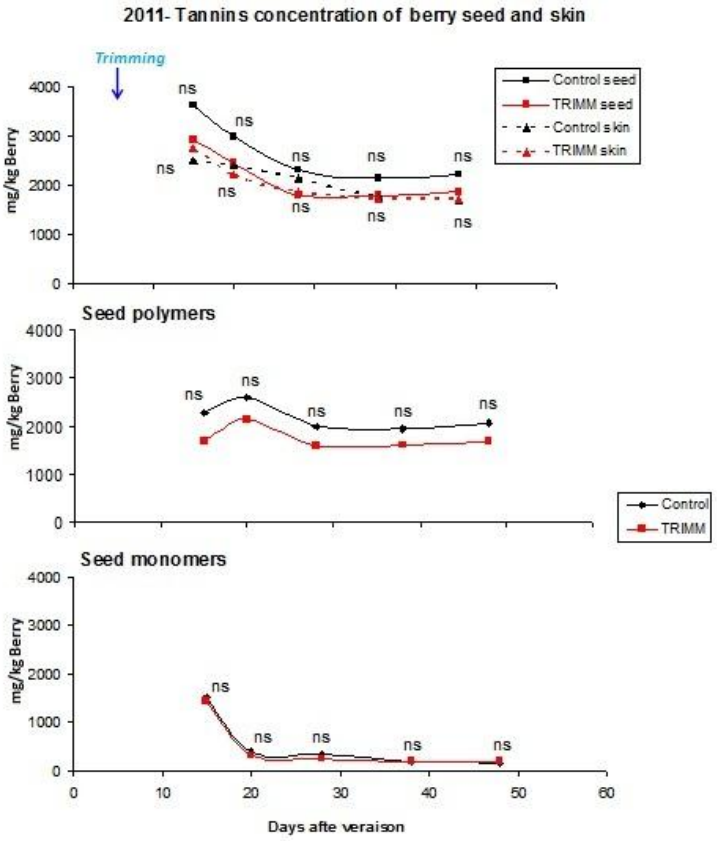

Fig. 47. The tannins concentration of berry seed and skin in the control and trimmed vines from veraison to harvest of the year 2011.

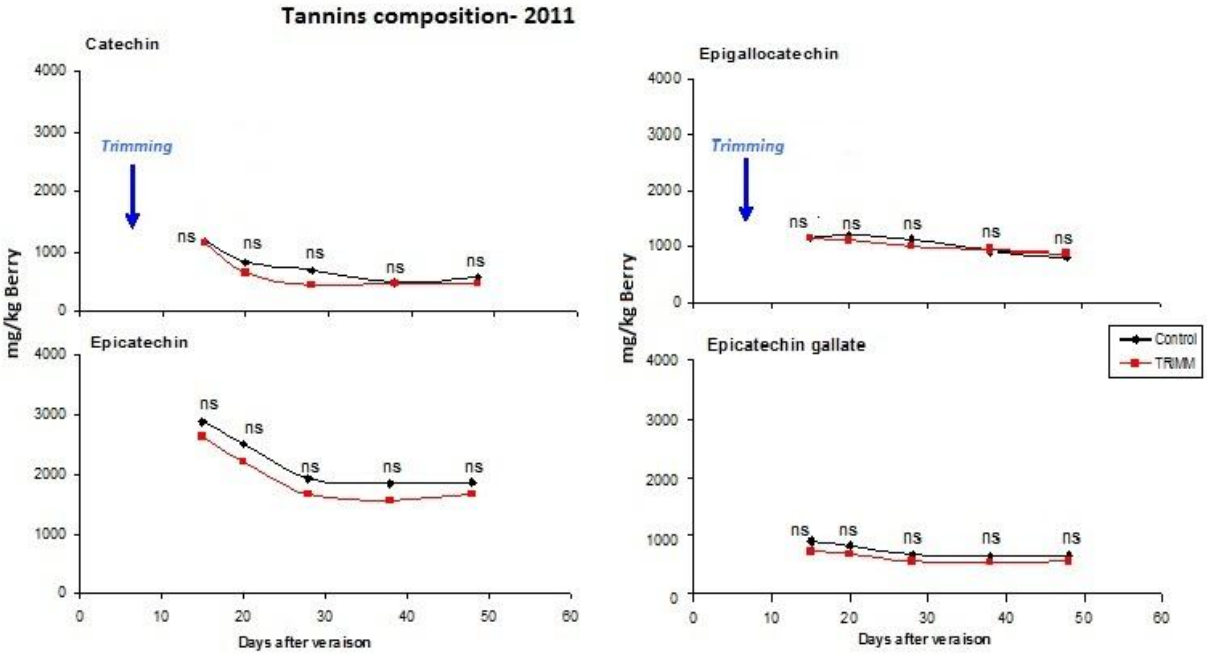

Fig. 48. The tannins composition in the control and trimmed vines from veraison to harvest of the year 2011.

Table 10. Berry composition in the control and trimmed vines at harvest of the years 2009, 2010 and 2011.

Parameters	2009		2010		2011		Year x Treatment Interaction
	C	TRIMM	C	TRIMM	C	TRIMM	
Soluble solids (°Brix)	25.2 a	24.6 b	23.7 a	23 b	26.4 a	27.2 a	ns
Total acidity (g/l)	7.38 a	7.23 a	7.79 a	7.96 a	8.44 a	8.91 a	ns
pH	3.53 a	3.47 a	3.39 a	3.36 a	3.39 a	3.36 a	ns
Total anthocyanins (mg/kg berries)	796.1 a	838.1 a	1117.7 a	1120.0 a	1708.0 a	1810.0 a	**
Total tannins	3506 a	3547 a	3330 a	3107 a	3699 a	3522 a	ns
Total tannins in skin	1006 a	1196 a	1466 a	1243 a	1697 a	1722 a	ns
Total tannins in seed	2600 a	2352 a	1864 a	1864 a	2002 a	1800 a	ns

4. CONCLUSION

The source limitation by trimming after veraison, showed some perspectives to achieve lower sugar accumulation in grape and lower alcohol potential in wines, without inducing modification in the evolution of the main assessed flavonoids compounds (skin anthocyanins, total seeds and skin tannins concentration and profile) in Sangiovese berries.

The efficiency of this technique seems to be strongly depending from the vines starch supply as the possible carbohydrate reserve depletion induced year over year by trimming, may be responsible for self regulation mechanism of yield constraint verified in the second and third year of the trial. In this condition the leaf area cutback after veraison is counterbalanced by the yield lowering and no effects is obtained in lowering sugar accumulation as the leaf area /yield ratio is maintained to high level. Probably the low fertility condition of the vineyard site and the lack of irrigation system may have promoted this mechanism.

Further researches on photosynthetic limitation after veraison to reduce sugar accumulation will be focused on vines in different soil fertility condition and on the evaluation of the extractability of tannins from seeds and skins in wines or wine-like solutions derived from this treatment.

5. GENERAL CONCLUSION

Wine grape must deal with serious problems which occur due to the unfavorable climatic conditions resulted from global warming.

The excessive elevated temperatures are critical for grapevine and contribute to degradation of grape and wine quality. As an illustration, elevated temperature is an important factor that can negatively affect anthocyanin accumulation in grape. Particularly, cv. Sangiovese, was identified to be very sensitive to such condition. The expressions of the genes involved in flavonoid biosynthesis were observed to be affected by temperature. The quantitative real-time PCR analysis showed that flavonoid biosynthetic genes were slightly repressed by high temperature. As well as that, the heat stress showed to repress the expression of the transcription factors VvMYBA1 that activates the expression of UFGT.

Moreover high temperatures showed to had repressing effects on the activity of the flavonoids biosynthetic enzymes PAL and UFGT.

Anthocyanin accumulation in berry skin is due to the balance between its synthesis and oxidation.

In grape cv. Sangiovese, the gene transcription and activity of peroxidases enzyme was found to be elevated by heat stress as a defensive mechanism of ROS-scavenging.

Even if different antioxidant enzymes have been recognized to be involved in flavonoids degradation under various abiotic stresses, this research identifies peroxidases as one of the best candidate to oxidize anthocyanins actively.

Between many isoforms of peroxidases genes, which are expressed in grape berry skin from veraison to harvest, one peroxidase gene (POD 1) induced in Sangiovese grape under thermal stress condition, was isolated and evaluated via the technique of genes transformation from grape to Petunia flower. Reduction in anthocyanins concentration in POD 1 transformed Petunia flowers was assessed after thermal stress conditioning compared to untrasformed control.

Biochemical and molecular studies confirmed the role of peroxidases enzyme in active anthocyanins degradation in Sangiovese cv. grape skin under temperature stress conditions. Based on our results, a similar mechanism that will be object of further investigation may be supposed for flavonols degradation. In conclusion this study showed that the temperature regimes after véraison have different effects on each flavonoid group. Although the anthocyanin, proanthocyanidin and flavonol share the main part of their biosynthetic pathway, no effects were obtained regarding any changes in the biosynthesis of the proanthocyanidins under these different thermal ripening conditions.

Moreover, the present study is the pioneer to approve that the mechanism of anthocyanin loss in grape skins due to high temperature involves not only the inhibition of anthocyanin biosynthesis, but also its enzymatic degradation.

What is more, in wine producing regions, it is inevitable for the grape growers to adopt some adaptive strategies to alleviate grape damages to abiotic stress conditions. It is necessary to ameliorate the vintage through improvement of management techniques to face the impacts of undesirable climatic conditions. Therefore, in this thesis, the technique of post veraison trimming was done to improve the coupling of the phenolic and sugar ripening in *Vitis vinifera* L. cultivar Sangiovese.

Trimming after veraison, allowed to limit the photosynthetic source and showed to be executable to slow down the rate of sugar accumulation in grape (to decrease the alcohol potential in wines) without inducing modification in the evolution of the main berry flavonoids compounds.

Moreover the present research showed that the efficiency of this technique applied year after year is related to the ability of the vines to recover charboidrate reserve to prevent self regulation mechanism of yield constraint.

Further researches need to be performed on the evaluation of the extractability of tannins from seeds and skins in wines or wine-like solutions derived from grape on vines subjected to post veraison photosynthetic limitation.

REFERENCES

Adams, D., 2006. Phenolics and ripening in grape berries. J. Am. J. Enol. Vitic. Vol. 57 (3): 249-256.

Alfenito, M.R., Souer, E., Goodman, C.D., 1998. Functional complementation of anthocyanin sequestration in the vacuole by widely divergent Glutathione Stransferases. J. The Plant Cell. Vol. 10:1135-1149.

Aron, P.M., Kennedy, J.A., 2008. Flavan-3-ols: Nature, occurrence and biological activity. J. Mol. Nutr.Food Res., Vol. 52: 79-104.

Bavaresco, L., Fregoni, C., 2001. Physiological role and molecular aspects of grapevine stilbenic compounds. In: K. A. Roubelakis-Angelakis (Ed.): Molecular biology and biotechnology of the grapevine, pp: 153-182.

Bennett, J., Jarvis, P.,Creasy, G., Trought, M. 2005. Influence of defoliation on overwintering carbohydrate reserves, return bloom, and yield of mature Chardonnay grapevines. J. Am. J. Enol. Vitic. Vol. 56(4): 386-393.

Bergqvist, J., Dokoozlian, N., Ebisuda, N., 2001. Sunlight exposure and temperature effects on berry growth and composition of Cabernet Sauvignon and Grenache in the central San Joaquin Valley of California. J. Amer. J. Enol. Vitic. Vol. 52 (1): 1-7.

Bindi, M., Fibbi, L., Gozzini, B., Orlandini, S., Miglietta, F., 1996. Modelling the impact of future climate scenarios on yield and yield variability of grapevine. J. Clim. Res. Vol. 7: 213–224.

Bogs, J., Downey, M.O., Harvey, J.S., Ashton, A.R., Tanner, G.J., Robinson, S.P., 2005. Proanthocyanidin Synthesis and Expression of Genes Encoding Leucoanthocyanidin Reductase and Anthocyanidin Reductase in Developing Grape Berries and Grapevine Leaves. J. Plant Physiol. Vol. 139: 652–663.

Bogs, J., Ebadi, A., McDavid, D., Robinson. S.P., 2006. Identification of the flavonoid hydroxylases from grapevine and their regulation during fruit development. J. Plant Physiol. Vol. 140(1): 279-291.

Boss, P.K., Davies, C., Robinson, S. P., 1996. Analysis of the expression of anthocyanin pathway genes in developing Vitis Vinifera L. cv Shiraz grape berries and the implications of pathway regulation. J. Plant Physiol. Vol. 111: 1059-1066.

Boulton, R., 2001. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. J. Am. J. Enol. Vitic. Vol. 52 (2): 67-87.

Braidot, E., Zancani, M., Petrussa, E., Peresson, C., Bertolini, A., Patui, S., Macrì, F., Vianello, A., 2008. Transport and accumulation of flavonoids in grapevine (Vitis vinifera L.). J. Plant signaling and behavior. Vol. 3(9): 626 - 632.

Broun, P., 2005. Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in Arabidopsis. J. Current Opinion in Plant Biology. Vol. 8(3): 272-279.

Bucchetti, B. 2004. Effetti biochimici e molecolari della luce sulla maturazione dell'uva nella cv. Sangiovese. Tesi di Dottorato di ricerca in Colture Arboree ed Agrosistemi Forestali e Paesaggistici, XVII ciclo, Universita di Bologna.

Bucchetti, B., Pastore, C., Filippetti, I., Intrieri, C., 2007. Effetti di diverse tecniche colturali sulla sintesi di antociani in Sangiovese. Poster VIII GIORNATE SCIENTIFICHE SOI. 8-11/05/2007 (SS).

Buchanan-Wollaston, V., Morris, K., 2000. Senescence and cell death in Brassica napus and Arabidopsis. In Programmed Cell Death in Animals and Plants (Garland, J.M., ed.), BIOS Scientific Publishers, pp: 163–174.

Butterfield, R.E., Gawith, M.J., Harrison, P.A., Lonsdale, K.J., Orr, J., 2000. Modelling climate change impacts onwheat, potato and grapevine in Great Britain: *in* Downing, T. E., Harrison, P. A., Butterfield, R. E. and Lonsdale, K.G., (eds.): Climate Change, Climatic Variability and Agriculture in Europe: An Integrated Assessment. Final Report: Environmental Change Institute, University of Oxford, 411 p.

Buttrose, M.S., Hale, C.R., Kliewer, W.M., 1971. Effect of temperature on composition of 'Cabernet Sauvignon' berries. J. Amer. J. Enol. Vitic. Vol. 22 (2): 71–75.

Calderon, A.A., Garciaflorenciano, E., Munoz, R., Barcelo, A. R., 1992. Gamay grapevine peroxidase its role in vacuolar anthocyani(di)n degradation. J. VITIS. Vol. 31(3): 139–147.

Candolfi-Vasconcelos, M., Candolfi, M., Kohlet, W. 1994.Retranslocation of carbon reserves from the woody storage tissues into the fruit as a response to defoliation stress during the ripening period in Vitis vinifera L.

Castellarin, S.D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., Di Gaspero, G., 2007. Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. J. Plant, Cell and Environment. Vol. 30: 1381–1399.

Castellarin, S.D., Di Gaspero, G., Marconi, R., Nonis, A., Peterlunger, E., Paillard, S., Adam-Blondon, A.F., Testolin, R., 2006. Colour variation in red grapevines (*Vitis vinifera* L.): genomic

organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin**. J.** *BMC Genomics.* Vol. 7(12): 1471-2164

Chen, G., Asada, K., 1989. Ascorbate Peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. J. plant cell physiol. Vol. 30 (7): 987-998.

Chen, J., Wen, P., Kong, W., Pan, Q., Wan, S., Huang, W. 2006. Changes and subcellular localizations of the enzymes involved in phenylpropanoid metabolism during grape berry development. J. Plant phisiol. Vol. 163 (2): 115-127.

Cheynier, V., Rigaud, J., 1986. HPLC separation and characterization of flavonols in the skin of Vitis Vinifera var. Cinsault. J. Am. J. Enol. Vitic. Vol. 37: 248-252.

Christie, P., Alfenito, M. R., Walbot, V., 1994. Impact of low temperature stress on general phenyl propanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. J. Planta. Vol. 194: 541–549.

Chua, C.S., Biermann, D., Goo, K, S., Sim, T., 2008. Elucidation of active site residues of Arabidopsis thaliana flavonol synthase provides a molecular platform for engineering flavonols. J. Phytochem. Vol. 69 (1): 66–75.

Cohen, S., Tarara, J., Kennedy, J. 2012. Diurnal temperature range compression hastens berry development and modifies flavonoid partitioning in grapes. J. Am. J. Enol. Vitic. vol. 63(1): 112-120.

Conde, C., Silva, P., Fontes, N., Dias, A., Tavares, R. M., Sousa, M., Agasse, A., Delrot, S., Gerós, H. 2007. Biochemical changes throughout grape berry development and fruit and wine quality; http://hdl.handle.net/1822/6820

Conner, A.J., Albert, N.W., Deroles, S.C., 2009. Transformation and regeneration of petunia. C Springer Springer Science+Business Media, LLC. Chapter 19. pp: 395-409.

Coombe, B. G., 1992. Research on Development and Ripening of the Grape Berry. J. Am. J. Enol. Vitic. Vol. 43(1): 101-110.

Cortell, J.M., M. Halbleib, A.V. Gallagher, T. Righetti, and J.A. Kennedy. 2007a. Influence of vine vigor on grape *{Vitis vinifera* L. cv. Pinot noir) anthocyanins. 1. anthocyanin concentration and composition in fruit. J. Agrie. Food Chem. 55:6575-6584

Darne´, G., 1993. New hypothesis on anthocyanin biosynthesis in berries and leaves of grapevine. J. Vitis. Vol. 32: 77-85.

Dat, J.F., Foyer, C.H., Scott, I.M., 1998. Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. J. Plant Physiol. Vol. 118: 1455–1461.

Davies, K.M., Schwinn, K.E., 2003. Transcriptional regulation of secondary metabolism. J. Functional Plant Biology. Vol. 30(9): 913 – 925.

Dela, G., Or, E., Ovadia, R., Nissim-Levi, A., Weiss, D., Oren- Shamir, M., 2003. Changes in anthocyanin concentration and composition in 'Jaguar' rose flowers due to transient hightemperature conditions. J. Plant Sci. Vol. 164: 333–340.

Deluc, L., Bogs, J., Walker, A.R., Ferrier, T., Decendit, A., Merillon, J.M., Robinson, S.P., Barrieu, F., 2008. The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. J. Plant Physiol. Vol. (147): 2041–2053.

Dokoozlian, N.K., 2000. Grape berry growth and development. Raisin Production Manual, Chapter 5. pp: 30- 37.

Downey, M.O., Dokoozlian, N.K., Krstic, M.P., 2006. Cultural Practice and environmental Impacts on the flavonoid composition of grapes and wine: A review of recent research. J. Amer. J. Enol. Vitic. Vol. 57 (3): 257-268.

Downey, M.O., Harvey, J.S., Robinson, S.P., 2003. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. J. Aust. J. Grape Wine Res. Vol. 9: 15-27.

Downey, M.O., Harvey, J.S., Robinson, S.P., 2003. Synthesis of flavonols and expression of flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay (Vitis Vinifera L.). J. Aust. J. Grape Wine Res. Vol. 9: 110-121.

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

Dry, I. B., Robinson, S. P., 1994. Molecular cloning and characterization of grape berry polyphenol oxidase. J. Plant Molecular Biology. Vol. 26: 495-502.

Duchene, E., Schneider, C., 2005. Grapevine and climatic changes: a glance at the situation in Alsace Agron. J. Sustain. Dev. Vol. 25: 93–99.

Fasoli, M., Dal Santo, S., Zenoni, S., Tornielli, G.B., Farina, L., Zamboni, A., Porceddu, A., Venturini, L., Bicego, M., Murino, V., Ferrarini, A., Delledonne, M., Pezzotti, M., 2012. The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a Maturation Program. J. The Plant Cell, Vol. 24: 3489–3505.

Ferrini, F., Mattii, G.B., Nicese, F.P., 1995. Effect of Temperature on Key Physiological Responses of Grapevine Leaf. J. Am. J. Enol. Vitic. Vol. 46(3): 375-379.

Filippetti, I., 2009. Technological and Phenolic ripening synchronization in winegrape. Data has not been published.

Filippetti, I., Ramazzotti, S., Centinari, M., Bucchetti, B., Intrieri, C. 2007. Effects of cluster thinning on grape composition: experience "Sangiovese" grapevines, Proc. Int. Workshop on Advance in Grapevine and Wine Research.

Fournand, D., Vicens, A., Sidhoum, L., Souquet, J.M., Moutounet, M., Cheynier, V., 2006. Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. J. Agric. Food Chem. Vol. 54: 7331-7338.

Fraser, E., 2008. "Crop yield and climate change", Retrieved on 2009-09-14.

Fujita, A., Soma, N., Goto-Yamamoto, N., Mizuno, A., Kiso, K., Hashizume, K., 2007. Effect of shading on proanthocyanidin biosynthesis in the grape berry. J. Japan. Soc. Hort. Sci., Vol. 76: 112-119.

Fujita, A., Goto-Yamamoto, N., Aramaki, I., Hashizume, K., 2006. Organ-Specific Transcription of Putative Flavonol Synthase Genes of Grapevine and Effects of Plant Hormones and Shading on Flavonol Biosynthesis in Grape Berry Skins. J. Biosci., Biotech., and Biochem. Vol. 70 (3): 632-638.

Gagné, S., Lacampagne, S., Claisse, O., Gény, L., 2009. Leucoanthocyanidin reductase and anthocyanidin reductase gene expression and activity in flowers, young berries and skins of *Vitis vinifera* L. cv. Cabernet-Sauvignon during development. J. Plant Physiol. & Biochem. Vol. 47 (4): 282–290.

Gatto, P., Vrhovsek, U., Muth, J., Segala, C., Romualdi, C., Fontana, P., Pruefer, D., Stefanini, M., Moser, C., Mattivi, F., Riccardo Velasco, R., **2008.** Ripening and genotype control stilbene accumulation in healthy grapes. J. Agric. Food Chem. Vol. 56 (24): 11773–11785.

Gollop, R., Even, S., Colova-Tsolova, V., Peri, A. 2002. Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. J. Exp Bot. Vol. 53: 1397–1409.

Gollop, R., Farhi, S., Perl, A., 2001. Regulation of the leucoanthocyanidin dioxygenase gene expression in *Vitis vinifera*. J. Plant Sci. Vol. 161 (3): 579–588.

Goto-Yamamoto, N., Mori, K., Numata, M., Koyama, K., Kitayama, M., 2009. Effects of temperature and water regimes on flavonoid contents and composition in the skin of red-wine grapes. J. Int. Sci. Vigne Vin, Vol. 43 (1): 75-80.

Goto-Yamamoto, N., Wan, G.H, Masaki, K., Kobayashi, S., 2002. Structure and transcription of three chalcone synthase genes of grapevine (Vitis vinifera). *J. Plant Sci*. Vol. 162: 867-872.

Greer, D. H., Weston, C., 2010. Heat stress affects flowering, berry growth, sugar accumulation and photosynthesis of *Vitis vinifera* cv. Semillon grapevines grown in a controlled environment. *J. Functional Plant Biol*. Vol. 37(3): 206–214.

Guidoni, S., Ferrandino, A., Novello, V. 2008. Effects of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. J.Am. J. Enol. Vitic. Vol. 59(1): 22-29.

Guidoni, S., Ferrandino, A., Novello, V., 2004. Ruolo del clima e degli interventi colturali sulla sintesi degli antociani nell'uva (Vitis Vinifera L. cv Nebbiolo). J. Quad. Vitic. Enol. Univ. Torino, 27.

Guidoni, S., P. Allara, and A. Schubert. 2002. Effect of cluster thinning on berry skin anthocyanin composition of *Vitis vinifera* cv. Nebbiolo. Am. J. Enol. Vitic. V. 53:224-226.

Harbertson, J.F., Kennedy, J.A., Adams, D.O., 2002, Tannin in skins and seeds of Cabernet Sauvignon, Syrah and Pinot noir berries during ripening. J. Am. J. Enol. Vitic. Vol. 53 (1): 54-59.

Hardie, W.J., O'Brien, T.P., Jaudzems, V.G., 1996. Morphology, anatomy and development of the pericarp after anthesis in grape, Vitis vinifera L. J. Aust. J. Grape Wine Res. Vol. 2: 97–142.

Haselgrove, L., Botting, D., Van Heeswijck, R., Høj, P.B., Dry, P.R., Ford, C., Iland, P.G., 2000. Canopy microclimate and berry composition: The effect of bunch exposure on the phenolic composition of Vitis vinifera L. cv. Shiraz grape berries. J. Aust. J. Grape Wine Res. Vol. 6: 141-149.

Hawker, J.S., 1969a. Changes in the activities of enzymes concerned with sugar metabolism during the development of grape berries. J. Phytochem. Vol. 8: 9-17.

Hawker, J.S., 1969b. Insoluble invertase from grapes: an artefact of extraction. J. Phytochem. Vol. 8: 337-344.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., Mullineaux, P.M., 2000. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. J. Plant Mol. Biol. Vol. 42: 819-832.

Hiratsuka, S., Onodera, H., Kawai, Y., Kubo, T., Itoh, H., Wada, R. 2001. ABA and sugar effects on anthocyanin formation in grape berry cultured in vitro. J. Scientia Horticulturae. Vol. 90 (1-2): 121-130.Hrazdina, G., Parsons, G.F., Mattick, L.R., 1984. Physiological and biochemical events during development and maturation of grape berries. J. Amer. J. Enol. Vitic. Vol. 35 (4): 220–227.

Huglin, P., 1978. Nouveau Mode d'Évaluation des Possibilités Héliothermiques d'un Milieu Viticole. C.R. Acad. Agr. France, Vol. 64: 1117-1126.

Hugueney, P., Provenzano, S., Verriès, C., Ferrandino, A., Meudec, E., Batelli, G., Merdinoglu, D., Cheynier, V., Schubert, A., Ageorges, A., 2009. A novel cation-dependent *o*-methyltransferase involved in anthocyanin methylation in grapevine. J. Plant Physiol. Vol. 150 (4): 2057- 2070.

Intrieri, C., 1994. Vini frizzanti: interventi agronomici per il controllo della maturazione. J. L'enotecnico, Vol. 9: 65-72.

Intrieri, C., Magnanini E., Silvestroni O., 1983, Effetti della potatura verde sul comportamento vegetativo e produttivo di Albana e Sangiovese. J. Vignevini, Vol. 5: 57-62.

Intrieri, C., Silvestroni, O., Poni, S. 1994 – Pluker: nuova defogliatrice polivalente per vigneti. Vignevini. Vol. 1-2: 25 – 31.

involvement of leucoanthocyanidin dioxygenase. J. Planta. Vol. 229(2): 427-445.

J. Planta. Vol. 192(4): 567-573.

Jackman, R.L., Smith, J.L., 1996. Anthocyanins and betalains. In: Hendry GAF, Houghton JD, eds. Natural food colorants, 2nd edn. London: Chapman & Hall, pp: 244–309.

Jeong, S.T., Goto-Yamamoto, N., Kobayashi, S., Esaka, M., 2004. Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. J. Plant Sci. Vol. 167: 247-252.

Jeong, S.T., Goto-Yamamoto, N., Hashizume, K., Esaka, M., 2006. Expression of the flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase genes and flavonoid composition in grape (*Vitis vinifera*). Plant Sci. Vol. 170 (1): 61–69.

Jiang, Y.M., Duan, X., Joyce, D., Zhang, Z., Li, J. 2004. Advances in understanding of enzymatic browning in harvested litchi fruit. J. Food Chem. Vol. 88: 443–446.

Jones, G.V., White, M.A., Cooper, O.R., Storchmann, K., 2005. Climate change and global wine quality. J. Climatic Change. Vol. 73: 319–343.

Kader, F., Haluk, J.P., Nicolas, J.P., Metche, M., 1998. Degradation of cyanidin 3-glucoside by blueberry polyphenol oxidase: kinetic studies and mechanisms. J. Agric. Food Chem. Vol. 46: 3060– 3065.

Kader, F., Nicolas, J.P., Metche, M., 1999. Degradation of pelargonidin 3-glucoside in the presence of chlorogenic acid and blueberry polyphenol oxidase. J. Sci. Food Agric. Vol. 79: 517–522.

Kader, F., Rovel, B., Girardin, M., Metche, M., 1997. Mechanism of browning in fresh highbush blueberry fruit (Vaccinium corymbosum L) Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. J. Sci. Food Agric. Vol. 74(1): 31–34.

Karimi, M., Inzé, D., Depicker, A., 2002. GATEWAY™ vectors for Agrobacterium-mediated plant transformation. J. Trends in Plant Sci. Vol. 7(5): 193–195.

Kemp, B.S. Harrison, R. Creasy, G.L. 2011. Effect of mechanical leaf removal and its timing on flavan-3-ol composition and concentrations in Vitis vinifera L. cv. Pinot Noir wine. J. Aust. J. Grape Wine Res. Vol. 17(2): 270–279.

Kennedy, J.A., Jones, G.V., 2001. Analysis of Proanthocyanidin Cleavage Products Following Acid-Catalysis in the Presence of Excess Phloroglucinol. Amer. J. Enol. Vitic. Vol. 49(4): 1740-1746.

Kennedy, J.A., Matthews, M.A., Waterhouse, A.L., 2000. Changes in grape seed polyphenols during fruit ripening. J. Phytochem. Vol. 55: 77-85.

Kennedy, J.A., Matthews, M.A., Waterhouse, A.L., 2002. Effect of maturity and vine water status on grape skin and wine flavonoids. J. Am. J. Enol. Vitic. Vol. 53: 268-274.

Kennedy, J.A., Saucier, C., Glories, Y., 2006. Grape and Wine Phenolics: History and Perspective. J. Am. J. Enol. Vitic. Vol. 57 (3): 239-248.

Kenny, G.J., Harrison, P.A., 1992. The effects of climate variability and change on grape suitability in Europe. J. Wine Res. Vol. 3: 163–183.

King, P., McClellan, D., Smart, R. 2012. Effect of severity of leaf and crop removal on grape and wine composition of Merlot vines in Hawke's bay vineyards. J. Am. J. Enol. Vitic. ajev.2012.12020.

Kliewer, W. M., 1970. Effect of day temperature and light intensity on coloration of Vitis vinifera grapes. J. Amer. Soci. Hort. Sci. Vol. 95: 693–697.

Kliewer, W. M., Torres, R. E., 1972. Effect of controlled day and night temperatures on grape coloration. J. Amer. J. Enol. Vitic. Vol. 23: 71–77.

Kliewer, W., Dokoozlian, N. 2005. Leaf area/crop weight ratios of grapevines: influence on fruit composition and wine quality. J. Am. J. Enol. Vitic. Vol. 56(2): 170-181.

Kliewer, W.M., 1966. Sugars and organic acids of *Vitis Vinifera*. J. Plant Physiol.Vol. 41: 923-931.

Kliewer, W.M., 1977. Influence of temperature, solar radiation and nitrogen on coloration and composition of Emperor Grapes. J. Am.J. Enol. Vitic. Vol. 28 (2): 96-103.

Kobayashi, S., Goto-Yamamoto, N., Hirochika, H., 2004. Retrotransposon-induced mutations in grape skin color. J. Sci. Vol. 304 (5673): 982 p.

Kobayashi, S., Ishimaru, M., Ding, C.K., Yakushiji, H., Goto, N., 2001. Comparison of UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) gene sequences between white grapes (*Vitis vinifera*) and their spots with red skin. J. *Plant Sci.* Vol. 160: 543-550.

Kobayashi, S., Ishimaru, M., Hiraoka, K., Honda, C., 2002. *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. J. *Planta*. Vol. 215: 924-933.

Kriedemann, P. E., 1968. Photosynthesis in vine leaves as a function of light intensity, temperature, and leaf age. J. Vitis. Vol. 7: 213-220.

Kuznetsov, V. V., Shevyakova, N. I. 2007. Polyamines and stress tolerance of plants. Global science books. Plant stress. Vol. 1(1): 50-71.

Laget, F., Tondut, J. L., Deloire, A., Kelly, M. T., 2008. Climate trends in a specific Mediterranean viticultureal area between 1950 and 2006. J. Int. Sci. Vigne Vin. Vol. 42 (3): 113-123.

Lakso, A. N., Kliewer, W. M., 1975. The Influence of Temperature on Malic Acid Metabolism in Grape Berries. J. Plant Physiol. Vol. 56 (3): 370-372.

Landy, A., 1989. Dynamic, structural, and regulatory aspects of site-specific recombination. J. Ann. Rev. Biochem. Vol. 58: 913–949.

Larronde, F., Krisa, S., Decendit, A., Chèze, C., Deffieux, G., Mérillon, J. M. 1998. Regulation of polyphenol production in Vitis vinifera cell suspension cultures by sugars. J.Plant Cell Reports. Vol. 17(12): 946-950.

Leyva, A., Jarillo, J., Salinas, J., Martinez-Zapater, M., 1995. Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of Arabidopsis thaliana in a light-dependent manner. J. Plant Physiol. Vol. 108: 39–46.

Lin-Wang, K., Micheletti, D., Palmer, J., Volz, R., Lozano, L. 2011. High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. J. Plant, Cell & Environment. Vol. 34(7): 1176–1190.

Lo Piero, A. R., Puglisi, I., Rapisarda, P., Petrone, G., 2005. Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage. J. Agric. Food Chem. Vol. 53: 9083–9088.

Lough, J. M., Wigley, T. M. L., Palutikof, J. P., 1983. Climate and climate impact scenarios for Europe in a warmer world. J. Clim. Appl. Meteorol. Vol. 22: 1673–1684.

Løvdal, T., Olsen, K. M., Slimestad, R., Verheul, M., Lillo, C. 2010. Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. J. Phytochemistry. Vol. 71(5–6): 605–613.

Makra, L., Vitányi, B., Gál, A., Mika, J., Matyasovszky, I., Hirsch, T., 2009. Wine Quantity and Quality Variations in Relation to Climatic Factors in the Tokaj (Hungary) Winegrowing Region. Amer. J. Enol. Vitic. Vol. 60 (3): 312-321.

Mann, M. E., Bradley, R. S., Hughes, M. K., 1998. Global-scale temperature patterns and climate forcing over the past six centuries. J. Nature. Vol. 392: 779-787.

Matsui, S., Ryugo, K., Kliewer, W. M., 1986. Growth inhibition of Thompson Seedless and Napa Gamay berries by heat stress and its partial reversibility by applications of growth regulators. J. Am. J. Enol. Vitic. Vol. 37 (1): 67-71.

Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., Velasco, R. 2006. Metabolite profiling of grape: Flavonols and anthocyanins. J. Agric. Food Chem. Vol. 54 (20): 7692-7702.

Mazza, G., Fukumoto, L., Delaquis, P., Girard, B., Ewert, B.1999. Anthocyanins, Phenolics, and Color 426 of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. J. Agric. Food Chem. 47:4009-427 4017. Menting, J. G. T., Scopes, R. K., Stevenson, T. W., 1994. Characterization of Flavonoid 3'5'-Hydroxylase in Microsomal Membrane Fraction of Petunia hybrida Flowers. J. Plany Physiol. Vol. 106 (2): 633- 642.

Mazza, G., Miniati, E., 1993. Color stabilization and intensification. In: Mazza G, Miniati E (eds) Anthocyanins in Fruits, Vegetables, and Grains. CRC Press, Boca Raton, pp 1–20.

Meyer, P., Heidmann, I., Forkmann, G., Saedler, H., 1987. A new petunia flower colour generated by transformation of a mutant with a maize gene. J. Nature. Vol. 330: 677-678.

Mol, J., 1998. How genes paint flowers and seeds. J. Trends in Plant Sci. Vol. 3: 212-217.

Morais, H., Ramos, C., Forgacs, E., Cserhati, T., Matos, N., Almeida, V., Oliveira, J., 2002. Stability of anthocyanins extracted from grape skins. J. Chromatographia. Vol. 56: 173–175.

Mori, K., Goto-Yamamoto, N., Kitayama, M., Hashizume, K., 2007a. Loss of anthocyanins in red-wine grape under high temperature. J. Experimental Botany. Vol. 58 (8): 1935-1945.

Mori, K., Goto-Yamamoto, N., Kitayama, M., Hashizume, K., 2007a. Effect of high temperature on anthocyanin composition and transcription of flavonoid hydroxylase genes in "Pinot noir" grapes (Vitis vionifera). J. Hortic. Sci. Biotechnol. Vol. 82 (2): 199-206.

Mori, K., Saito, H., Goto-Yamamoto, N., Kitayama, M., Kobayashi, S., Sugaya, S., Gemma, H., Hashizume, K., 2005a. Effects of abscisic acid treatment and night temperatures on anthocyanin composition in 'Pinot noir' grapes. J. Vitis. Vol. 44: 161–165.

Mori, K., Sugaya, S., Gemma, H., 2005b. Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. J. Sci. Hortic. Vol. 105: 319–330.

Movahed, N., , Maeiyat, M.M., 2009. Phytoremediation and sustainable urban design methods (Low Carbon Cities through Phytoremediation). 45th ISOCARP Congress. Disponible en: http://www.isocarp.net. Fecha de consulta 04 Septiembre del 2009.

Movahed, N., Masia, A., Cellini, A., Pastore, C., Valentini, G., Allegro, G., Filippetti, I. 2011. Biochemical approaches to study the effects of temperature on grape composition in cv. Sangiovese. The 17th International GIESCO Symposium, Asti, Italy; The proceeding: PP: 393- 397.

Munne´-Bosch, S., Alegre, L., 2004. Die and let live: leaf senescence contributes to plant survival under drought stress. J. Funct. Plant Biol. Vol. 31: 203-216.

Nissim-Levi, A., Kagan, S., Ovadia, R., Oren-Shamir, M., 2003. Effects of temperature, UV-light and magnesium on anthocyanin pigmentation in cocoplum leaves. J. Hortic. Sci. Biotechnol. Vol. 78: 61-64.

Oren-Shamir, M., Levi-Nissim, A., 1997a. Temperature effects on the leaf pigmentation of Cotinus coggygria 'Royal Purple'. J. Hortic. Sci. Vol. 72: 425–432.

Oren-Shamir, M., Levi-Nissim, A., 1997b. UV-light effect on the leaf pigmentation of Cotinus coggygria 'Royal Purple'. J. Sci. Hortic. Vol. 71: 59–66.

Oren-Shamir, M., Nissim-Levi, A., 1999. Temperature and gibberellin effects on growth and anthocyanin pigmentation in Photinia leaves. J. Hortic. Sci. Biotechnol. Vol. 74: 355–360.

Palliotti, A., Poni, S., Berrios, J.G., Bernizzoni, F., 2010. Vine performance and grape composition as affected by early-season source limitation induced with anti-transpirants in two red Vitis vinifera L. cultivars. J. Aust. J. Grape Wine Res. Vol. 16: 426–433.

Pastore, C., 2010. Reserches on berry composition in red grape: agronomical, biochemical and molecular approaches. Tesi di Dottorato di ricerca in Colture Arboree ed Agrosistemi Forestali e Paesaggistici, XXII ciclo, Faculta di Agraria. Universita di Bologna.

Pereira, G.E., Gaudillere, J.P., Pieri, P., Hilbert, G., Maucourt, M., Deborde, C., Moing, A., Roil, D., 2006. Microclimate influence in mineral and metabolic profiles of grape berries. J. Agric. Food Chem. Vol. 54: 6765-6775.

Pirie, A., Mullins, M.1976. Changes in anthocyanin and phenolics content of grapevine leaf and fruit tissues treated with sucrose, nitrate, and abscisic acid. J. Plant Physiol. Vol. 58(4): 468-472.

Pourcel, L., Routaboul, J. M., Cheynier, V., Lepiniec, L., Debeaujon, I., 2006. Flavonoid oxidation in plants: from biochemical properties to physiological functions. J.Trends in Plant Sci. Vol. 12 (1): 29-36.

Price, S.F., Breen, P.J., Valladao, M., Watson, B.T., 1995. Cluster sun exposure and quercetin in Pinot noir grapes and wine. Am.J. Enol. Vitic. Vol. 46: 187-194.

Quattrocchio, F., Baudry, A., Lepiniec, L., Grotewold, E., 2006. The regulation of flavonoid biosynthesis. J. The science of flavonoids. C Springer, Chapter 4, pp: 97–122.

Rebucci, B., Poni, S., Intrieri, C., Magnanini, E., Lakso, A. N., 1997. Effects of manipulated grape berry transpiration on post-veraison sugar accumulation. J. Aust. J. Grape Wine Res. Vol. 3: 57-65.

Reynolds, A., Price, S., Wardle, D., Watson, B. 1994. Fruit environment and crop level effects on Pinot noir. I. vine performance and fruit composition in British Columbia. J. Am. J. Enol. Vitic. Vol. 45(4): 452-459.

Reynolds, A., Wardle, D. 1989. Influence of fruit microclimate on monoterpene levels of Gewürztraminer. J. Am. J. Enol. Vitic. Vol. 40(3): 149-154.

Ristic, R., Bindon, K., Francis, L.I., Herderich, M. J., Iland, P.G., 2010, Flavonoids and C13-norisoprenoids in Vitis vinifera L. cv. Shiraz: relationships between grape and wine composition, wine colour and wine sensory properties. J. Aust. J. Grape Wine Res. Vol. 16: 369–388.

Ristic, R., Downey, M., Iland, P., Bindon, K., Francis, I., Herderich, M., Robinson, S. 2007. Exclusion of sunlight from Shiraz grapes alters wine colour, tannin and sensory properties. J. Aust. J. Grape Wine Res. Vol. 13(2):53-65.

Robinson, S. P., Davies, C., 2000. Molecular biology of grape berry ripening. J. Aust. J. Grape Wine Res. Vol. 6: 175-188.

Robinson, S., 2006. Colour and tannin during berry development and ripening. In "Finishing the job". ASVO proceedings held 21 July 2006, Mildura, Vic., Australia. pp: 11-14.

Roby, G., Harbertson, J. F., Adams, D. A., Matthews, M. A., 2004. Berry size and vine water deficits as factors in winegrape composition: anthocyanins and tannins. J. Aust. J. Grape Wine Res. Vol. 10: 100- 107.

Rolland, F., Baena-Gonzalez, E., Sheen, J., 2006. Sugar sensing and signaling in plants: Conserved and novel mechanisms. J. Ann. Rev. Plant Biol. Vol. 57: 675-709.

Romero, C., Bakker, J., 2000. Effect of storage temperature and pyruvate on kinetics of anthocyanin degradation, Vitisin A derivative formation, and color characteristics of model solutions. J. Agric. Food Chem. Vol. 48: 2135–2141.

Sadras, V. 2007. Evolutionary aspects of the trade-off between seed size and number in crops. J. Field crop research. Vol. 100(2-3): 125-138.

Sadras, V.O., Petrie, P. R., Moran, M. A. 2013. Effects of elevated temperature in grapevine. II juice pH, titratable acidity and wine sensory attributes. Australian Journal of Grape and Wine Research. Vol. 19 (1): 107–115.

Sarni, P., Fulcrand, H., Souillol, V., Souquet, J. M., Cheynier, V., 1995. Mechanisms of anthocyanin degradation in grape must-like model solutions. J. Sci. Food Agr. Vol. 69(3): 385–391.

Scholefield, P.B., May, P., Neales, T.F. 1977. Harvest-pruning and trellising of 'Sultana' vines. II. Effects on early spring development. Sci. Hortic. Vol. 7:123-132.

Schultz, H. R., 1995. Grape canopy structure, light microclimate and photosynthesis. I. A two dimensional model of the spatial distribution of surface area densities and leaf ages in two canopy systems. J. Vitis. Vol. 34: 211-215.

Schultz, H. R., 2000. Climate change and viticulture: a European perspective on climatology, carbon dioxide and UV-B effects. Aust. J. Grape Wine Res. Vol. 6: 2–12.

Schultz, H. R., 2003. Extension of a Farquhar model for limitations of leaf photosynthesis induced by light environment, phenology and leaf age in grapevines (Vitis vinifera L. cvv. White Riesling and Zinfandel).J. Functional Plant Biol. Vol. 30(6): 673 – 687.

Sepúlveda, G., Kliewer, W. M., Ryugo, K., 1986. Effect of high temperature on grapevines (*Vitis vinifera* L.). I. translocation of ^{14}C-photosynthates. J. Am. J. Enol. Vitic. Vol. 37 (1): 13-19.

Shaked-Sachray, L., Weiss, D., Moshe, R., Nissim-Levi, A., Oren- Shamir, M., 2002. Increased anthocyanin accumulation in aster flowers at elevated temperatures due to magnesium treatment. J. Physiol Plant. Vol. 114: 559–565.

Shvarts, M., Borochov, A., Weiss, D., 1997. Low temperature enhances petunia flower pigmentation and induces chalcone synthase gene expression. J. Physiol. Plant. Vol. 99: 67–72.

Sipiora, M. J., Gutie´rrez-Granda, M. J., 1998. Effects of pre-veraison irrigation cutoff and skin contact time on the composition, color, and phenolic content of young Cabernet Sauvignon wines in Spain. J. Amer. J. Enol. Vitic. Vol. 49: 152–162.

Smart, R. E., 1985. Principles of grapevine canopy microclimate manipulation with implication for yield and quality. J. Amer. J. Enol. Vitic. Vol. 36: 230-239.

Smith, G. J., Markham, K. R., 1998. Tautomerism of flavonol glucosides - relevance to plant UV protection and flower colour. J. Photochem. Photobiol. A – Chem. Vol. 118: 99-105.

Soar, C. J., Collins, M. J., Sadras, V. O., 2009. Irrigated Shiraz vines (*Vitis vinifera*) up regulate gas exchange and maintain berry growth in response to short spells of high maximum temperature in the field. J. *Functional Plant Biology.* Vol. 36(9): 801–814.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., Tonelli, C., 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (Vitis Vinifera L.). J. Plant Molecular Biology. Vol. 24: 743-755.

Spayd, S. E., Tarara, J. M., Mee, D. L., Ferguson, J. C., 2002. Separation of sunlight and temperature effects on the composition of Vitis vinifera cv. Merlot berries. J. Amer. J. Enol. Vitic. Vol. 53:171-182.

Spelt, C., 2002. Anthocyanin1 of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. The plant cell online. Vol. 14: 2121-2135.

Stafford, H. A., 1990. Flavonoid Metabolism; CRC Press: Boca Raton, FL: 298 pp.

Stracke, R., De Vos, R. C. H., Bartelniewoehner, L., Ishihara, H., Sagasser, M., Martens, S., Weisshaar, B., 2009. Metabolomic and genetic analyses of flavonol synthesis in *Arabidopsis thaliana*support the in vivo

Taureilles-Saurel, C., Romieu, C. G., Robin, J. P., Flanzy, C., 1995. Grape (*Vitis vinifera* L.) malate dehydrogenase. II. Characterization of the major mitochondrial and cytosolic isoforms and their role in ripening. J. Am. J. Enol. Vitic. Vol. 46 (1): 29-36

Tomana, T., Utsunomiya, N., Kataoka, I., 1979. The effect of environmental temperatures on fruit ripening on the tree. II. The effect of temperatures around whole vines and clusters on the coloration of 'Kyoho' Grapes. Japan. Soc. Hort. Sci. Vol. 48: 261-266.

Treutter, D., 2006. Significance of flavonoids in plant resistance: a review. J. Environ. Chem. Lett. Vol. 4: 147–157.

Ubi, B.W., Honda, C., Bessho, H., Kondo, S., Wada, M., Kobayashi, S., Moriguchi, T., 2006. Expression analysis of anthocyanin biosynthetic genes in apple skin: effect of UV-B and temperature. J. Plant Sci. Vol. 170: 571–578.

Ushimaru, T., Maki, Y., Sano, S., Koshiba, K., Asada, K., Tsuji, H., 1997. Induction of enzymes involved in the ascorbate-dependent antioxidative system, namely, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase, after exposure to air of rice (Oryza sativa) seedlings germinated under water. J. Plant Cell Physiol. Vol. 38: 541-549.

Vaknin, H., Bar-Akiva, A., Ovadia, R., Nissim-Levi, A., Forer, I., Weiss, D., Oren-Shamir, M., 2005. Active anthocyanin degradation in Brunfelsia calycina (yesterday-today-tomorrow) flowers. J. Planta. Vol. 222: 19–26.

Venturini, L., 2009, Study of some gene families involved in anthocyanin accumulation during Vitis vinifera berry ripening. Tesi di laurea specialistica in biotechnologie agro-industriali; faculta di scienze matematiche, fisichie e naturali; Universita degli studi di Verona. pp:7-12, 30-51.

Wahid, A., Perveen, M., Gelani, S., Basra, S.M.A., 2007. Pretreatment of seed with H_2O_2 improves salt tolerance of wheat seedlings by alleviation of oxidative damage and expression of stress proteins. J. Plant Physiol. Vol. 164 (3): 283–294.

Walker, A.R., Lee, E., Bogs, J., McDavid, D. A. J., Thomas, M. R., Robinson, S. P., 2007. White grapes arose through the mutation of two similar and adjacent regulatory genes. The Plant journal: for cell and molecular biology. Vol. 49: 772-85.

Walker, J. R., Ferrar, P. H., 1998. Diphenol oxidases, enzymecatalysed browning and plant disease resistance. J. Biotechnol. Genet. Eng. Rev. Vol. 15: 457–498.

Waterhouse, A. L., 2002. Wine Phenolics, Ann. N.Y. Acad. Sci. Vol. 957: 21-36.

Watson, R.T., Rodhe, H., Oeschger, H., Siegenthaler, U., 1990. Greenhouse gases and aerosols. http://rivernet.ncsu.edu

Welinder K. G., Justesen A. F., Kjærsga I. V. H., Jensen R. B., Rasmussen S. K., Jespersen H. M., Duroux L., 2002. Structural diversity and transcription of class III peroxidases from Arabidopsis thaliana. J. Eur. J. Biochem. Vol. 269: 6063–6081.

Wen, Y., Zhang, Y., Pan, Q., 2009. Progress of organic acids in grape berries. Natural science journal of Hainan University. S663.1

Williams, R. J., Spencer, J. P., Rice-Evans, C., 2004. Flavonoids: antioxidants or signaling molecules? J. Free Radic. Biol. Med. Vol. 36: 838–849.

Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. J. Current opinion in plant biology Vol. 5: 218-23.

Winkler, A. J., Cook, J. A., Kliewer, W. M., Lider, L. A., 1962. Development and composition of grapes. General viticulture. Berkeley, CA: University of California Press. pp: 141–196.

Xie, D.Y., Sharma, S. B., Paiva, N. L., Ferreira, D., Dixon, R. A., 2003. Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis. J. Science. Vol. 299: 396–399.

Yamane, T., Jeong, S. T., Goto-Yamamoto, N., Koshita, Y., Kobayashi, S., 2006. Effects of temperature on anthocyanin biosynthesis in grape berry skins. J. Amer. J. Enol. Vitic. Vol. 57: 54–59.

Yamasaki, H., Sakihama, Y., Ikehara, N., 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H2O2. J. Plant Physiol. Vol. 115: 1405-1412.

Yokotsuka, K., Singleton, V. L., 1997. Disappearance of anthocyanins as grape juice is prepared and oxidized with PPO and PPO substrates. J. Am. J. Enol. Vitic. Vol. 48 (1): 13-25.

Yoruk, R., Marshal, R. M., 2003. Physiocochemical properties and function of plant polyphenol axidase. A review. J. Food Biochem. Vol. 27: 361-422.

Yu, D. J., Kim, S. J., Lee, H. J., 2009. Stomatal and non-stomatal limitations to photosynthesis in field-grown grapevine cultivars. J. Biologia Plantarum. Vol. 53 (1): 133-137.

Zsófi, Z., Váradi, G., Bálo, B., Marschall, M., Nagy, Z., Dulai, S., 2009. Heat acclimation of grapevine leaf photosynthesis: mezo- and macroclimatic aspects. J. *Functional Plant Biology*. Vol. 36(4): 310–322.